

Hypothesis

Sequence similarity of mammalian epoxide hydrolases to the bacterial haloalkane dehalogenase and other related proteins

Implication for the potential catalytic mechanism of enzymatic epoxide hydrolysis

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Abstract

Direct comparison of the amino acid sequences of microsomal and soluble epoxide hydrolase superficially indicates that these enzymes are unrelated. Both proteins, however, share significant sequence similarity to a bacterial haloalkane dehalogenase that has earlier been shown to belong to the α/β hydrolase fold family of enzymes. The catalytic mechanism for the dehalogenase has been elucidated in detail [Verschuere et al. (1993) *Nature* 363, 693–698] and proceeds via an ester intermediate where the substrate is covalently bound to the enzyme. From these observations we conclude (i) that microsomal and soluble epoxide hydrolase are distantly related enzymes that have evolved from a common ancestral protein together with the haloalkane dehalogenase and a variety of other proteins specified in the present paper, (ii) that these enzymes most likely belong to the α/β hydrolase fold family of enzymes and (iii) that the enzymatic epoxide hydrolysis proceeds via a hydroxy ester intermediate, in contrast to the presently favoured base-catalyzed direct attack of the epoxide by an activated water.

Key words: α/β Hydrolase fold, Hydrolase, Lipase, Esterase, Luciferase, Chelatase, Peroxidase

1. Introduction

Epoxide hydrolases (EH, EC3.3.2.3) comprise a group of enzymes that are functionally related in that they catalyze the hydrolytic cleavage of oxirane compounds to yield the corresponding diols. Two major, well characterized EHs are present in the liver of all animal species so far investigated.

The first EH, called microsomal epoxide hydrolase (mEH), the broad-specific mEH with the diagnostic substrate benzo[a]pyrene-4,5-oxide, is located in the endoplasmic reticulum. Its major function is thought to be the hydrolysis of epoxides derived from xenobiotics, in-

cluding polycyclic aromatic hydrocarbons [1]. This catalytic breakdown protects essential biomolecules like nucleic acids and proteins from the electrophilic attack by these reactive molecules and therefore represents an important protective metabolic pathway. The biochemical properties of mEH_h have been studied in detail and the primary structure and the genomic organization were elucidated already in the mid eighties [2–4]. On the other hand, our understanding of the overall structure of the mature protein is presently restricted to speculation about the mode of membrane association/integration of the enzyme [3,5].

The other major EH is the soluble epoxide hydrolase (sEH), that has often been referred to as cytosolic epoxide hydrolase (cEH). Because accumulating evidence has been presented for a simultaneous localization of this enzyme in both the cytosol and the peroxisomal matrix of liver cells [6–8] the older term ‘soluble epoxide hydrolase’ seems to more precisely characterize the enzyme and is now recommended. While the sEH, like the mEH_h, accepts some xenobiotic-derived epoxides as substrates

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Abbreviations: EH, epoxide hydrolase, sEH, soluble epoxide hydrolase, mEH_h, microsomal epoxide hydrolase, HALO, haloalkane dehalogenase, DMPD, 2-hydroxyomuconic semialdehyde hydrolase isoenzyme DMPD_PSEPU, XYLF, 2-hydroxyomuconic semialdehyde hydrolase isoenzyme XYLF_PSEPU

[9,10], it appears that its primary function might be the metabolic transformation of epoxides arising from endogenous substrates, as suggested by the significant enzymatic activity of the enzyme with fatty acid epoxides [11,12]. Recent work of our groups has elucidated the primary structure of sEH from rat, mouse and man [13–15]. Initially, direct comparison of these sequences to that of mEH_b did not reveal a significant amount of similarity among these enzymes, at first sight. However, as will be detailed later in this paper, we hypothesize that there is a distant relationship among these enzymes that is too obscure to be picked up by standard similarity analysis.

A third epoxide hydrolase, the mEH_{ch}, that is different from the mEH_b and is also present in the endoplasmic reticulum specifically hydrolyzes cholesterol-5,6-epoxides [16]. Little information is available on this enzyme, and its relationship to the major EHs is thus largely unclear. Two additional epoxide hydrolases are specifically involved in the metabolism of certain intermediates of the arachidonic acid cascade. Of these, the leukotriene A₄ hydrolase (LTA₄ hydrolase) is especially well characterized [17,18]. In contrast to the situation with other EHs, hydrolysis by the LTA₄ hydrolase does not result in the formation of vicinal diols. The two hydroxyl groups introduced during the transformation of LTA₄ to LTB₄ have a spatial distance of eight carbon atoms. The available primary sequence of LTA₄ hydrolase does not show significant similarity to either sEH or mEH_b. The fifth enzyme, the hepoxiline hydrolase, is as yet not sufficiently characterized to evaluate its relationship to other epoxide hydrolases [19].

The mechanism of EH-catalyzed epoxide hydrolysis has not been completely elucidated. In the case of mEH_b, the importance of one specific histidine residue for maintenance of catalytic activity could be demonstrated [20,21]. At present, the most accepted hypothesis is that the reaction proceeds via a general base catalysis by the histidine that is thought to abstract a proton from a water molecule. The resulting hydroxyl anion subsequently is thought to attack the oxirane ring at one of the two carbon atoms, the stereochemistry of the resulting product being subject to the sterical and electronical constraints of the parent compound. Several mechanistic studies with the sEH are consistent with this mechanism as well [22,23].

2. Materials and methods

Protein sequence retrieval and analysis was carried out employing a Macintosh IIvx computer. Similarity searches of the SWISS-PROT library, release 25, were performed using the FASTA program, V 1.6c22 [24]. Dot matrix analysis was conducted using the GeneWorks software package, V 2.1 (IntelliGenetics, Mountain View, CA). The same software was used for sequence alignments. However, this was mainly done by hand as this yielded better results in the present case, due to the comparatively low degree of similarity.

3. Results and discussion

3.1 Soluble and microsomal epoxide hydrolase are both related to the same bacterial proteins

As already pointed out there is no evident similarity between sEH and mEH_b when compared directly on the basis of their amino acid sequences. Instead, screening of the SWISS-PROT library with both protein sequences from the respective rat enzymes resulted in the identification of three bacterial proteins as candidates showing a significant yet marginal relationship to sEH, namely the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (HALO) and two different isoforms of the 2-hydroxymuconic semialdehyde hydrolases from *Pseudomonas putida* (DMPD and XYLF), while no mEH_b-related sequences (only the mEH_b from rat, rabbit and human) were picked up.

Interestingly, some degree of similarity between the human mEH_b and HALO has earlier been described by others [25]. Due to minor differences between the rat and human mEH_b sequence this similarity was not noticed by our first similarity search. When the match between the dehalogenase and mEH_b was taken as the core alignment and the corresponding fragments of sEH, DMPD and XYLF were aligned to this, the interrelationship of all five proteins became apparent (Fig. 1). Over a stretch of about eighty amino acids, none of the possible comparisons of any two of the sequences gave a result below 20% of similarity. With the introduction of only two alignment gaps, six residues were conserved completely throughout all of these proteins in the respective region and a further fifteen residues were identical in four of the five proteins.

3.2 Soluble and microsomal epoxide hydrolase potentially belong to the α/β hydrolase-fold family of enzymes – implication for the putative reaction mechanism

The key result of the above alignment is the observation that the dipeptide DW (see Fig. 1) is conserved in a similar sequence context among sEH, mEH_b and HALO, because the specific aspartic acid residue herein has been identified as the major constituent of the catalytic center of the haloalkane dehalogenase. Two years ago, the crystal structure of HALO was determined at a resolution of 2.4 Å [26]. This led to the classification of the enzyme as a member of the α/β hydrolase-fold family. This class of enzymes comprises a group of proteins that are structurally, functionally and mechanistically related [27]. All of them are hydrolytic enzymes that share the same three-dimensional core structure, the α/β hydrolase-fold. While their catalytic specificities are radically different – lipids, peptides, haloalkanes, lactones and acetylcholine are the substrates for the different enzymes – their enzymatic mechanisms appear to be rather similar. They all possess an active center at corresponding

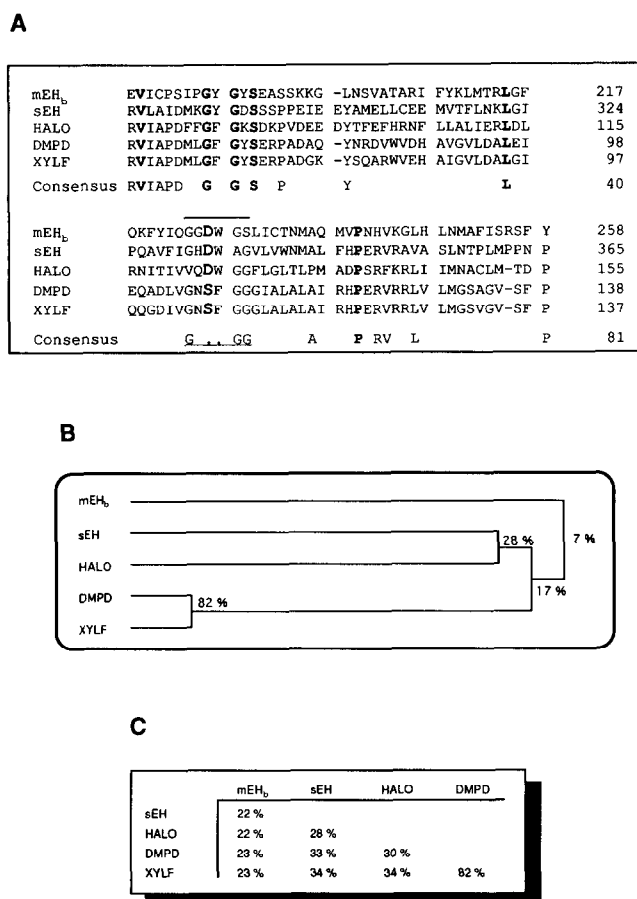


Fig 1 Analysis of the similarity of epoxide hydrolases, haloalkane dehalogenase and 2-hydroxymuconic semialdehyde hydrolases in the region of the potential catalytic nucleophile of the enzymes (A) Sequence alignment. The core regions around the potential nucleophiles of the different proteins were aligned by hand. Residues conserved in all sequences are printed in bold letters. The potential catalytic nucleophiles are printed in big bold letters. Residues printed in the consensus are conserved in at least four out of the five sequences. Dots in the consensus indicate positions where three out of the five sequences share identical residues. (B) Similarity tree. The comparison of the different fragments is presented structured in subalignments to exemplify their relationship. The numbers give the percentage identity of the respective subalignment. (C) Individual comparison of the different fragments. The sequence similarity of each individual comparison of the different fragments is given in percentage identity. mEH_b = Microsomal epoxide hydrolase from rat, sEH = soluble epoxide hydrolase from rat, HALO = haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10, DMPD = 2-hydroxymuconic semialdehyde hydrolases from *Pseudomonas putida*, isoform, XYLF = 2-hydroxymuconic semialdehyde hydrolases from *Pseudomonas putida*, isoform.

positions in the α/β hydrolase-fold motif bearing three largely conserved amino acid residues that form a catalytic triad (these amino acids are separated from each other by a variable number of other residues in the primary structure of the different enzymes but are, in each case, in vicinity to each other in the correctly folded proteins). The first of these amino acids represents a nucleophile that covalently binds to the substrate in the

first step of the enzymatic reaction. In the esterase/amidase-type enzymes this is a serine or cysteine that forms esters or thioesters with the carboxylic component of the substrate, respectively. In the dehalogenase the nucleophile is the above mentioned aspartic acid residue that, in analogy, replaces the halide of its substrates thereby forming an ester bond to the remaining alkyl. The ester intermediates formed by all members of the α/β hydrolase-fold family are subsequently hydrolyzed by a water molecule cooperatively activated by the two other conserved residues, a histidine and an acidic amino acid, either aspartic or glutamic acid. The single steps in the enzymatic mechanism have recently been confirmed for the haloalkane dehalogenase in an elegant study employing X-ray analysis of the trapped covalent intermediate [28].

The sequence similarity among the EHs and HALO, including the aspartic acid of the dehalogenase active center, suggests that the EHs potentially belong to the α/β hydrolase-fold family. Some additional arguments support this hypothesis. First, the other related proteins, the semialdehyde hydrolases, that do not have the conserved aspartate residue but a serine instead, catalyze the hydrolytic cleavage of a C-C bond by removing the aldehyde moiety of the 2-hydroxymuconic semialdehyde to give 2-oxopent-4-enoate and formic acid. The serine is a very good candidate for the nucleophile to form a formylester intermediate. All five enzymes of the above alignment fit very well in the proposed consensus for the nucleophile in the α/β hydrolase-fold that is sm-x-nu-x-sm-sm, where nu is the nucleophile, sm is a small amino acid (preferentially glycine), and x is any amino acid (see Table 1). Second, the importance of a histidine, the second important constituent of the catalytic triad of α/β hydrolase-fold enzymes, for the EH catalysis has long been accepted. For the mEH_b, the specific histidine essential for the enzymatic reaction has recently been identified by both biochemical analysis and site-directed mut-

mEH _b	MERGGHFAAFEEP	438
sEH	IEDCGHWTQIEKP	530
HALO	IADAGHFVQEFGE	296
DMPD	FGQCGHWTQIEHA	263
XYLF	FGQCGHWTQIEHA	262
Consensus	.GH . . Q.E	

Fig 2 Sequence alignment of epoxide hydrolases, haloalkane dehalogenase and 2-hydroxymuconic semialdehyde hydrolases in the region of the potential catalytic histidine of the enzymes. Residues conserved in all sequences are printed in bold letters. Residues printed in the consensus are conserved in at least four out of the five sequences. Dots in the consensus indicate positions where three out of the five sequences share identical residues. Note that sEH, DMPD and XYLF are identical in the eight central residues of the displayed region.

agenesis [20,21] Like the catalytic histidines of the α/β hydrolase-fold family members its position (His⁴³¹) is close to the C-terminus of the enzyme. Similarly, a histidine close to the C-terminus of the sEH (His⁵²³) is an especially good candidate for the member of a postulated catalytic triad of this enzyme as it is situated within a stretch of eight amino acid residues that is perfectly conserved among the sEH and the bacterial muconic semialdehyde hydrolases (Fig 2) The constraints for the acidic residue (Asp or Glu) that interacts with the His in the water activation are too poorly defined by the present data to identify favourable candidates for this residue in the mEH_b or sEH sequence, yet there are several residues in each of the sequences that would fit with the model of a catalytic triad

The observed sequence similarity of the epoxide hydrolases to the haloalkane dehalogenase implies that the epoxide hydrolysis catalyzed by these enzymes proceeds via an as yet unexpected two-step mechanism involving a covalent enzyme-substrate hydroxy ester intermediate (Fig 3) This hydroxy ester intermediate can be seen as the inverse equivalent to the acyl enzyme of serine hydrolases since the acyl group is contributed by the enzyme rather than by the substrate

3.3 Other related enzymes

An additional screening of the SWISS PROT data library with the five protein sequences of the above alignment identified further enzymes that display similarity to the alignment in Fig 1 All of these share the above described consensus around a potential nucleophilic center that lies 40 to 50 amino acids carboxyterminal to a short sequence loosely fitting with the motif RVIAPD that corresponds to β -strand 4 of the haloalkane dehalogenase [26] A third common feature is a short motif

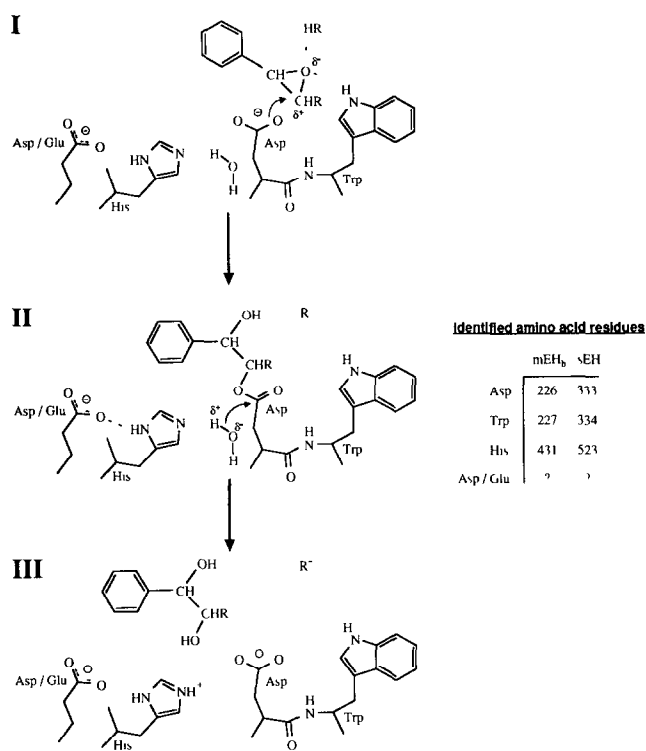


Fig 3 Potential catalytic mechanism of epoxide hydrolases The proposed reaction mechanism is based on the findings of Verschuere et al [28] for the dehalogenase After entering the active center the epoxide is polarized by hydrogen bonding to the hydrogen of a proton donor HR yet to be identified and possibly to the tryptophane adjacent to the nucleophile (I) On attack of the nucleophile the epoxide ring opens under proton abstraction leading to the postulated hydroxy ester intermediate (II) In the following step the water activated by the His-Asp/Glu couple hydrolyzes the ester bond resulting in the release of the formed diol (III) A final necessary regeneration step (not shown) is the transfer of the proton from the His to the R⁻ to reconstitute the proton donor The table specifies the respective residues so far identified by either sequence similarity or experimental work

Table 1

Comparison of the nucleophile-motif of α/β hydrolase-fold enzymes to the corresponding regions of the proteins of the sequence alignment in Fig 1

	SWISS-PROT entry	Protein	Source	Nucleophile-motif (sm-x-nu-x-sm-sm) ^a
α/β hydrolase-fold enzymes (structure resolved by X-ray analysis)	ACES_TORCA	acetylcholine esterase	<i>Torpedo californica</i>	GESAGG
	LIP1_GEOCN	lipase	<i>Geotrichum candidum</i>	GESAGA
	CBP2_WHEAT	carboxypeptidase	<i>Triticum aestivum</i>	GESTAG
	CLCD_PSEPU	dienelactone hydrolase	<i>Pseudomonas putida</i>	GTCLGG
	HALO_XANAU	haloalkane dehalogenase	<i>Xanthobacter autotrophicus</i>	VQDWGG
Enzymes identified by sequence similarity to the soluble epoxide hydrolase	DMPD_PSEPU	hydroxy-muconic semi-aldehyde hydrolase	<i>Pseudomonas putida</i>	GNSFGG
	XYLF_PSEPU	hydroxy-muconic semi-aldehyde hydrolase	<i>Pseudomonas putida</i>	GNSFGG
	HYEP_RAT	microsomal epoxide hydrolase	<i>Rattus norvegicus</i>	GCDWGS
	HYES_RAT	soluble epoxide hydrolase	<i>Rattus norvegicus</i>	GHDWAG

^a Amino acid composition of the motif is given in one-letter code except sm = small amino acid, and nu = nucleophile

between these two with the consensus GxGxS, that is quite well conserved among the different proteins in both, sequence and distance from the other two common features (see Table 2) The majority of the proteins identified this way clearly have known hydrolytic function which fits with the catalytic concept of the α/β hydrolase-fold enzymes The possible enzymatic functions of the magnesium chelatase, the luciferase of *Renilla reniformis* and the bromoperoxidase that relates them to hydrolases, however, are as yet obscure

On the basis of these observations we re-examined the possible relationship of LTA₄ hydrolase with mEH_b and sEH As shown in Table 2 there is a region in the protein sequence of the LTA₄ hydrolase that loosely fits with the above described pattern In contrast to all other enzymes addressed above, though, alignments and dot matrix comparisons of the LTA₄ hydrolase sequence of the region in question to that of the other epoxide hydrolases and the haloalkane dehalogenase did not give conclusive results Thus we cannot rule out a sparse relationship of LTA₄ hydrolase to the other epoxide hydrolases but also have no convincing evidence for it

Very recently, Cygler et al. [29] have identified a large number of proteins, mainly esterases and lipases, that are structurally related to the acetylcholine esterase from *Torpedo californica* and the lipase from *Geotrichum candidum*, the two α/β hydrolase-fold enzymes related by sequence similarity. The α/β hydrolase-fold family of enzymes therefore appears to be a rapidly growing group

of proteins comprising a variety of different hydrolytic enzymes including – according to our deductions – also epoxide hydrolases.

In conclusion, sequence comparisons have revealed a significant relationship of the microsomal and the soluble epoxide hydrolase to an array of mostly bacterial proteins. The similarity among these sequences is mainly restricted to a specific region that corresponds to an important part of the active center of the haloalkane dehalogenase, a member of the α/β hydrolase-fold family of enzymes We conclude from our observations that (i) there is a distant evolutionary relationship between these sequences and that (ii) probably all these proteins belong to the α/β hydrolase-fold enzymes, as it is generally accepted that the three-dimensional structure of proteins related in function is stronger preserved in evolution than their primary sequence [27]. Thus, the present observations strongly suggest that the enzymatic hydrolysis of epoxides proceeds via an intermediate covalently bonded to the enzyme by an α -hydroxy ester linkage Such a mechanism has already been considered as a possible alternative to the base catalysis by Armstrong et al [30] but these authors themselves strongly favour the direct hydrolytic cleavage of epoxides [30,31].

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Table 2
Sequences depicted by similarity to the alignment in Fig 1

SWISS-PROT Entry	Protein	Source	RVIAPD ^a -Motif	Distance to Next Motif	GxGxS-Motif	Distance to Next Motif	Nucleophile-to Next Motif
HYES_RAT	soluble epoxide hydrolase	<i>Rattus norvegicus</i>	RVLAIID	2	GYGDS	33	GHDWAG
HYEP_RAT	microsomal epoxide hydrolase	<i>Rattus norvegicus</i>	EVICPS	2	GYGYS	32	GGDWGS
HALO_XANAU	haloalkane dehalogenase	<i>Xantobacter autotrophicus</i>	RVIAPD	2	GFGKS	33	VQDWGG
DMPD_PSEPU	hydroxyumuconic semialdehyde hydrolase	<i>Pseudomonas putida</i>	RVIAPD	2	GFGYS	32	GNSFGG
XYLF_PSEPU	hydroxyumuconic semialdehyde hydrolase	<i>Pseudomonas putida</i>	RVIAPD	2	GFGYS	32	GNSFGG
TODF_PSEPU	heptadienoate hydrolase	<i>Pseudomonas putida</i>	RVIAPD	2	GFGFT	32	GNSFGG
TPES_PSEPU	tropinesterase	<i>Pseudomonas putida</i>	RYLALD	2	GHGGT	31	GHSMGS
BPHD_PSES1	biphenyl hydrolase	<i>Pseudomonas</i> sp	RVLLPD	2	GFNKS	32	GNSMGG
PHAB_PSEOL	lipase	<i>Pseudomonas oleovorans</i>	EVIAPD	2	GVGGS	31	GVSWGG
ACOC_ALCEU	(probable) dihydroliipoamide acetyltransferase	<i>Alcaligenes eutrophus</i>	TVVALD	2	GHGQS	31	GHSMGG
LIP3_MORSP	lipase	<i>Moraxella</i> sp	HLIIPD	2	GFGNS	33	GNSMGG
BCHO_RHOCA	magnesium chelatase	<i>Rhodobacter capsulatus</i>	RVIVPD	2	GHGCS	32	GHSAGG
LUCI_RENRE	luciferase	<i>Renilla reniformis</i>	RCIIPD	2	GMGKS	33	GHDWGA
BPA2_STRAU	bromoperoxidase	<i>Streptomyces aureofaciens</i>	RVITYD	2	GFGQS	31	GFSMGT
LKHA_RAT ^b	leukotriene A4 hydrolase	<i>Rattus norvegicus</i>	RVPIPC	7	GALES	32	AEDLGG

^a Amino acid composition of the motifs is given in one-letter code, ^b not picked up by similarity search (see text)

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Note added in proof

After submission of the present manuscript Lacourciere and Armstrong have published experimental results which strongly support the enzymatic mechanism proposed here (G M Lacourciere and R N Armstrong (1993) *J Am Chem Soc* 115, 10466-10467)