Biochemical Evidence for the Involvement of Tyrosine in Epoxide Activation during the Catalytic Cycle of Epoxide Hydrolase*

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Epoxide hydrolases (EH) catalyze the hydrolysis of epoxides and arene oxides to their corresponding diols. The crystal structure of murine soluble EH suggests that Tyr^{465} and Tyr^{381} act as acid catalysts, activating the epoxide ring and facilitating the formation of a covalent intermediate between the epoxide and the enzyme. To explore the role of these two residues, mutant enzymes were produced and the mechanism of action was analyzed. Enzyme assays on a series of substrates confirm that both Tyr^{465} and Tyr^{381} are required for full catalytic activity. The kinetics of chalcone oxide hydrolysis show that mutation of Tyr^{465} and Tyr^{381} decreases the rate of binding and the formation of an intermediate, suggesting that both tyrosines polarize the epoxide moiety to facilitate ring opening. These two tyrosines are, however, not implicated in the hydrolysis of the covalent intermediate. Sequence comparisons showed that Tyr^{465} is conserved in microsomal EHs. The substitution of analogous Tyr^{374} with phenylalanine in the human microsomal EH dramatically decreases the rate of hydrolysis of cis-stilbene oxide. These results suggest that these tyrosines perform a significant mechanistic role in the substrate activation by EHs.

Epoxide hydrolases (EH, EC3.3.2.3) hydrolyze epoxides and arene oxides to their corresponding diols (1). These enzymes are widely distributed among many species, including bacteria, fungi, plants, insects, and mammals (2–5). In mammals, there are two major classes of EH with broad and complementary substrate selectivity, soluble EH (sEH) and microsomal EH (mEH) (6). sEH participates not only in xenobiotic detoxification but also endogenous lipid metabolism, acting on epoxides of linoleic acid (leukotxin and isoleukotxin) (7) and arachidonic acid (cis-epoxycisatrienoic acid) (8). Elevated titers of linoleate and arachidonic acid diols are, respectively, thought to be associated with the inflammatory disorder known as acute respiratory distress syndrome (7) and pregnancy-induced hypertension (9). Inhibition of epoxide hydration may accordingly have a therapeutic value for these two serious disorders. Alternately, mEH appears to be mainly involved in the metabolism of xenobiotic epoxides (6). A protein-reactive and cytotoxic epoxide, naphthalene epoxide for example, is converted to the less toxic diol by this enzyme (10). The mEH is also related to activation of other arene oxides such as 7,12-dimethylbenzanthracene, a member of the polycyclic aromatic hydrocarbon class of chemical carcinogens (11). To understand xenobiotic toxicity, metabolic aberrations associated with pathological disorders, and to develop possible therapies against these, it is important to elucidate the molecular basis of EH catalysis.

The EHs belong to the a/β hydrolase fold family. These enzymes characteristically employ a two-step mechanism in which a catalytic nucleophile of the enzymes attacks a polarized electrophilic substrate, and the covalent intermediate is subsequently hydrolyzed (Fig. 1) (6). The mechanism of murine sEH has been elucidated from a series of experiments utilizing heavy isotopes, protein mass spectrometry, and site-directed mutagenesis (12, 13). They indicated that Asp^{333} acts as a catalytic nucleophile and that a water molecule is activated by the nearby His^{525} and Asp^{396} pair (Fig. 1). This mechanism was extended to other EHs (2, 14–18). However, one or more additional amino acids are likely involved in the catalytic cycle especially in the activation of the epoxide ring (5), based on the mechanism of the haloalkane dehalogenase, HLD1, from Xanthobacter autotrophicus G10, a related a/β hydrolase (6). Kinetics of the hydrolysis of chalcone oxide by sEH support this hypothesis. Thus a generalized scheme is postulated in which one or more amino acid(s) may polarize the epoxide oxygen by an acid-like mechanism, weakening the C-O epoxide bond and facilitating the attack on the carbon of the epoxide ring by a nucleophile, such as the conjugate base, Asp^{333} (19). Recently, the crystal structure of murine sEH has been determined at 2.8 Å resolution (20). The structure supports the previously proposed mechanism and suggests that Tyr^{465} and Tyr^{381} are the possible acid catalysts that activate the epoxide ring (Fig. 1). Additionally, analysis of the crystal structure of EH from Agrobacterium radiobacter, AD1, likewise suggested that Tyr^{392}-Tyr^{315} in this prokaryotic enzyme may have the same function (21). Thus, biochemical evidence is required to verify the mechanism of epoxide activation and to complement structure-based prediction.

In this study, site-directed mutagenesis was utilized to explore the role of Tyr^{465} and Tyr^{381} in the catalytic mechanism...
of murine sEH. The sEH variants were subjected to a series of enzyme assays and kinetic studies to assess their impact on epoxide activation. Additionally, this work was extended to Tyr774 of human mEH. Interpretation in view of the EH crystal structure, these results clearly support the role of active site tyrosine residues in epoxide activation by eukaryotic sEH and mEH.

EXPERIMENTAL PROCEDURES

Chemicals—The following compounds were previously synthesized in our laboratory: racemic 4-nitrophenol-2,3-epoxy-9-phenylpropyl carbonate (NEP2C, compound 1) (22); [9H]trans-1,3-diphenylpropane oxide (tDPOO, compound 2); [14C]cis-9,10-epoxyoctane acid (ESA, compound 3); [3H]trans-stilbene oxide (ISO, compound 4) (23); [3H]cis-stilbene oxide (cSO, compound 5) (24); para-substituted chalcone oxides (compounds 6-11) (19); compounds 13 and 15 (25) and compound 14 (26). [3H]Juvenile hormone III (JH III, compound 5) was obtained from NEN Life Science Products. Compound 12 was purchased from Aldrich.

Construction of Mutant Murine sEH—Sequence numbers are based on the murine sEH sequence (GenBank TM/EMBL Data Bank accession number L05781) and human mEH sequence (X07936). Murine sEH cDNA or human mEH cDNA (27) was inserted into the site of baculovirus expression vector, pFastBac1 (Life Technologies, Inc.). Cells from High5 (500 ml, 5 x 10^5 cells/ml) were infected with virus solution at a multiplicity of infection of 0.1. Three days postinfection, the cells were resuspended in 20 ml of 0.1 M sodium phosphate buffer (pH 7.4) (buffer A) containing 1 mM phenylmethylsulfonyl fluoride, EDTA, and dithiothreitol, and homogenized with a Polytron. The crude extract was centrifuged at 100,000 g for 1 h. For murine sEH, the resulting supernatant (cytosol fraction) was stored at 80 °C.

Protein Analysis—Protein concentrations were determined with the Pierce BCA assay (Pierce) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (28) using a 10% resolving gel. Assays for murine sEH, employing Western blot techniques, were performed using the correspond-

Catalytic Mechanism of Epoxide Hydrolase
The nonlinear regression of the initial rate of EC formation (\( \rho \)) versus inhibitor concentration ([I]) permits the calculation of \( K_d \) and \( k_{\text{cat}} \) (19). The slope of the EC complex decomposition, \( \ln(A_0 - A) \) versus time, permits the calculation of \( k_3 \) (19). Derived kinetic constants are listed in Table II.

Both mutations of Tyr^{465} and of Tyr^{381} by phenylalanine resulted in increases in the \( K_d \) values up to 50-fold, with the exception of compound 7 and the Tyr^{381} mutant (Table II). These results demonstrate that both tyrosines are implicated in the binding of the substrate. The change in \( K_d \) is well correlated (\( r^2 = 0.91 \)) with the \( \sigma \) values of the para-substitution of chalcone oxides for Y465F, whereas no relationships were found for Y381F. The observed increased \( K_d \) in mutant enzymes reflect the smaller interaction between the enzyme and the inhibitor. The linear relation observed between \( K_d \) and \( \sigma \) for Y465F highlights the possible bond between Tyr^{465} and epoxide moiety, as stated (26).

The rate of alkylation (\( k_d \)) is also greatly altered in both mutations (Table II). The values of \( k_d \) for Y465F decreased increasingly from 2- to 30-fold for compounds 11 to 6, respectively. For Y381F such trends in the decreased \( k_d \) were observed only from compound 9 to 6, whereas compounds 11 to 9 showed a 7-fold decrease in their \( k_d \) values. The Hammett plot (Fig. 3) shows the logarithm of the relative rates (\( k_{\text{cat}}^\text{mut} / k_{\text{cat}}^\text{wt} \)) versus the constant \( \sigma^+ \) for each 4-position substituent illustrates this influence. These results were correlated with \( \sigma^+ \) for the three enzymes, indicating that development of a relative positive charge at the reactant center is important for the alkylation step. For the wild-type enzyme, a linear relationship (\( r^2 = 0.93 \)) with a slope of \(-0.56\) was obtained, indicating a push-pull mechanism, in which the epoxide oxygen is activated by protonation facilitating a nucleophilic attack on the carbon of the epoxide ring by the Asp^{383} carboxylate anion (19). A linear relationship (\( r^2 = 0.91 \)) was obtained for Y465F, indicating a consistent mechanism operating throughout the chalcone oxide series. However, the sign of the slope (\(+0.27\)) is inverted from the slope of the of the wild-type enzyme, indicating a different mechanism. This value is very close to the value (\(+0.32\)) found for a general basic mechanism of opening of similar epoxides (36). This result strongly suggests that a simple nucleophilic mechanism is implied in the action of Y465F and that Tyr^{465} is directly related to the polarization of the epoxide moiety. A bell shaped relationship was obtained for Y381F, indicating a change of mechanism operating throughout the chalcone oxide series. A slope (\(+0.26\)) similar to one of Y465F is observed for the electron donating para-substitutions (6 to 9), whereas a slope (\(-0.51\)) similar to the one for wild type is obtained for electron withdrawing para-substitutions (9 to 11). Therefore, Tyr^{381} participates in the polarization of the epoxide moiety. However, its role is less clear than that of Tyr^{465}.

The rate of dealkylation \( k_3 \) was less influence by either of the tyrosine mutations; the decreased \( k_3 \) between 1- and 3-fold are observed for Y465F and Y381F compared with the wild type.

RESULTS

Expression and Activities of Tyrosine Mutants of Murine sEH—To explore the role of each of the tyrosines of murine sEH, we prepared four different mutant constructs, Y381F, Y465F, Y465A, and Y381F/Y465F. The enzymes were subsequently produced using baculovirus expression system as described under “Experimental Procedures.” In the purification of murine sEH, the ligand (Fig. 2A) interacts with the putative hydrophobic pocket near the active site (12, 34). Wild-type and mutant protein were detected at similar expression level in the cytosol fraction and not detected in the flow through fraction (Fig. 2C) indicating that the mutant enzyme probably maintained its structural integrity. The mutant enzymes were eluted in low yield with 1 mM 4-fluorochalcone oxide (FCO), a selective inhibitor for sEH, indicating probable lower affinity for the inhibitor (Fig. 2, D–E). Total protein eluted by FCO followed by SDS (Fig. 2E) was similar for both wild and mutant enzymes, indicating same overall binding for each.

The specific activities of the wild-type and mutant enzymes in the hydrolysis of five substrates are summarized in Table I. These substrates are classified, and ordered from the more reactive (NEP2C (compound 1)) to the less reactive (JH III (compound 5)). The specific activities of wild-type enzyme were similar to previously published results (19, 23). NEP2C and JH III are known to be hydrolyzed by esterases; however, the esterase-dependent hydrolysis was not detectable in these experiments (data not shown). In mutant enzymes, the activities were lower than those of the wild-type. Substrates containing less reactive epoxides resulted in larger changes in activity between the wild-type and the mutant enzymes, suggesting that hydrolysis of less reactive epoxides is dependent on activation by tyrosine(s). The Y381F/Y465F mutant demonstrated no detectable catalytic activity with any of the substrates used. These results suggest that both Tyr^{381} and Tyr^{465} are required for full activity of the murine sEH. This is consistent with the results of analogous experiments with bacterial EH showing that active site tyrosines Tyr^{152} and Tyr^{215} are important for catalysis (35).

Kinetics of Inhibition—To further investigate the role of the two residues Tyr^{381} and Tyr^{465} in the catalytic mechanism, we determined the kinetic constants of their inhibition by chalcone oxides. Chalcone oxides are in fact poor EH substrates that inhibit the enzyme by forming a stable covalent intermediate (19). Their action is described by the following equation.

\[
E + I \rightleftharpoons EI \rightarrow EC \rightarrow E + P
\]  

(Eq. 1)

The rate of dealkylation (\( k_3 \)) is also greatly altered in both mutations (Table II). The values of \( k_3 \) for Y465F decreased increasingly from 2- to 30-fold for compounds 11 to 6, respectively. For Y381F such trends in the decreased \( k_3 \) were observed only from compound 9 to 6, whereas compounds 11 to 9 showed a 7-fold decrease in their \( k_3 \) values. The Hammett plot (Fig. 3) shows the logarithm of the relative rates (\( k_{\text{cat}}^\text{mut} / k_{\text{cat}}^\text{wt} \)) versus the constant \( \sigma^+ \) for each 4-position substituent illustrates this influence. These results were correlated with \( \sigma^+ \) for the three enzymes, indicating that development of a relative positive charge at the reactant center is important for the alkylation step. For the wild-type enzyme, a linear relationship (\( r^2 = 0.93 \)) with a slope of \(-0.56\) was obtained, indicating a push-pull mechanism, in which the epoxide oxygen is activated by protonation facilitating a nucleophilic attack on the carbon of the epoxide ring by the Asp^{383} carboxylate anion (19). A linear relationship (\( r^2 = 0.91 \)) was obtained for Y465F, indicating a consistent mechanism operating throughout the chalcone oxide series. However, the sign of the slope (\(+0.27\)) is inverted from the slope of the of the wild-type enzyme, indicating a different mechanism. This value is very close to the value (\(+0.32\)) found for a general basic mechanism of opening of similar epoxides (36). This result strongly suggests that a simple nucleophilic mechanism is implied in the action of Y465F and that Tyr^{465} is directly related to the polarization of the epoxide moiety. A bell shaped relationship was obtained for Y381F, indicating a change of mechanism operating throughout the chalcone oxide series. A slope (\(+0.26\)) similar to one of Y465F is observed for the electron donating para-substitutions (6 to 9), whereas a slope (\(-0.51\)) similar to the one for wild type is obtained for electron withdrawing para-substitutions (9 to 11). Therefore, Tyr^{381} participates in the polarization of the epoxide moiety. However, its role is less clear than that of Tyr^{465}.

The rate of dealkylation \( k_3 \) was less influence by either of the tyrosine mutations; the decreased \( k_3 \) between 1- and 3-fold are observed for Y465F and Y381F compared with the wild type.
Moreover, no relation was found between the intensity of the change in $k_3$ and the nature of the para-substitution for Y465F and Y381F. These results indicate that neither tyrosine directly influences the hydrolysis of the enzyme-inhibitor covalent intermediate. This agrees with prediction from the crystal structure (20). Additionally, changes in specific activities for compounds 1-5 probably came from the changes in $K_d$ and $k_2$, because $k_3$ was unchanged for compounds 6-11.

Inhibition of Tyrosine Mutants by Ureas and Carbamate—

The crystal structure of sEH-$N$-cyclohexyl-$N_9$-decyl urea (compound 14) complex shows that Tyr 465 and Tyr 381 provide hydrogen bond interaction with the carbonyl group of the urea (26). To evaluate this apparent binding trend with the tyrosines, IC$_{50}$ values were determined for wild type and for both tyrosine mutants (Table III). Substitution of Tyr 381 by phenylalanine resulted in an enzyme with 8–88-fold higher IC$_{50}$ values for compounds tested. Replacement of Tyr 465 by phenylalanine increased IC$_{50}$ by 2–13-fold. These results suggest that both Tyr 381 and Tyr 465 interact with these inhibitors. Particularly, Tyr 381 seems more important than Tyr 465 for binding with compounds 13 and 14. Tyr 465 may contribute less to the free energy of binding for these inhibitors than Tyr 381.

**Mutagenesis of Tyr$^{774}$ in Human mEH—** Although human mEH has 21% sequence identity with murine sEH (5), the catalytic triad (Asp$^{226}$, Glu$^{404}$, and His$^{431}$) is conserved when compared with sEH (6). Sequence alignment shows that Tyr$^{774}$ in human mEH seems to be analogous to Tyr$^{465}$ in murine sEH (2). The tyrosine is conserved in all sequenced mEHs (20, 21). As shown in Fig. 4A, the linear distance between these amino acids is similar in the primary structures of murine sEH and human mEH. Tyr$^{283}$ or Tyr$^{293}$ in human mEH could correspond to Tyr$^{381}$ in murine sEH. However, the tyrosines are not conserved in all mEHs (20, 21). Thus only the Tyr$^{774}$ mutant was made to explore the role of the residue in this enzyme. As shown in Fig. 4B, wild-type and Y374F enzymes were expressed in the microsomal fraction at similar levels. Specific activity was determined for the hydrolysis of cis-stilbene oxide (compound 16), which is selective for mEH. Compared with wild-type enzyme, the Y374F mutant had dramatically decreased activity (Fig. 4D). The recent crystal structure of EH from Aspergillus niger, a soluble member of mEH, showed that Tyr$^{314}$ likely plays a significant role in epoxide activation (37). The authors suggested that Tyr$^{314}$ appears to be equivalent to Tyr$^{374}$ in human mEH based on the sequence comparison between these two enzymes. Taken together, mutation of Tyr$^{374}$ likely affects polarization of epoxide moiety rather than some other step in the catalytic cycle. This result suggests that involvement of tyrosine in the catalytic mechanism is common in both sEH and mEH.

## DISCUSSION

In the present study, we examined the role of the tyrosines in the catalytic mechanism of EH. It had been proposed that the epoxide oxygen might be activated by a possible acid catalyst to
wild-type enzyme (34). Y381F/Y465F was eluted by 1 mM FCO for the enzymes. Such an effect also was observed for thehowever, the decreased solubility overcame increased affinity23086

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Tyr465 and Tyr381 are implicated in the binding of substratelargely from a loss of the hydroxyl group(s) in the active site.

decreased specific activities in the mutants (Table I) resultmational changes in the active site. We hypothesize that thedata suggest that the mutations do not cause dramatic confor-
mimics the transition state for epoxide ring opening in sEH

weaken the C-O bond of the epoxide ring and facilitate theattack by a nucleophilic aspartate (6). However, a varietyof early studies showed that the reaction intermediate lacked full
carbocation character (reviewed in Ref. 6). Based on the crystalstructure of murine sEH (20, 26), we tested the hypothesis that Tyr465 and Tyr381 are involved in epoxide activation by examiningthe mutant enzymes.

The substitution of tyrosine with phenylalanine is notexpected to create a significant conformational change, even lo-

cally, because the body of the tyrosine and phenylalanine areboth partially buried and unchanged, whereas the changedregion, the phenolic hydroxyl group, is in the solvent accessibleregion of the active site. We tested the hypothesis by showing thatall mutant enzymes bind to the active site-directed affinitycolumn with similar efficiency. This would not be expected ifmutation caused a major alteration in the conformation of theenzyme. Our results are consistent with the prediction. The sEHvariants bound on the affinity column were eluted by 1 mMFCO with lower recovery than the wild type. Because theaffinity ligand is thought to interact with the putative hydro-


distances of these two tyrosines are suitable to activateepoxide oxygen in concert with nucleophilic attack by Asp333.

The kinetics of hydrolysis of chalcone oxides suggests bothTyr465 and Tyr381 are implicated in the binding of substrateand in the formation of a hydroxyalkyl intermediate. Particu-

larly, Tyr465 showed a linear relationship in decreased Kd andk2 with para-substitution of chalcone oxides, indicating theamino acid directly polarizing epoxide moiety. In the complexof sEH and the potent urea inhibitor, N-cyclohexyl-N'-decyl urea, the carbonyl oxygen accepts the hydrogen bond from Tyr465 and Tyr381, and one of the NH groups accepts the hydrogen bond from Asp333. The side chain Gln382 additionally participates in a hydrogen bond interaction with the carbonyl oxygen of the urea (Fig. 5A). The binding nature suggests that the inhibitor mimics the transition state for epoxide ring opening in sEHcatalysis (26). Our results are consistent with this structure-
based prediction. Both mutations of the two tyrosines increasedIC50 values for ureas tested (Table III). Because the epoxidesubstrates are susceptible to an S_{N}2 backside attack on thecarbon of epoxide ring by nucleophilic Asp333 (13), the angleand distance of these two tyrosines are suitable to activateepoxide oxygen in concert with nucleophilic attack by Asp333.

Analysis of small molecule crystal structures contained in the Cambridge Structural Data base (38) yields 15 independentexamples of hydrogen bonds donated from hydroxyl groups toepoxide oxgens with an average O-O separation of 2.8 Å. Thescatter plot in Fig. 5B reveals that the hydrogen bond stereo-

chemistry between the phenolic hydroxyl groups of Tyr465,
Tyr\textsuperscript{381}, and the substrate epoxide oxygen modeled into the sEH active site (26) is quite consistent with the preferred stereochemistry of such interactions reflected by the examples from the Cambridge Structural Data base. Epoxide-phenol hydrogen bond stereochemistry is also consistent with the preferred stereochemistry of hydrogen bonds to tyrosines in refined protein structures as outlined by Ippolito et al. (39). In the crystal structure of murine sEH, Tyr\textsuperscript{465} is flanked by edge to face interactions with Trp\textsuperscript{334} and Phe\textsuperscript{385} (Fig. 5C). The interaction is thought to stabilize phenolate anion in the transition state (20). Similar interaction is found in the recent crystal structures of bacterial EH and fungus EH (21, 37). Our biochemical results highlight the significance of Tyr\textsuperscript{465} in the epoxide activation.

Our preliminary results suggest that Tyr\textsuperscript{374} in human mEH is analogous to Tyr\textsuperscript{465} in murine sEH. The tyrosine is absolutely conserved in all EHs sequenced (20, 21). Although it is not known if Tyr\textsuperscript{374} forms edge to face interaction with other aromatic amino acids, sequence alignment shows that Trp\textsuperscript{227} and Phe\textsuperscript{534} in human mEH likely correspond to Trp\textsuperscript{334} and Phe\textsuperscript{385} in murine sEH. Additional experiments will be required to address the question if a corresponding second tyrosine exists for mEH. Tyr\textsuperscript{291} and Tyr\textsuperscript{381} in human mEH may be equivalent to Tyr\textsuperscript{381} in murine sEH on the basis of distance comparison (Fig. 4A). However, both tyrosines are not conserved in all mEHs (20, 21). Tyr\textsuperscript{291} was suggested to be a possible second tyrosine in human mEH, based on the sequence comparison with fungus sEH. This fungus sEH belongs to the same class as mammalian mEH, although the sequence relationships were weak (37). There is another possibility that mEH may have only one tyrosine for activation. Genetic relationships suggest that mEH and sEH likely have diverged from common ancestor (5). Both enzymes are distantly related and have the same major amino acid side chain involved in hydrating epoxides. However, they are separated by a vast evolutionary distance (5) and have different substrate preference; sEH has an activity on less reactive epoxides such as arachidonic acid epoxides, and mEH on reactive epoxides such as arene oxides. The sEH is more active on epoxide substrates on linear system as one would anticipate from its active site being a tunnel rather than a groove (20).

EHs are members of the a/b hydrolase fold family-like esterases and haloalkane dehalogenases (5, 40). EHs and esterases have a common mechanism in hydrating substrates but a different nucleophile, aspartate and serine, respectively. The D333S mutant of murine sEH has no activity on epoxides (12). It was hypothesized that the mutant enzyme might acquire esterase activity with serine as a nucleophile. However, it had no hydrolytic activity on several esters.\textsuperscript{2} The ester will be trigonal moving to tetrahedral as a transition state and transient intermediate interacting with NH moiety of two glycines and one alanine (41, 42). In contrast, the epoxide will be tetrahedral in the transition state interacting with OH moiety of two tyrosines. The absence of esterase activity in D333S can be explained in part by having an incorrect angle of the activating groups. Genetic relationships reveal that haloalkane dehalogenase and the sEH C-terminal catalytic domain are more related and might have been diverged from common ancestor (5). Both enzymes have a common nucleophilic aspartate (6). In haloalkane dehalogenase HLD1, however, two tryptophans (Trp\textsuperscript{125} and Trp\textsuperscript{175}) are responsible for the activation of the halide-leaving group (43). As shown in Fig. 5A, Trp\textsuperscript{284} and Trp\textsuperscript{524} conserved in the EH family are located near the active site in murine sEH. In the past, these two residues were proposed to activate the epoxide ring (14, 15). However, the orientation, angle, and distance are not suitable to activate the substrate epoxide, supporting the previous mutagenesis results of these triptophans (14, 15). In addition, an epoxide likely needs more activation than a halide group does. These data suggest the possibility that EHs have acquired a unique mechanism for substrate activation through evolution. Alternately, lysine was

\textsuperscript{2} B. D. Hammock, unpublished data.
previously postulated to be a proton-donating group (2, 5, 44). This residue is, however, not present in the active site (Fig. 5A).

In conclusion, we demonstrated the involvement of tyrosine in the activation of epoxide in the catalytic cycle of EH. The activation mechanism is apparently conserved within EH family and is likely unique in the αβ hydrolase fold family.

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REFERENCES

1. Oesch, F. (1973) Xenobiotica 3, 305–340
2. Rink, R., Fennema, M., Smidt, M., Dehnel, U., and Janssen, D. (1997) J. Biol. Chem. 272, 14650–14657
3. Arand, M., Hemmer, H., Durk, H., Baratti, J., Archelas, A., Furstoss, R., and Oesch, F. (1999) Biochem. J. 344, 273–280
4. Debernard, S., Morisseau, C., Severson, T. F., Feng, L., Wojtasek, H., Prestwich, G. D., and Hammock, B. D. (1998) Insect Biochem. Mol. Biol. 28, 409–419
5. 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
6. Hammock, B., Grant, D., and Storms, D. (1997) in Comprehensive Toxicology (Sipes, I., McQueen, C., and Gandolfi, A., eds) Vol. 3, pp. 283–305, Pergamon, Oxford
7. Moghaddam, M. F., Grant, D. F., Cheek, J. M., Greene, J. F., Williamson, K. C., and Hammock, B. D. (1995) Anal. Biochem. 230, 176–187
8. Tingle, M., Pirmohamed, M., Templeton, E., Wilson, A., Madden, S., and Hammmock, B. D. (1997) J. Biol. Chem. 272, 2805–2904
10. Tingle, M., Pirmohamed, M., Templeton, E., Wilson, A., Madden, S., Kitteringham, N., and Park, B. (1993) Biochem. Pharmacol. 46, 1529–1538
11. Phillips, D., and Grover, P. (1994) Drug Metab. Rev. 26, 449–467
12. Pinot, F., Grant, D. F., Beetham, J. K., Parker, A. G., Borhan, B., Landt, S., Jones, A. D., and Hammock, B. D. (1995) J. Biol. Chem. 270, 7968–7974
13. Borhan, B., Jones, A. D., Pinot, F., Grant, D. F., Kurth, M. J., and Hammock, B. D. (1995) J. Biol. Chem. 270, 29823–29830
14. Arand, M., Wagner, H., and Oesch, F. (1996) J. Biol. Chem. 271, 4223–4229
15. Laughlin, L. T., Tseng, H. F., Lin, S., and Armstrong, R. N. (1998) Biochemistry 37, 2905–2911
16. Tseng, H. F., Laughlin, L. T., and Armstrong, R. N. (1998) Biochemistry 37, 2905–2911
17. Arand, M., Muller, F., Mecky, A., Hinz, W., Urban, P., Pompon, D., Kellner, R., and Oesch, F. (1999) Biochem. J. 337, 37–43
18. Lacourciere, G. M., and Armstrong, R. N. (1993) J. Am. Chem. Soc. 115, 4664–4670
19. Morisseau, C., Du, G., Newman, J. W., and Hammock, B. D. (1998) Arch. Biochem. Biophys. 356, 214–221
20. Argiriadi, M., Morisseau, C., Hammock, B., and Christianson, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10845–10850
21. Nardini, M., Zou, J., Hallberg, B., and Oesch, F. (1999) J. Biol. Chem. 274, 14579–14586
22. Dietze, E., Kuwano, E., and Hammock, B. D. (1994) Anal. Biochem. 216, 176–187
23. Borhan, B., Mebrahtu, T., Nazarian, S., Kurth, M. J., and Hammock, B. D. (1995) Anal. Biochem. 231, 188–200
24. Gill, S. S., Ota, K., and Hammock, B. D. (1983) Anal. Biochem. 131, 273–282
25. Morisseau, C., Goodrow, M., Dowdy, D. L., Zheng, J., Greene, J., Sanborn, J., and Hammock, B. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8849–8854
26. Argiriadi, M. A., Morisseau, C., Goodrow, M. H., Dowdy, D. L., Hammock, B. D., and Christianson, D. W. (2000) J. Biol. Chem. 275, 15265–15270
27. Grant, D. F., Greene, J. F., Pinot, F., Borhan, B., Moghaddam, M. F., Hammock, B. D., McCutchen, B., Ohkawa, H., Luo, G., and Guenthner, T. M. (1996) Biochem. Pharmacol. 51, 503–515
28. Laennili, U. (1970) Nature 227, 680–685
29. Mumbry, S. M., and Hammock, B. D. (1979) Anal. Biochem. 92, 16–21
30. Beetham, J. K., Tian, T., and Hammock, B. D. (1993) Arch. Biochem. Biophys. 306, 197–201
31. Guex, N. (1996) Experientia 52, 26 (abstr.)
32. Guex, N., and Peitsch, M. C. (1996) Protein Data Bank Quarterly Newsletter 77, 7
33. Guex, N. and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
34. Wixtrom, R. N., Silva, M. H., and Hammock, B. D. (1988) Anal. Biochem. 169, 71–80
35. Rink, R., Spelberg, J. H., Pieters, R. J., Kingma, J., Nardini, M., Kellogg, D. B., and Dijkstra, B. W. (1999) J. Am. Chem. Soc. 121, 7417–7418
36. Blumenstein, J., Ukachukwu, V., Mehan, R., and Whalen, D. (1993) J. Org. Chem. 58, 924–932
37. Oesch, F., Belknap, W. R., Shinozaki, K., and Hammock, B. D. (1995) Anal. Biochem. 230, 176–187
38. Allen, F. H., and Kennard, O. (1993) Chem. Design Automation News 111, 31–37
39. Ipoptito, J. A., Alexander, R. S., and Christianson, D. W. (1990) J. Mol. Biol. 215, 457–471
40. Ollis, D., Cheah, E., Cigler, M., Dijkstra, B., Frolow, F., Franken, S., Harel, M., Remington, S., Silinan, I., Schrag, J., Sussman, J., Verschueren, K., and Goldman, A. (1992) Protein Eng. 5, 197–211
41. Sussman, J., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Siliman, I. (1991) Science 253, 872–879
42. Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B., and Shafferman, A. (1998) J. Biol. Chem. 273, 19505–19517
43. Verschueren, K., Seljee, F., Rozeboom, H., Kalk, K., and Dijkstra, B. (1993) Nature 363, 693–698
44. Bell, P. A., and Kasper, C. B. (1983) J. Biol. Chem. 268, 14011–14017