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DRUG METABOLISM REVIEWS
Vol. 35, No. 4, pp. 365–383, 2003

The Telltale Structures of Epoxide Hydrolases

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ABSTRACT

Traditionally, epoxide hydrolases (EH) have been regarded as xenobiotic-metabolizing enzymes implicated in the detoxification of foreign compounds. They are known to play a key role in the control of potentially genotoxic epoxides that arise during metabolism of many lipophilic compounds. Although this is apparently the main function for the mammalian microsomal epoxide hydrolase (mEH), evidence is now accumulating that the mammalian soluble epoxide hydrolase (sEH), despite its proven role in xenobiotic metabolism, also has a central role in the formation and breakdown of physiological signaling molecules. In addition, a certain class of microbial epoxide hydrolases has recently been identified that is an integral part of a catabolic pathway, allowing the use of specific terpens as sole carbon sources. The recently available x-ray structures of a number of EHs mirror their respective functions: the microbial terpen EH differs in its fold from the canonical α/β hydrolase fold of the

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DOI: 10.1081/DMR-120026498
0360-2532 (Print); 1097-9883 (Online)
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xenobiotic-metabolizing mammalian EHs. It appears that the latter fold is the perfect solution for the efficient detoxification of a large variety of structurally different epoxides by a single enzyme, whereas the smaller microbial EH, which has a particularly high turnover number with its prefered substrate, seems to be the better solution for the hydrolysis of one specific substrate. The structure of the sEH also includes an additional catalytic domain that has recently been shown to possess phosphatase activity. Although the physiological substrate for this second active site has not been identified so far, the majority of known phosphatases are involved in signaling processes, suggesting that the sEH phosphatase domain also has a role in the regulation of physiological functions.

Key Words: Mechanism; Detoxification; Crystallography; Xenobiotic metabolism; Phosphate.

EPOXIDE HYDROLASE FUNCTION

Epoxide hydrolases (EHs) catalyze the hydrolytic scission of oxirane derivatives. Three general functions have been attributed to EH so far (Fig. 1). One important task of EH certainly is the detoxification of potentially genotoxic epoxides. Furthermore, EH have evolved as regulators of physiological functions of certain epoxides that act as signaling molecules. Very recently, EH have also been identified as essential components in microbial catabolic pathways.

Defense Against Genotoxic Epoxides

Epoxide hydrolases were first identified as detoxification enzymes (Oesch, 1973). Epoxides, being three-membered heterocycles that include unfavorable bond angles,

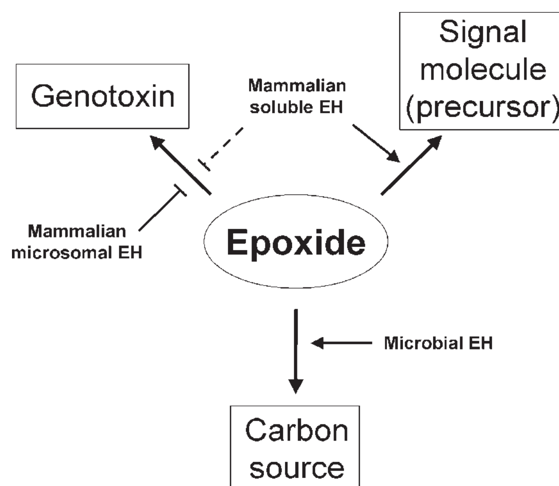

Figure 1. Different functions of epoxide hydrolases.

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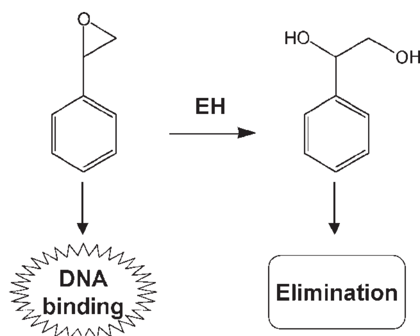

Figure 2. Epoxide hydrolases detoxify genotoxic epoxides.

are highly strained compounds. In combination with the polarization of the C–O bonds in the cycle, this usually leads to a significant electrophilic reactivity, the strength of which is modulated by the substitution pattern of the epoxide in question. Reactive electrophiles are potentially genotoxic because chemical components of nucleic acids, such as the exocyclic amino groups of nucleotides or the N^7 moiety of purines, represent vulnerable targets that can react with those compounds. If not repaired in time, the resulting modifications can alter the base-pairing behavior of the affected residue, giving rise to misincorporation during the next DNA duplication which, after a second round of DNA replication, can result in a permanent mutation. If such a sequence modification occurs in the relevant region of a protooncogene or a tumor suppressor gene, it can trigger the formation of a tumor. Therefore, many epoxides are carcinogens, and their metabolic control by EH is a crucial defense mechanism against chemical carcinogenesis (Fig. 2).

Epoxides are frequent intermediate metabolites in the course of xenobiotic metabolism, formed from arene or alkene derivatives by many different oxygenase isozymes, the most important of these being isozymes of the cytochrome P450-dependent monooxygenase (CYP) superfamily. In contrast, a single mammalian enzyme, the microsomal epoxide hydrolase (mEH), seems to be responsible for the detoxification of most epoxides challenging the organism. The mEH has a rather broad substrate specificity and can metabolize a wide range of very different epoxides (Fig. 3), many of those with—in regard of their structural diversity—surprisingly high apparent affinity. One important restriction, however, is that *trans*-1,2-disubstituted or trisubstituted epoxides are processed very poorly or not at all. In such cases, the soluble epoxide hydrolase (sEH) can sometimes take over. This enzyme prefers slim substrates and seems to preferentially act on lipid epoxides, although a number of xenobiotic-derived compounds, such as *trans*-stilbene oxide or *trans*-ethylstyrene oxide, are also very good substrates. The properties of these two mammalian enzymes and their protective role in chemical carcinogenesis are well described in a number of recent reviews (Arand amd Oesch, 2002; Armstrong and Cassidy, 2000; Fretland and Omiecinski, 2000; Hammock et al., 1997).

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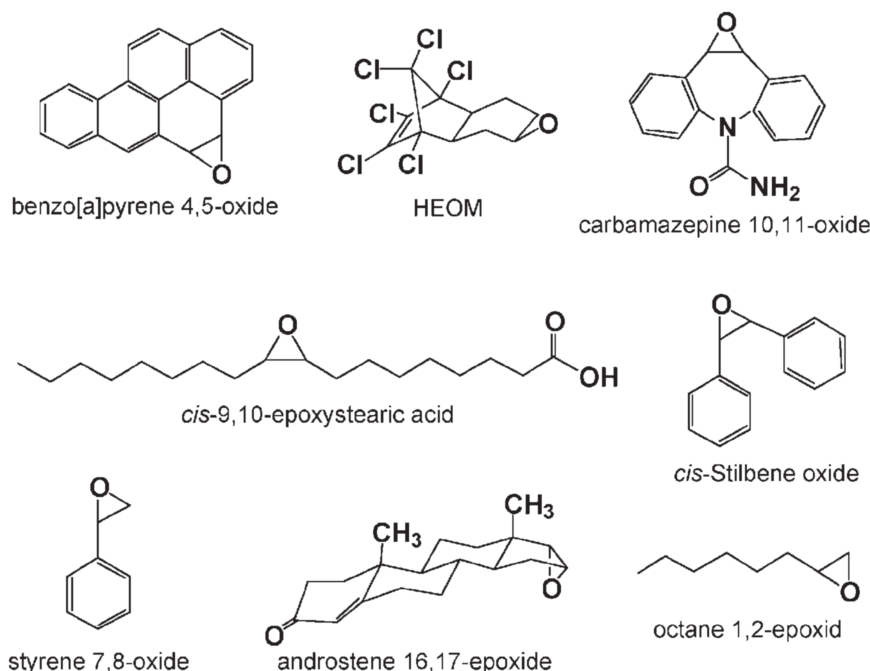

Figure 3. Structural diversity of epoxides hydrolyzed by the mammalian microsomal epoxide hydrolase.

Processing of Signal Molecules

Although sEH seems to be of lesser importance than mEH for the detoxification of xenobiotic epoxides, it has turned out to be a key player in the processing of lipid epoxides that act as signaling molecules or precursors thereof. Hammock and colleagues presented convincing evidence that leukotoxin diol, a fatty acid metabolite generated by sEH, is the mediator of multiple organ failure or acquired respiratory dystress syndrom (ARDS), the fatal pathophysiological reactions frequently observed after severe burns (Moghaddam et al., 1997; Zheng et al., 2001). Furthermore, sEH has been shown to be involved in the breakdown of vasoactive arachidonic acid epoxides (Sinal et al., 2000; Yu et al., 2000). The arachidonic acid epoxides in question act as hyperpolarizing factors and reduce the vascular resistance (Fig. 4). Consequently, enhanced sEH activity leads to increased blood pressure.

Use of Carbon Sources

In 1998, a bacterial epoxide hydrolase of unusually small molecular weight was described (van der Werf et al., 1998); it turned out to be part of a novel bacterial degradation pathway for limonene (van der Werf et al., 1999) (Fig. 5). By rapidly hydrolyzing limonene 1,2-oxide, the first metabolite in this pathway, the enzyme

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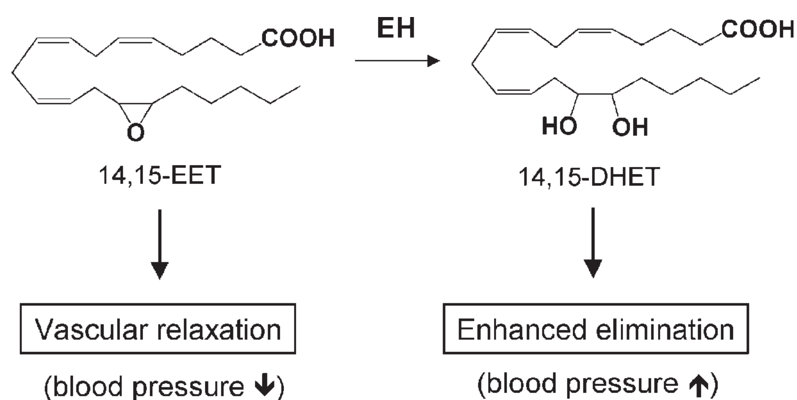

Figure 4. Mammalian soluble epoxide hydrolase participates in blood pressure regulation via hydrolysis of vasoactive arachidonic acid epoxides. EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid.

actually serves two functions. First, it is an essential link in the metabolic chain leading to the complete breakdown of the compound. Second, being orders of magnitude more active than the respective monooxygenase in bacteria grown on limonene, it keeps the level of limonene 1,2-epoxide very low (van der Werf

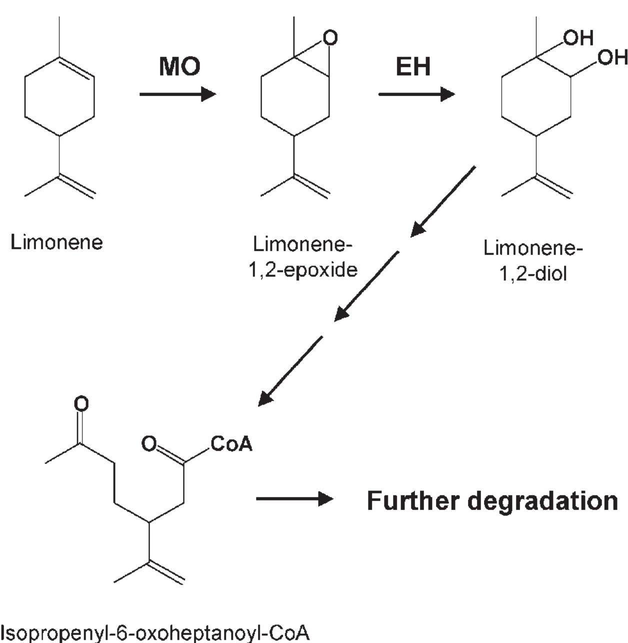

Figure 5. Bacterial limonene epoxide hydrolase is essential for the use of limonene as carbon source.

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et al., 1999), thus minimizing the potential genotoxic damage possibly caused by this metabolite.

EPOXIDE HYDROLASE STRUCTURES

Sequence Similarities Among Epoxide Hydrolases

The primary structure of a mammalian mEH was first determined 20 years ago (Heinemann and Ozols, 1984), yet little information on folding and enzymatic mechanism could be extracted from the data available at that time. Five years later, Janssen and colleagues realized a marginal sequence similarity between mEH and a bacterial haloalkane dehalogenase (HAD) (Janssen et al., 1989), but it took several more years to appreciate the significance of this finding. When the sEH cDNA sequence became available (Arand et al., 1991; Beetham et al., 1993; Grant et al., 1993; Knehr et al., 1993), no similarity of the encoded enzyme to mEH was evident from direct comparison of the sequences. However, both mEH and sEH shared a weak but significant sequence similarity to the bacterial HAD, which led to the conclusion that the three enzymes share the same overall tertiary structure (Arand et al., 1994; Beetham et al., 1995; Lacourciere and Armstrong, 1994; Pries et al., 1994). This was a key observation: the 3D structure of HAD had already been solved (Franken et al., 1991) and its catalytic mechanism had just been elucidated (Verschueren et al., 1993). Thus, its structural similarity to the epoxide hydrolases paved the way for the biochemical analysis of the epoxide hydrolase enzymatic mechanism (see below).

The sequence similarity between mammalian epoxide hydrolases and HAD is represented in Fig. 6. Both sEH and mEH have N-terminal extensions that have neither a counterpart in HAD nor similarities to each other. The N-terminal domain of sEH, on the basis of sequence similarity, has been assigned to the large group of haloacid dehalogenase-like enzymes (Koonin and Tatusov, 1994). Haloacid dehalogenase itself is a bacterial enzyme related to HAD by function but not by structure (Ridder et al., 1997). Thus, the present architecture of mammalian sEH is obviously the result of a gene fusion event (Beetham et al., 1995), the significance of which is not yet understood. Very recently, a phosphatase activity was identified as the enzymatic function of the sEH N-terminal domain (Cronin et al., 2003; Newman et al., 2003). The smaller N-terminal extension of mEH has no match in the databases with respect to sequence similarity. However, we recently cloned an EH from *Aspergillus niger* (Arand et al., 1999a) that displays a similar extension, with the exception that the membrane insertion signal present at the extreme N-terminus of mEH is missing in the fungal enzyme. This is in accord with observations that the latter protein is not membrane-bound (Morisseau et al., 1999).

In addition to the EH proteins with structural similarity to HAD, two other types of epoxide hydrolases have been cloned and sequenced. The first is the leukotriene A_4 (LTA_4) hydrolase (Funk et al., 1987), an enzyme with atypical EH activity. It introduces the hydroxy group at the end of a π electron system composed of three conjugated C–C double bonds. These shift one carbon unit along the chain as the epoxide ring opens. Thus, the reaction product leukotriene B_4 is not a vicinal diol,

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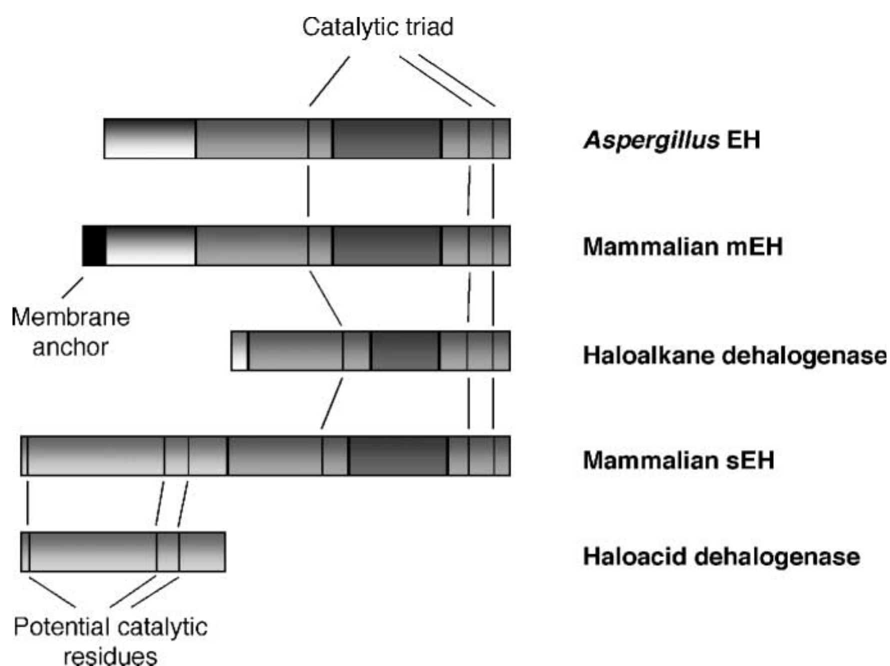

Figure 6. Sequence similarity between epoxide hydrolases and dehalogenases. The primary sequences of the compared enzymes are represented as horizontal bars. Segments building the α/β hydrolase fold are shaded in gray. Within a given sequence, they are interrupted by the segment forming the lid domain (dark gray). The N-termini that vary in size and structure are presented in light gray, with different modes of shading used to indicate the degree of relationship.

like all other EH products known so far; instead, the two hydroxy groups resulting from hydrolysis are eight carbon atoms apart from each other. This functional difference is reflected in the lack of structural similarity to the HAD-related EHs (Arand et al., 1994). A third type of enzyme, the bacterial limonene 1,2-epoxide hydrolase, has also been cloned and sequenced (Barbirato et al., 1998) and lacks any similarities to either HAD-type enzymes or the LTA_4 hydrolase.

Three-Dimensional Structures of Epoxide Hydrolases

The first epoxide hydrolase 3D structure to be published (Nardini et al., 1999) was that of a bacterial EH that shared sequence similarity with the mammalian EHs (Rink et al., 1997), most particularly sEH. It revealed that the catalytic unit of EHs is composed of an α/β hydrolase fold and a lid domain, with a catalytic triad sitting on top of the hydrolase fold underneath the lid (Fig. 7A), as was hypothesized earlier for this type of EH on the basis of the sequence similarity to HAD (Arand et al., 1994; Beetham et al., 1995; Lacourciere and Armstrong, 1994; Pries et al., 1994). The

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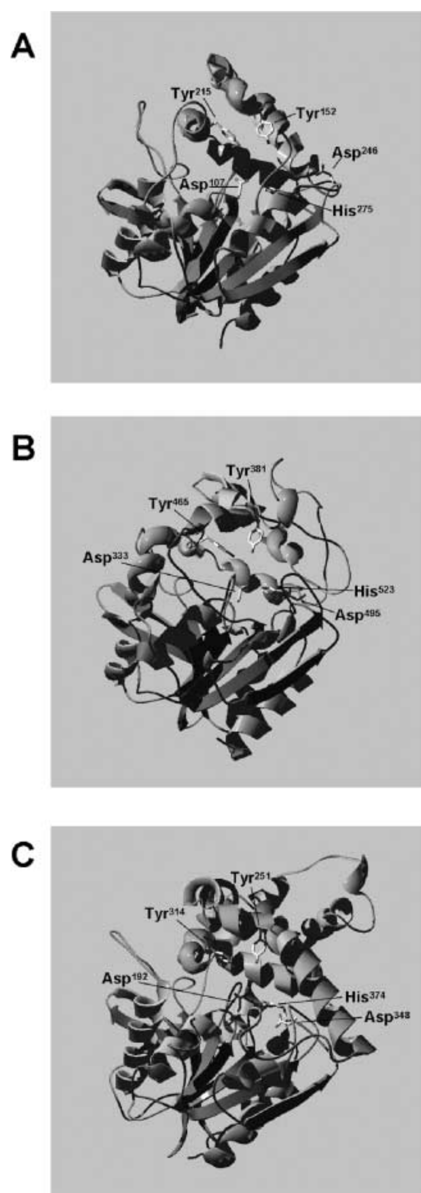

Figure 7. Three-dimensional structure of epoxide hydrolases with α/β hydrolase fold. (A) Epoxide hydrolase from *Agrobacterium radiobacter*. (B) C-Terminal domain of mouse soluble epoxide hydrolase (sEH). (C) Epoxide hydrolase from *Aspergillus niger*. The enzymes are displayed from the same viewing angle with respect to the conserved α/β hydrolase fold. The positions of the catalytic residues are indicated. Note the differences in the lid architecture between the three enzymes.

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most important new information obtained from this structure was the identification of two tyrosine residues pointing from the lid toward the catalytic triad; these residues are pertinent to epoxide hydrolysis in particular.

At about the same time, the structure of the mouse sEH was solved (Argiriadi et al., 1999). The C-terminal epoxide hydrolase domain displayed an overall architecture similar to that of the bacterial EH (Fig. 7B). The nature, order, and spatial arrangement of secondary structure elements in the α/β hydrolase fold domains of the two enzymes were very similar. Some differences in the arrangement of the α-helices building the lid domains of the two enzymes were observed, but the two tyrosines previously identified in the bacterial enzyme were well conserved in position and orientation in the mammalian enzyme, further supporting their role in catalysis. The authors identified an L-shaped substrate binding cavity formed between the α/β hydrolase fold domain and the lid domain that is perfectly suited to accommodate long-chain fatty acid epoxides. To the N-terminal domain, dubbed the vestigial domain by the authors due to the lack of a known function at that time, a structural function was assigned: in the sEH homodimer, the vestigial domain of one subunit interacts with the EH domain of the second subunit, thereby constituting the major part of the dimerization interface. The third determined EH 3D structure was that of the *Aspergillus* EH (Zou et al., 2000), which although it is more closely related to mEH, lacks a membrane anchor and, therefore, is a soluble enzyme (Arand et al., 1999a). Again, this structure shows the well-conserved α/β hydrolase fold containing the catalytic triad as well as two tyrosine residues reaching from the lid into the active site cavity (Fig. 7C). The significance of the two tyrosines is further underlined by the fact that their spatial position and secondary structural context is well conserved, despite major differences in the lid geometry between the *Aspergillus* enzyme and the two other EH structures described above. The N-terminal extension typical for mEH-like epoxide hydrolases turned out to form a long meander that starts at the bottom of the α/β hydrolase fold and then wraps around the molecule to the upper part of the lid, thereby apparently holding the lid down to the α/β hydrolase fold and winds back close to the point where it started from. Thus, it seems to stabilize the subunit structure of the protein. In addition, together with the lid surface, it forms the dimerization interface of the enzyme. Molecular modeling studies with human mEH, based on the structure of the *Aspergillus* enzyme, are compatible with a dimeric structure of the mammalian mEH (Zou et al., 2000).

In 2001, the first structure of an epoxide-hydrolyzing enzyme that lacked the canonical α/β hydrolase fold was reported, namely, that of LTA_4 hydrolase (Thunnissen et al., 2001). It is composed of three domains (Fig. 8); the central domain harbors the catalytically important residues and has a fold similar to thermolysin, a bacterial zink-dependent protease. Modeling studies, as well as site-directed mutagenesis experiments, suggest that LTB_4 formation is afforded by Zn-mediated epoxide ring opening, followed by addition of a hydroxy group in the 12-position which is catalyzed by Asp^{375}-dependent proton abstraction from the catalytic water (Rudberg et al., 2002). This enzymatic mechanism is rather specific and precisely tailored for the substrate LTA_4. Very recently, we solved the 3D structure of the limonene 1,2-epoxide hydrolase (LEH), an EH with yet another fold (Arand et al., 2003a). The enzyme has a single α/β barrel

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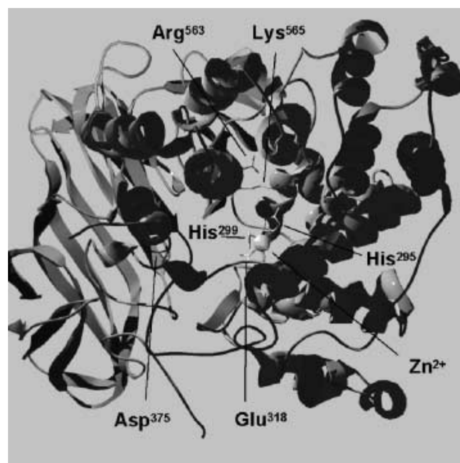

Figure 8. Three-dimensional structure of leukotriene A_4 hydrolase. The positions of potential catalytic residues are indicated.

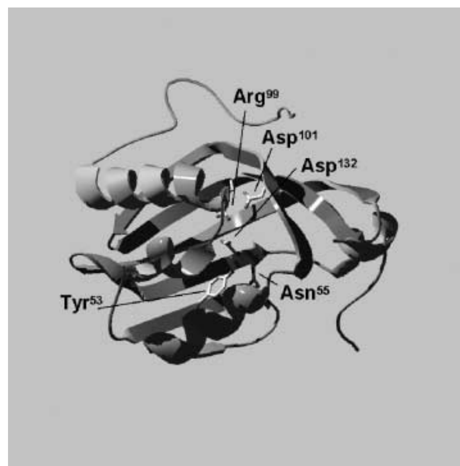

Figure 9. Three-dimensional structure of limonene epoxide hydrolase from *Rhodococcus erythropolis*. The positions of catalytic residues are indicated. Note the particularly small size of the enzyme.

domain, with the lumen of the barrel forming the substrate-binding cavity (Fig. 9). Given the observed high turnover number of LEH with its preferred substrate 1*R*,2*S*,4*R*-limonene 1,2-epoxide, its reductionistic architecture obviously represents an extremely economic solution to the task of hydrolyzing one specific epoxide.

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Enzymatic Mechanisms of Epoxide Hydrolysis

At present, three different mechanisms for epoxide hydrolysis have been elucidated by a combination of biochemical and x-ray structure analysis, namely, those of the α/β hydrolase fold EHs, of LEH, and of LTA_4 hydrolase. Of these, the α/β hydrolase fold EH mechanism has been studied most extensively (Arand et al., 1996, 1999b; Argiriadi et al., 1999, 2000; Hammock et al., 1994; Lacourciere and Armstrong, 1993; Laughlin et al., 1998; Müller et al., 1997; Nardini et al., 1999; Pinot et al., 1995; Rink and Janssen, 1998; Rink et al., 1997, 2000; Tzeng et al., 1996, 1998; Yamada et al., 2000; Zou et al., 2000). Substrate hydrolysis in this case proceeds essentially in two steps as detailed in Fig. 10. Epoxide hydrolysis by LEH also produces a vicinal *trans*-diol, but it proceeds in a single step (Arand et al., 2003a), as shown in the same figure. Comparison of these two mechanisms further improves our understanding of why the two-step catalysis apparently is nature's preferred solution to the hydrolysis of potentially genotoxic xenobiotic epoxides. On the one hand, the mode of action of LEH provides evidence that fast enzymatic hydrolysis of epoxides is easily achieved in a single step. On the other hand, this seems to go hand in hand with comparatively narrow substrate specificity. The efficient direct hydrolysis involves the tight spatial fixation of the hydrolytic water—the catalytic nucleophile in this reaction mechanism—by residues Tyr^{53}, Asn^{55}, and Asp^{132}. Thus, the substrate has to bind with a specific orientation and distance to allow an efficient reaction. In contrast, the catalytic nucleophile of the α/β hydrolase fold EH, invariably an aspartic acid residue, sits on the tip of the nucleophile ellbow, a sharp kink between a β-strand and an α-helix (Ollis et al., 1992). The degree of freedom of this catalytic nucleophile is somewhat higher than that of the catalytic water in the LEH active site, and it is well conceivable that it can adapt to the position of its substrate in the active site with reasonable flexibility. Initial non-covalent binding of the substrate is directed by hydrogen bonding of the epoxide oxygen to the two tyrosines of the lid as well as by hydrophobic interactions with residues lining the active site cavity. These latter interactions are obviously dependent on the pattern of substituents at the epoxide ring and will modulate the position of the reactive epoxide carbon in space, which apparently can be compensated for very well by the inferred flexibility of the catalytic nucleophile. Thus, a large variety of structurally different compounds may be processed in the same catalytic site, as long as no major steric constraints prohibit their binding. The next step of catalysis, the hydrolysis of the ester intermediate, is probably much less suited to spatial adaptation, because the involved charge relay system with its network of hydrogen bonds represents a more rigid structure than the catalytic nucleophile. This may well be the reason for the observation that the hydrolytic step of the reaction is rate limiting and usually orders of magnitude slower than the first step (Müller et al., 1997; Tzeng et al., 1996). For the detoxication process, the slowness of the hydrolytic step is of minor consequence, because the first step already neutralizes the toxic potential of the substrate. The fact that mEH, the EH important for detoxification of most epoxides, is usually present in significantly higher concentrations than its substrates renders the regeneration of enzyme for the next catalytic cycle a process of lower priority. The kinetics of this reaction mechanism are discussed in detail elsewhere (Arand et al., 2003b).

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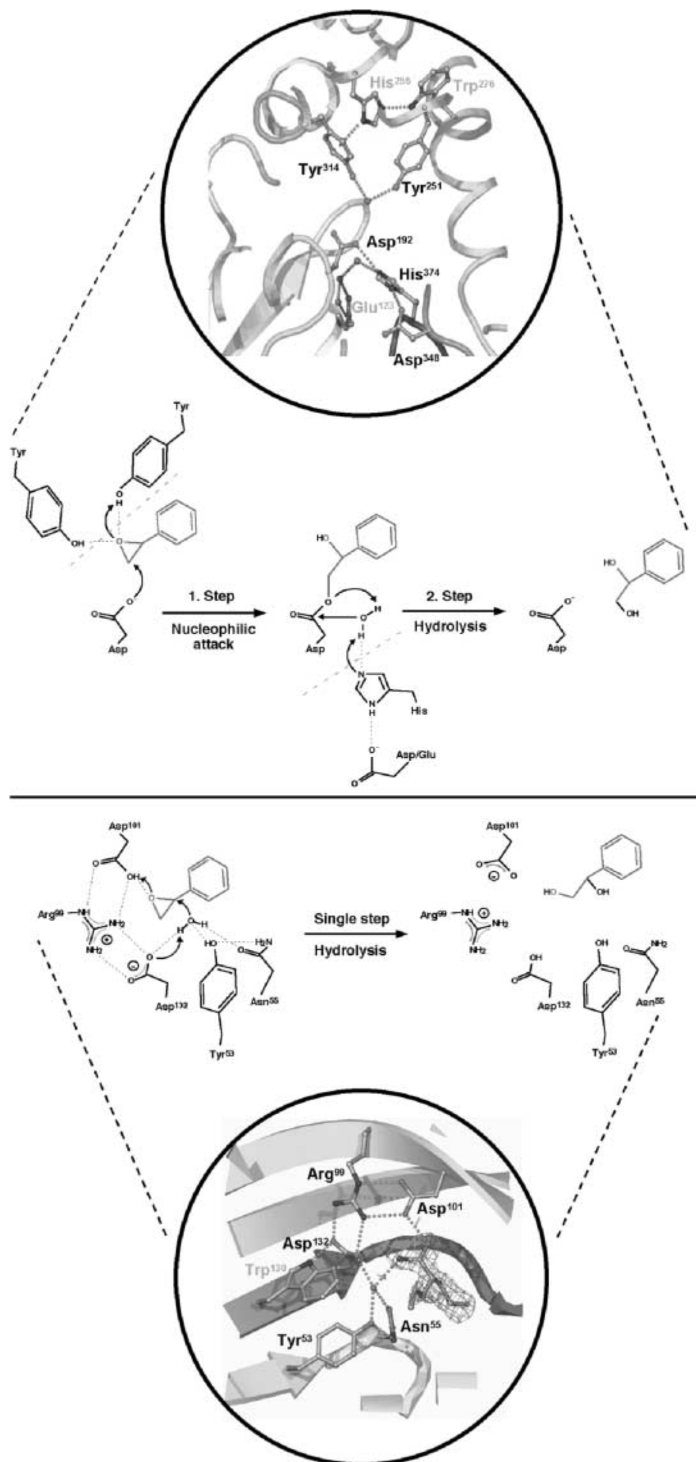

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The LTA4 hydrolase mechanism has already been briefly described above and is of, as yet, minor interest with respect to general epoxide hydrolysis, because it represents a rather specialized function, with the hydroxy group being introduced into the substrate molecule far away from the epoxide moiety. Therefore, it has not been further discussed here in the comparison of general EH mechanisms.

Other Enzymatic Functions

Despite serious attempts to identify non-epoxide substrates for the epoxide hydrolase domain of mEH, sEH, and LEH, no such compound could be identified to date (M. Arand, unpublished). Structural analysis of the *Aspergillus* EH revealed that the N-terminal extension of mEH does not form a separate domain (Zou et al., 2000); therefore, an additional enzymatic function attributable to this part of the enzyme is highly unlikely. In contrast, the N-terminal extension of sEH does represent a separate domain, related in sequence (Beetham et al., 1995; Koonin and Tatusov, 1994) and structure (Argiriadi et al., 1999) to the large group of haloacid dehalogenase-like enzymes. In addition, the sEH structure revealed a potential substrate-binding cavity at the position of the well-conserved active site residues. Based on these observations, we and others could recently show that the N-terminal domain of sEH has a phosphatase activity (Fig. 11) (Cronin et al., 2003; Newman et al., 2003).

Why should a phosphatase activity be associated with an epoxide hydrolase function within the same enzyme? While we found phosphatase activity toward the generic substrates 4-nitrophenyl phosphate and 4-methylumbelliferyl phosphate (Cronin et al., 2003), Newman and colleagues identified the monophosphate esters of 9,10-dihydroxy octadecanoic acid as substrates (Newman et al., 2003). This latter

Figure 10. Comparison of the enzymatic mechanisms of α/β hydrolase fold epoxide hydrolases and limonene epoxide hydrolase. The upper half of the figure presents the mechanism of the α/β hydrolase fold enzymes; the lower half shows the mechanism of limonene epoxide hydrolase. The two circles show active site views of *Aspergillus* epoxide hydrolase (top) and limonene epoxide hydrolase (bottom), respectively. The two-step mechanism of the α/β hydrolase fold EHs proceeds as follows. On substrate docking, the two tyrosines from the lid hydrogen bond to the epoxide oxygen, thereby positioning and further activating the epoxide for the nucleophilic attack by the catalytic aspartic acid residue. The reaction between the aspartate and the epoxide leads to an enzyme substrate ester intermediate (note that the configuration of this ester is inverse compared to the respective intermediate formed by esterases). Subsequent hydrolysis is achieved by water activation through the His/Asp charge relay system, leading to product formation and reconstitution of the active enzyme. In contrast, the limonene epoxide hydrolase performs the hydrolysis in a single step. The hydrolytic water is activated through proton abstraction by Asp^{132} and tightly fixed in its spatial position via hydrogen bonding to Tyr^{53} and Asn^{55}. On docking of the substrate, Asp^{101} provides a proton to the epoxide oxygen which facilitates the direct hydrolysis of the oxiran ring by a classical push-pull mechanism. Arg^{99} mediates between the two catalytic aspartic acid residues and most likely serves as the proton shuttle to regenerate the protonated Asp^{101}.

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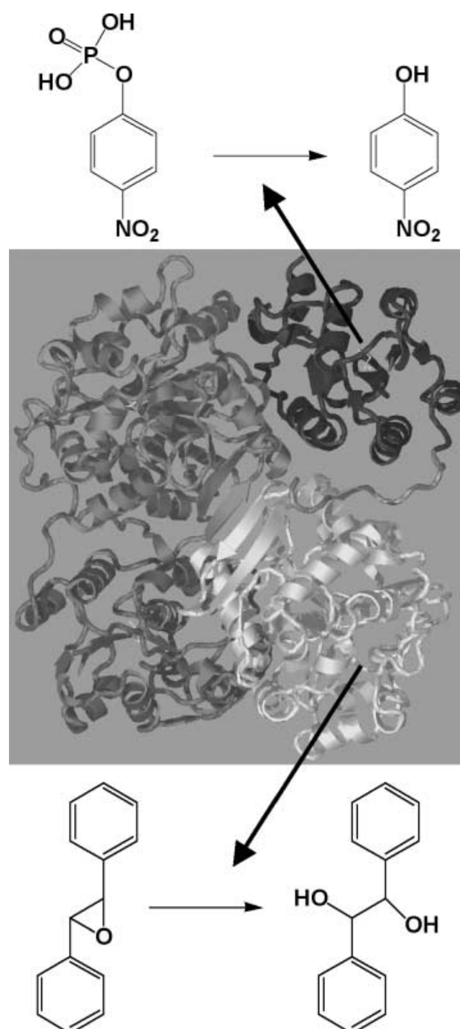

Figure 11. Domain structure of soluble epoxide hydrolase. The center of the figure shows the dimeric structure of mouse soluble epoxide hydrolase. One subunit is represented in gray (left side). The N-terminal domain of the other subunit is shown in dark gray (upper right side), and the C-terminal domain is shown in white (lower right side). The enzymatic reactions catalyzed by the respective subunit are symbolized with the diagnostic substrates 4-nitrophenyl phosphate for the N-terminal phosphatase domain (top) and *trans*-stilbene oxide for the C-terminal epoxide hydrolase domain (bottom).

finding is particularly intriguing because with these substrates, the N-terminal sEH domain generates the same reaction product as the C-terminal domain does on the hydrolysis of octadecanoic acid 9,10-epoxide. However, this class of hydroxy fatty acid phosphate esters first needs to be identified as physiological metabolites before major conclusions can be drawn from this observation.

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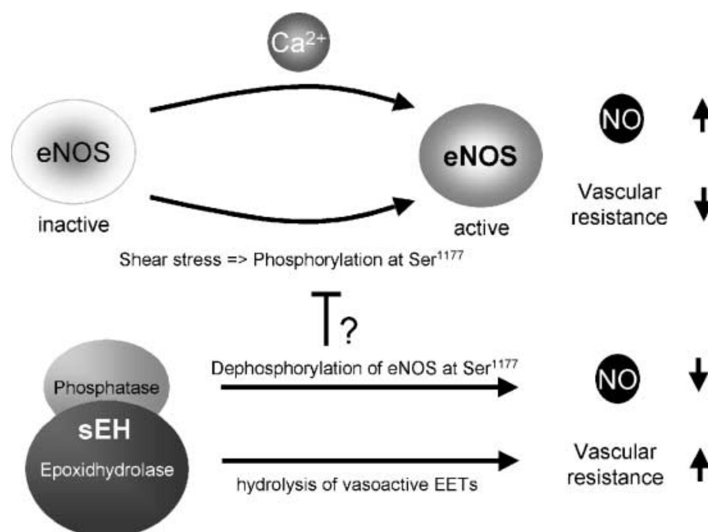

Figure 12. Hypothetical cross-talk between soluble epoxide hydrolase and endothelial NO synthase in the regulation of blood pressure.

Another possible reason for the combination of an epoxide hydrolase and a phosphatase in a single enzyme could be a functional synergism. The recent observation, that the endothelial NO synthase (eNOS), an enzyme with central function in the regulation of blood pressure, can be activated by phosphorylation (Dimmeler et al., 1999), invites one to speculate about a possible cross-talk between eNOS and sEH (Fig. 12). Shear stress-induced activation of eNOS through phosphorylation at position Ser^{1177} leads to vascular relaxation, due to the increased formation of NO. Thus, if sEH was able to dephosphorylate eNOS at Ser^{1177} by the activity of its N-terminal domain, this would lead to an increase in blood pressure, which would be synergistic to the blood pressure effects of the C-terminal domain that hydrolyzes vasorelaxing epoxides. So far, high expression of sEH in endothelial cells, the location of eNOS, has been shown (Fang et al., 2001), but no experimental data of a direct interaction between sEH and eNOS are as yet available.

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