Asp\(^{333}\), Asp\(^{495}\), and His\(^{523}\) Form the Catalytic Triad of Rat Soluble Epoxide Hydrolase*

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On the basis of the sequence similarity between mammalian epoxide hydrolases and bacterial haloalkane dehalogenase reported earlier (Arand, M., Grant, D. F., Beetham, J. K., Friedberg, T., Oesch, F., and Hammock, B. D. (1994) FEBS Lett. 338, 251–256; Beetham, J. K., Grant, D., Arand, M., Garbarino, J., Kiyosue, T., Pinot, F., Oesch, F., Belknap, W. R., Shinozaki, K., and Hammock, B. D. (1995) DNA Cell. Biol. 14, 61–71) we selected candidate amino acid residues for the putative catalytic triad of the rat soluble epoxide hydrolase. The predicted amino acid residues were exchanged by site-directed mutagenesis of the epoxide hydrolase cDNA, followed by the expression of the respective mutant enzymes in Escherichia coli. A total of 25 different mutants were analyzed for their epoxide hydrolase activity toward the model substrate trans-stilbene oxide. In case of impaired catalytic activity of a given mutant, the structural integrity of the recombinant enzyme protein was assessed either by its ability to covalently bind the substrate trans-stilbene oxide or by affinity purification on benzyl thio-Sepharose, using the soluble epoxide hydrolase-specific competitive inhibitor 4-fluorochalcone oxide to release the bound enzyme from the affinity matrix. Of the mutants under investigation, only those with changes in the positions Asp\(^{333}\), Asp\(^{495}\), and His\(^{523}\) were completely inactive toward the model substrate trans-stilbene oxide while retaining the proper protein fold. These amino acids were exactly those previously predicted by sequence alignment. Exchange of the amino acid residues flanking the catalytic nucleophile Asp\(^{333}\) significantly changed the kinetic properties of the enzyme. Mutation of His\(^{523}\) to Gln had no apparent effect on the \(K_m\) but led to a heavily reduced \(V_{max}\) (5% that of the wild type) of the mutant enzyme, while the exchange of Trp\(^{540}\) against Phe strongly increased the \(K_m\) (7-fold) and also moderately enhanced the \(V_{max}\) (2-fold) of the corresponding mutant. Mutation of Trp\(^{540}\) apparently had a strong effect on the protein conformation.

Epoxide hydrolases (EH; EC 3.3.2.3) are a group of functionally related enzymes that catalyze the addition of water to oxirane compounds (epoxides), thereby generating vicinal trans-diols (1, 2). This enzymatic reaction represents a detoxification step of central importance, because (i) many oxirane derivatives are reactive electrophiles, due to the high tension of the C-O bonds, and (ii) epoxides are frequent intermediary metabolites arising during the biotransformation of foreign compounds (2). EH have been found in all types of living organisms, including mammals, invertebrates, plants, fungi, and bacteria and may thus be regarded as ubiquitous. In mammals, the two EH implicated in the xenobiotic metabolism are the microsomal epoxide hydrolase (mEH) (3) and the soluble epoxide hydrolase (sEH) (4). These two enzymes are distinct by substrate specificity, subcellular distribution and inducibility by foreign compounds (5, 6).

Very recently, much progress has been made in understanding the structural relationship between these two mammalian EH. They share amino acid sequence similarity to a region around the active center of the bacterial haloalkane dehalogenase (7), an enzyme with a known three-dimensional structure that belongs to the family of \(\alpha/\beta\) hydrolase fold enzymes (8). This led us and others to hypothesize that EHs also belong to the \(\alpha/\beta\) hydrolase fold family (9–11). The common feature of this family of proteins is a highly conserved protein fold in the so-called domain I, composed of a central sheet of parallel \(\beta\)-strands that is sandwiched by \(\alpha\)-helices (12) (see Fig. 1). This domain provides the framework for a catalytic triad, similar to that of serine proteases, with which these enzymes perform their catalytic task. The catalytic mechanism involves two steps: in a first reaction, an ester bond is formed between enzyme and substrate by attack of the substrate with a nucleophilic amino acid residue (Ser, Cys, or Asp; see N in Fig. 1), and this ester is subsequently hydrolyzed in a second step by a water molecule that has been activated via proton abstraction by a His-Asp/Glu pair (H and A in Fig. 1). The reaction mechanism for the sEH, as inferred from the analogy to that of haloalkane dehalogenase (13), is shown in Fig. 2. First experimental evidence for the correctness of this proposed mechanism was brought about by Lacourciere and Armstrong for the mEH (14) and Hammock et al. for the sEH (15). Lacourciere and Armstrong showed that rat mEH incorporates \(^{18}\)O during substrate turnover in \(H_2\(^{18}\)O\) and is subsequently capable of transferring this \(^{18}\)O into the product of the enzymatic reaction in the complete absence of \(H_2\(^{18}\)O\). The stoichiometry of the reaction is best explained by the intermediate formation of an enzyme-substrate ester. Hammock et al. could unequivocally demonstrate intermediate covalent binding of the substrate juvenile hormone III to murine sEH by precipitation of the enzyme-substrate ester and subsequent microchemical analysis.

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‡ The only known exception is the leukotriene A4 hydrolase that forms a dial with a distance of eight carbon atoms between the two created hydroxyl groups.
Fig. 1. General three-dimensional structure of $\alpha/\beta$ hydrolase fold enzymes. This is a schematic representation of the structurally conserved domain I of $\alpha/\beta$ hydrolase fold enzymes as described by Ollis et al. (12). $\alpha$-Helices are shown as spirals, and $\beta$-strands are symbolized by flat arrows. Helices and strands are marked with letters and numbers, respectively, according to Ref. 12. The important elements of the active center are as follows: nucleophile (Ser, Cys, or Asp) (N); water-activating histidine (H); and acidic residue of the charge relay system (Asp or Glu) (A). Domain II is not structurally conserved among all $\alpha/\beta$ hydrolase fold enzymes, but it is present between strand 6 and helix D in the haloalkane dehalogenase (8) and the bromoperoxidase (34), the two $\alpha/\beta$ hydrolase fold enzymes of solved three-dimensional structure with sequence similarity to epoxide hydrolases.

The aim of the present study was to provide experimental proof for the composition of the catalytic triad of rat soluble epoxide hydrolase as predicted by sequence similarity analysis (for a condensed sequence alignment, see Fig. 3). Our strategy was to substitute the amino acid residues in question by site-directed mutagenesis and to analyze the resulting recombinant proteins after expression in bacteria for their catalytic activities and their structural integrity. Those mutants with lost enzymatic activity but apparently retained structural conformation were directly involved in the catalytic mechanism. While this study was underway, Pinot et al. (16) reported that the mutation of murine sEH at positions Asp$^{333}$ and His$^{342}$ leads to inactive enzyme and that these residues, therefore, are part of the catalytic triad of sEH, corresponding to N and H in Fig. 1. In the present report we identify Asp$^{333}$ as the third member (A) of the catalytic triad of the rat sEH, in addition to confirm the function of Asp$^{333}$ and His$^{342}$ also in the rat enzyme. Furthermore, we describe altered kinetic properties of mutant rat sEH due to the exchange of amino acids neighboring the catalytic nucleophile Asp$^{333}$.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Site-directed mutagenesis of the sEH coding sequence (17) was performed via polymerase chain reaction (18) using Ampli-Taq (Perkin-Elmer) as the thermostable DNA polymerase. The amplified fragments were introduced either by using flanking restriction enzyme recognition sites present in the wild type cDNA (MsdI for the mutants of amino acids His$^{332}$, Asp$^{333}$, and Trp$^{334}$; BbsI for the mutants of amino acid His$^{342}$) or by the method of Tomic et al. (19). This latter procedure employs restriction enzymes that do not cut within their recognition sequence but at a position of fixed distance downstream of the latter, thereby creating a unique 5'-overhang. Regenerative recognition sites are introduced in the 5'-region of oligonucleotides used for DNA amplification and are subsequently removed from the amplified fragments by digestion with the restriction enzyme in question. The remaining sequence, including its 5'-overhang, is devoid of any sequence material not present in the original template, with the only exception being the wanted mutation. Two fragments need to be amplified per mutation, one carrying the desired sequence alteration and to assess the correctness of the other inclusion the sites of ligation, in order to monitor the introduction of the respective expression vector. We introduced the desired mutations using oligonucleotides with BbsI restriction sites at their 5'-ends (see Table I; for further details of the procedure see Ref. 19). Most of the mutation primers were designed ambiguous at the site of desired mutation to allow for the simultaneous generation of alternative amino acid exchanges at the same position using a single mutation primer. After polymerase chain reaction and restriction enzyme digest, the mutation-harboring fragments were inserted together with the respective adaptor fragment into the HindIII (1212)/BamHI (1526) site in the case of the mutants for the amino acids Glu$^{332}$, Asp$^{333}$, Glu$^{342}$, and Asp$^{342}$ or into the BamHI (1526)/EcoRI (1932) site in the case of the mutants for amino acids His$^{332}$, Lys$^{334}$, and Tyr$^{342}$. The positions of the mutated amino acid residues and of the employed restriction enzyme recognition sites within the sEH sequence are presented in Fig. 4. The resulting expression constructs were analyzed by dideoxy sequencing (20) over the entire range of the amplified sequences, including the sites of ligation, in order to monitor the introduction of the desired sequence alteration and to assess the correctness of the other part of the sequence.

Bacterial Expression of sEH Mutants—Expression of sEH was performed using the plasmid vector pRSET B (Invitrogen) in Escherichia coli JM109 (50 ml of culture volume/expression) as described earlier (17). Accumulation of recombinant protein was allowed for 16 h at 37 °C after switching to permissive growth conditions. Thereafter, cells were harvested by centrifugation at 4 °C and 3000 × g for 10 min. The resulting pellet was washed once in STE buffer (10 mM Tris-Cl, pH 8.1, 0.1 M NaCl, 0.01 M EDTA).
mg EDTA, 150 mg NaCl), reconsititured under the same conditions, and resuspended in 1.8 ml of STE buffer containing lysosome, 100 μg/ml. After incubation on ice for 15 min, 0.2 ml of diithiothreitol solution, 50 mΜ, was added and the cells were broken by sonication with a Branson Cell Disruptor B15 sonifier by four subsequent series of four pulses each with the microtip, while intermittently cooling the sample on ice. The homogenate was centrifuged at 4°C and 13,000 x g for 30 min. The resulting supernatant was used directly for measuring the epoxide hydrolase activity or as the source for further purification of the recombinant enzymes.

Analysis of the Epoxide Hydrolase Mutants—The success of the expression of recombinant protein was monitored by immunoblotting. In order to obtain an SEH-specific immunological probe the BamHI (31)/BamHI (734) fragment of the SEH coding cDNA encoding the full-length enzyme domain (see Fig. 4) was expressed as a recombinant β-galactosidase/SEH fusion protein after insertion into the pEZ2 expression vector, and a rabbit immunosorbsent was raised against the purified fusion protein according to procedures described earlier (21, 22). SDS-polyacrylamide gel electrophoresis of SEH mutants was performed on 10% acrylamide gels essentially as described by Laemmli (23). Protein transfer from the gels to nitrocellulose sheets and subsequent immunodetection was carried out according to Towbin et al. (24). The above specified rabbit antisem was used in a dilution of 1:1000 for the detection of SEH protein, and the resulting immunocomplex was visualized with an alkaline phosphatase-coupled anti-rabbit immunoglobulin antibody using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium as chromogenic substrates. The resulting color signals were digitized employing an Eagle Eye II still video system (Stratagene), and the absorbance signals were subsequently calculated by using the NIH Image public domain package (version 1.49), created by Wayne Rasband. 

Table I

| Site of mutation | Mutation primer | Adapter primer |
|-----------------|-----------------|---------------|
| His332          | CATTTGCACGACCTGGGGCTTGT | Not required  |
| Asp333          | CATTTGCATGCTGGGCTTGTG    | Not required  |
| Thr334          | CATTTGCAATGCTGGGCTTGTGCT | Not required  |
| Asp334          | tgaagaacccCGGATCTGAGGCTTGGT | Not required  |
| Glu339          | KGAAGACCATGCTGCTGTGCTGCT | Not required  |
| Asp339          | KGAAGACCATGCTGCTGTGCTGCT | Not required  |
| His347          | KGAAGACATGCTGCTGTGCTGCT | Not required  |
| His383          | TCAAGACTGCAATGCTGCTGCTGCT | Not required  |
| Lys399          | gcgaagaactcATGCTGCTGCTGCT | Not required  |
| Trp340          | gcgaagaactcATGCTGCTGCTGCT | Not required  |
| Lys382          | gcgaagaactcATGCTGCTGCTGCT | Not required  |

FIG. 3. Alignment of important conserved amino acid sequence elements of selected SEH-related proteins. Amino acid sequences are given in the one-letter code. The positions of the residues involved in the catalytic mechanism are marked with arrows. Asterisks indicate residues in the SEH sequence that were mutated in the present study. Numbers in italics designate the position of the adjacent amino acid in the respective sequence. Numbers between back slashes represent the number of connecting amino acid residues not shown in the alignment. Numbers in brackets represent the total number of amino acids of the respective protein. In case of sequence identity ≥ 50% among the 10 sequences at a given position, the common amino acid residue is boxed in the respective sequences and printed in the consensus, with the exception of the catalytic nucleophile that is marked by a plus sign in the consensus. Note that sequence identity is never below 30% in the displayed range. The horizontal bars at the bottom of the diagram represent the position of the reported secondary structural elements of haloalkane dehalogenase (13) and bromoperoxidase (34). SEH, rat soluble epoxide hydrolase (17); mEH, rat microsomal epoxide hydrolase (37), SEH, soluble epoxide hydrolase from Arabidopsis thaliana (33); pEH, soluble epoxide hydrolase from Solanum tuberosum (32); HALO, haloalkane dehalogenase from Xanthobacter autotrophicus GP10 (7); BPH-RP, human biphenyl hydrolase-related protein (38); BPH, biphenyl hydrolase from P. putida (50); BPA2, 2-hydroxyxynamic semi-aldehyde hydrolase from P. putida (40); XYL2, 2-hydroxyxynamic semi-aldehyde hydrolase from P. putida (41); BPA2, bromoperoxidase A2 from S. aureofaciens (34); Con, consensus sequence; N, nucleophile; H, water-activating histidine; A, acidic residue of the charge relay system.

*Oligonucleotides used for the introduction of specific mutations.*

Note that sequence identity is never below 30% in the displayed range. The horizontal bars at the bottom of the alignment represent the position of the reported secondary structural elements of haloalkane dehalogenase (13) and bromoperoxidase (34). SEH, rat soluble epoxide hydrolase (17); mEH, rat microsomal epoxide hydrolase (37), SEH, soluble epoxide hydrolase from Arabidopsis thaliana (33); pEH, soluble epoxide hydrolase from Solanum tuberosum (32); HALO, haloalkane dehalogenase from Xanthobacter autotrophicus GP10 (7); BPH-RP, human biphenyl hydrolase-related protein (38); BPH, biphenyl hydrolase from P. putida (50); BPA2, 2-hydroxyxynamic semi-aldehyde hydrolase from P. putida (40); XYL2, 2-hydroxyxynamic semi-aldehyde hydrolase from P. putida (41); BPA2, bromoperoxidase A2 from S. aureofaciens (34); Con, consensus sequence; N, nucleophile; H, water-activating histidine; A, acidic residue of the charge relay system.
mixed by vortexing. Phase separation was initiated by the addition of 1.5 ml of distilled water and thorough mixing. After centrifugation at 1,500 \( \times g \) for 5 min most of the upper aqueous phase was removed without touching the protein precipitate at the interphase. After the addition of 1.5 ml methanol, mixing, and centrifugation at 1,500 \( \times g \) for 5 min the supernatant was removed, and the remaining pellet was washed twice with 1 ml of ice-cold acetone and dried in a desiccator under vacuum for 10 min. To the dry pellet, 100 \( \mu l \) of distilled water were added and maceration was allowed for 30 min at room temperature. Thereafter, the precipitate was completely dissolved in 500 \( \mu l \) of Protosol (DuPont NEN) by incubation in a water bath for several hours. The complete mixture was added to 7 ml of Rotiszint 2200 (Roth), and analysis was performed in a Packard Tri-Carb 300C liquid scintillation counter.

RESULTS

Construction and Expression of the sEH Mutants—By introducing site-specific mutations into polymerase chain reaction we obtained 21 different recombinants (see Fig. 5), each with the single mutation being located in the desired position as directed by the respective mutation primer. In addition, a number of mutants were isolated that displayed additional sequence alterations including double mutations and frameshifts, due to the notorious error rate within in vitro amplified DNA sequences. Furthermore, we isolated a clone with the MscI fragment in the opposite orientation as compared with the wild type. The inversion of this part of the sequence retains the open reading frame but leads to substitution of the amino acid sequence of the putative \( \alpha \)-helix C and \( \beta \)-strand 6 (Fig. 1) of sEH, including the catalytic nucleophile Asp\(^{333} \), as predicted from the sequence comparison to haloalkane dehalogenase.

The expression of the different sEH constructs in E. coli J M109 resulted in the massive production of respective fusion proteins in the bacteria. The non-EH part of the recombinant proteins was a vector-derived N-terminal peptide of 41 amino acids, containing a (His)\(_{6}\)-tag to facilitate purification of the recombinant proteins by immobilized metal affinity chromatography. Immunoblot analysis of the expression products in the bacterial 13,000 \( \times g \) supernatants using purified native rat sEH as a standard revealed the accumulation of immunoreactive protein with an apparent molecular mass of 67 kDa, which corresponds to the expected increase in molecular mass of about 5 kDa as compared with the native enzyme. The amount of soluble recombinant protein obtained was remarkably con-
sEHs—We employed two different procedures in order to determine whether the loss of enzymatic activity observed with a variety of mutants was due to the direct role of the exchanged amino acid in the catalytic process or if an impaired protein folding was the primary cause for the impaired function. Based on the fact that the catalysis by sEH proceeds in two steps, with the intermediate formation of a covalent bond between sEH and the substrate (14), we concluded that the exchange of amino acid residues important for the second step should result in the accumulation of the enzyme-substrate ester during incubation of the mutant enzymes with radioactive labeled substrate and trapped the respective intermediates by methanol/chloroform precipitation. The results obtained with mutants that previously displayed strongly impaired enzymatic activity are shown in Fig. 6, in comparison with results obtained with the wild type (WT) and the mutant with the inverted MscI fragment that was intended to serve as a negative control (NC). The substrate binding observed with the mutants D333G, H517Y/C521Y, H523D, W540L, and W540S did not exceed the binding obtained with the negative control. In several independent experiments the mutant D333C precipitated with slightly more radioactive material bound to it as compared with the negative control, yet this trend failed to be statistically significant (p > 0.05 in Student’s t test). All other mutants displayed significant substrate binding (p < 0.01) in the range of 40–115% of that observed with the wild type.

Based on sequence alignments (1, 9, 11) (Fig. 3), Asp333 is predicted to be the nucleophile that is directly involved in the ester formation between the enzyme and the substrate during the first step of the enzymatic reaction (Fig. 2). Therefore, a mutant enzyme with an exchange of Asp333 against a nonacidic amino acid may not be expected to covalently bind the substrate. To prove the structural integrity of the Asp333 mutants we tested their capability to bind to benzyl thio-Sepharose, the affinity matrix used for the specific retention of sEH in a one-step purification procedure (26). Both the D333G and the D333C mutants were specifically retained by the affinity matrix and eluted like the wild type enzyme when the washing buffer (75 mM Tris–Cl, pH 7.4) was fortified with 0.5 mM 4-fluorochalcone oxide, a potent competitive inhibitor of sEH, while the mutant with the inverted MscI fragment did not bind to benzyl thio-Sepharose. However, while the Gly mutant behaved indistinguishably from the wild type, significantly less protein of the Cys mutant was retained by the affinity material in parallel experiments.

Kinetic Properties of sEH Mutants—The W334F mutant had retained surprisingly much of the sEH activity, despite the indication for an important function of Trp334 in the catalytic process as suggested by its high degree of conservation among epoxide hydrolases and haloalkane dehalogenase. We therefore investigated whether the kinetic properties of the mutant deviated significantly from those of the wild type. Fig. 7A shows the Lineweaver-Burk plot of the mutant W334F as compared with the wild type. The mutation resulted in an increase of the $K_m$ from 8 µM to 56 µM and also an increase in the $V_{max}$ from 340 to 650 nmol/mg/min. The same analysis was performed with the mutants H332Q and W540L in order to decide whether the strong decrease in enzymatic activity observed with them was due to a change in their substrate affinity (Fig. 7B). However, the $K_m$ deviated only slightly from that of the
FIG. 7. Kinetic analysis of selected sEH mutants. Lineweaver-Burke representation of the substrate dependence of the catalytic activity of the sEH mutants W334F (A), H332Q (B), and W540L (B), in comparison with that of the wild type recombinant enzyme (A). Note the different scales in A and B. WT, wild type recombinant sEH.

wild type (8.5 μM for H332Q, 6 μM for W540L), while the low activity obtained in the previous analysis was confirmed by a V_max of 17 nmol/mg/min for H332Q and of 15 nmol/mg/min for W540L.

DISCUSSION

The recent advances in molecular cloning of soluble epoxide hydrolases from several different mammalian (17, 29–31) and plant (32, 33) species have significantly enhanced the understanding of the relationship between the different epoxide hydrolases and other proteins with structural similarity. The similarity of the C-terminal part of sEH and mEH to the bacterial haloalkane dehalogenase led us to postulate that EH as well as a number of other enzymes that share this sequence similarity belong to the α/β hydrolase fold family of enzymes (9). Interestingly, the three-dimensional structure for one of these other enzymes, namely the bromoperoxidase A2 from Streptomyces aureofaciens, has recently been elucidated and confirmed the assignment of the enzyme to the α/β hydrolase fold family that we had previously predicted (34).

By similarity analysis (1, 9–11), Asp333 could be almost unequivocally identified as the nucleophile necessary for the first step of the sEH-mediated epoxide hydrolysis. The role of His523 for the water activation during the second step of catalysis was strongly suggested by the exceptionally high conservation between the amino acid sequence around His523 in sEH and the corresponding region in the hydroxyxymuconic semialdehyde hydrolases from Pseudomonas putida and, to a somewhat lesser extent, the haloalkane dehalogenase (9). The prediction of the third member of the potential catalytic triad by sequence comparison was more ambiguous because of a lower degree of sequence conservation around the respective acidic residue. However, two independent reports both came to the conclusion that Asp495 was the best candidate for this catalytic residue (1, 11).

Stil obscure is the function of the N-terminal sEH sequence that is 230 residues in length and shows significant similarity to bacterial haloacid dehalogenases (1, 35). In the present study, we investigated the catalytic properties of mutants that were specifically designed to prove or disprove the role of the above named amino acid residues in the catalytic process. In addition to the identified candidate residues we exchanged additional amino acids that were possible alternatives for the former, although of much lower probability. Of the five acidic residues we exchanged individually, three turned out to be without major importance for the catalytic activity, namely Glu433, Asp434, and Glu493, while substitution of the two amino acids predicted to be part of the catalytic triad, Asp495 and Asp496, resulted in completely inactive mutants.

Covalent substrate binding in the case of Asp495His and the capability to bind to the affinity purification matrix benzyl thio-Sepharose in the case of Asp333Gly and Asp333Cys demonstrated that the loss of enzymatic activity was not due to an improper folding of the proteins but must be explained by the direct involvement of the two mutated residues in the catalytic mechanism. Three histidines were substituted: the major candidate His523, His517 as the one being most proximal to it, and His332 that directly precedes the catalytic nucleophile Asp333.

The three mutants with an exchanged His523 were all enzymatically inactive, and two of them, H523N and H523Y, were able to covalently bind the substrate trans-stilbene oxide. The third mutant, H523D, did not coprecipitate any substrate, most likely due to a loss of structural integrity. This is readily explained by the potential electrostatic repulsion taking place by replacing the basic histidine, that is sandwiched in the catalytic triad between two aspartic acids, with a third acidic residue. While the mutant H517D retained about 50% of the enzymatic activity as compared with the wild type, two His517 double mutants were enzymatically inactive. The one with an additional C521Y mutation could not covalently bind the substrate, while the other one with the additional mutation H523N did. These findings were perfectly in line with our expectations, because complete loss of activity was only associated with retained structure, as judged by substrate binding, in the case of a His523 mutation. The third histidine exchange, H332Q, resulted in a strongly impaired yet detectable enzymatic activity of the resulting mutant. Neither the K_m nor the ability to covalently bind the substrate was impaired for the mutant as compared with the wild type, which indicates that the second step of the enzymatic reaction, i.e. the ester hydrolysis, must be affected by the exchange. Trp334, the other amino acid residue flanking the nucleophile of the catalytic triad, is highly conserved among the epoxide hydrolases and the haloalkane dehalogenase. In the latter enzyme, a role of this tryptophan in hydrogen bonding the chloride released from 1,2-dichloroethane by the haloalkane dehalogenase has been reported, and we have hypothesized that this tryptophan might, in analogy, support the opening of the epoxide ring by sEH via hydrogen bonding. 
bonding the oxirane oxygen. However, exchange of Trp\textsuperscript{340} to
phenylalanine did not reduce the enzymatic activity but enhanced \( K_m \) by a factor of 7 and \( V_{\text{max}} \) by a factor of 2. A similar result was recently reported for the analogous mutation of halofantrine dehalogenase (36). We conclude that Trp\textsuperscript{340} is not
mechanistically involved in the catalytic process of epoxide hydrolysis but is of some importance for the binding of the substrate.

Our first attempt to align the amino acid sequences of mEH and seE suggested a small motif near the carboxyl termini of both enzymes, namely the tetrapeptide Lys-Trp-Leu-Lys/Lys-Trp-Val-Lys corresponding to positions 539–542/411–414 of seE/mEH, respectively.\textsuperscript{3} Thus, Lys\textsuperscript{539}, Trp\textsuperscript{540}, and Lys\textsuperscript{542} were the first positions where we constructed mutants. Exchange of either of the lysine residues against arginine or methionine did not affect the enzymatic activity to a great extent. On the other hand, the mutation of the tryptophan had a strong effect. Exchange against leucine or serine strongly reduced the enzymatic activity, and introducing a stop codon at this position completely abolished the catalytic activity. In contrast, a frame shift mutation leading to a different amino acid sequence from position 545 on had no appreciable effect on the enzymatic activity. Neither of the Trp\textsuperscript{540} mutants displayed a detectable extent of covalent substrate binding. On the other hand, the apparent \( K_m \) had decreased rather than increased with the mutant W540L. Sequence alignment (Fig. 3) offers a possible explanation for these observations. Trp\textsuperscript{540} is amino acid number 17 C-terminal to the catalytic histidine. At the corresponding position, all other sequences of the alignment have a highly conserved phenylalanine. This position is at the end of the last \( \alpha \)-helix of the two sequences of known three-dimensional structure and is oriented toward the \( \beta \)-sheet. The behavior of the Trp mutants is consistent with a role of the conserved aromatic amino acid residue in fixing helix F with the conserved catalytic histidine His\textsuperscript{523} close to its N terminus (see Fig. 3) in a position to retain the proper conformation of the active center. A fraction of 5% of the mutant proteins being in the active conformation would not have been picked up by our substrate binding assay, due to the relatively low sensitivity of this analytical procedure.

In conclusion, our findings demonstrate that (i) the catalytic triad of rat seE is composed of the residues Asp\textsuperscript{133} (the nucleophile), Asp\textsuperscript{495} (the histidine-supporting acid), and His\textsuperscript{523} (the water-activating histidine) (ii) the amino acid residues flanking the nucleophile significantly influence kinetic parameters of the enzymatic reaction, and (iii) Trp\textsuperscript{540} has an important function in stabilizing the structure of the enzyme.

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