Stereochemical features of the hydrolysis of 9,10-epoxystearic acid catalysed by plant and mammalian epoxide hydrolases

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cis-9,10-Epoxystearic acid was used as a tool to probe the active sites of epoxide hydrolases (EHs) of mammalian and plant origin. We have compared the stereochemical features of the hydrolysis of this substrate catalysed by soluble and membrane-bound rat liver EHs, by soluble EH (purified to apparent homogeneity) obtained from maize seedlings or celeriac roots, and by recombinant soybean EH expressed in yeast. Plant EHs were found to differ in their enantioselectivity, i.e. their ability to discriminate between the two enantiomers of 9,10-epoxystearic acid. For example, while the maize enzyme hydrated both enantiomers at the same rate, the EH from soybean exhibited very high enantioselectivity in favour of 9*R*,10*S*-epoxystearic acid. This latter enzyme also exhibited a strict stereoselectivity, i.e. it hydrolysed the racemic substrate with a very high enantioconvergence, yielding a single chiral diol product, *threo*-9*R*,10*R*-dihydroxystearic acid. Soybean EH shared these distinctive stereochemical features with the membrane-bound rat liver EH. The stereochemical outcome of these enzymes probably results from a stereoselective attack by the nucleophilic residue on the oxirane ring carbon having the (*S*)-configuration, leading to the presumed (in plant EH) covalent acyl–enzyme intermediate. In sharp contrast, the reactions catalysed by cytosolic rat liver EH exhibited a complete absence of enantioselectivity and enantioconvergence; this latter effect might be ascribed to a regioselective formation of the acyl–enzyme intermediate involving C-10 of 9,10-epoxystearic acid, independent of its configuration. Thus, compared with soybean EH, the active site of rat liver soluble EH displays a very distinct means of anchoring the oxirane ring of the fatty acid epoxides, and therefore appears to be a poor model for mapping the catalytic domain of plant EHs.

Key words: enantioconvergence, enantioselectivity, enzyme purification, epoxy fatty acids, expression analysis.

INTRODUCTION

Epoxide hydrolases (EHs) are ubiquitous enzymes which hydrate epoxides to form vicinal diols. In mammals they exist as soluble and microsomal (membrane-bound; mEH) forms, which are both involved in the detoxification of xenobiotics. In addition, soluble EHs were also shown to participate actively in the metabolism of epoxide derivatives of endogenous lipids such as arachidonic acid and linolenic acid [1–3]. In plants, EHs have also been detected as cytosolic [4–9] and particulate enzymes. The membrane-associated forms are likely to be involved in constitutive plant defence mechanisms, since they catalyse the hydration of, for example, epoxy-fatty acids involved in the biosynthesis of cutin monomers [10,11]. However, the physiological roles of the soluble forms of plant EHs remain quite unclear, although these enzymes appear to be of considerable importance, as indicated by their wide distribution in different organs from various plants.

At first, EHs, which do not require any cofactors or metals for their activity, were assumed to function by a direct attack of a water molecule on the epoxide via a general acid/base-catalysed mechanism. However, from sequence alignments of the cloned proteins, evidence has emerged that EHs are members of the α/β-hydrolase-fold superfamily [12,13]. These hydrolytic enzymes are known to share not only a similar topology but also a preserved arrangement of a catalytic triad consisting of a nucleophile (serine, cysteine or aspartic acid), an acidic residue and a strictly conserved histidine [14]. Accordingly, it was demonstrated that mammalian EHs catalyse the hydration of epoxides according to a two-step reaction mechanism involving: (i) an initial attack of the oxirane ring by the carboxylic group of an aspartate residue, yielding a covalent acyl–enzyme intermediate; followed by (ii) a general-base-catalysed activation of a water molecule by a histidine residue, with the assistance of a second aspartate as a charge-relay system, resulting in the hydrolysis of the acyl linkage and liberation of the diol product [15–18]. Extensive stereochemical and mechanistic studies on mammalian EHs have shown that the nucleophilic attack resulting in the epoxide opening often occurs within the active site of mEH with remarkable regio- and enantio-selectivities; in sharp contrast, in the case of the soluble EHs this step is strongly dependent on the structure of the substrate [19–21].

In contrast with the wealth of information available on mammalian EHs, in higher plants this class of enzymes has received little attention until recently. Amino acid sequence alignments of the cloned plant EHs from potato, *Arabidopsis* and tobacco [22–24] have revealed a high degree of conservation of the three residues that form the catalytic triad in mammalian EHs. Thus it is likely that the plant EH-catalysed hydrolysis of epoxides may also proceed through the formation of an acyl–enzyme intermediate, although such a mechanism awaits definitive experimental proof. On the other hand, we have reported high enantioselectivity in the transformation of fatty acid epoxides catalysed by soybean EH that was accompanied by a remarkable stereoselectivity resulting in a deracemization, i.e. racemic mixtures of epoxides, after complete hydrolysis, yielded a single diol enantiomer with an enantiomeric excess (*e.e.*) $>99\%$ [25,26]. Thus soybean EH is potentially a highly valuable tool

Abbreviations used: *e.e.*, enantiomeric excess; EH, epoxide hydrolase; mEH, membrane-bound epoxide hydrolase; TMV, tobacco mosaic virus.

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in the field of biotransformation chemistry. However, it has not been shown whether this stereocontrolled epoxide hydration mechanism is shared by other plant enzymes, or if it is restricted to the soybean species. In other words, does the active site of plant EHs resemble that of the soluble or membrane-bound mammalian (i.e. hepatic) enzymes? What are the topological and substrate-binding characteristics of the active sites of the EHs of different origin? These questions are crucial because they should yield interesting comparisons between the mammalian and plant EH active sites, a better understanding of their catalytic mechanisms, and, finally, provide valuable information on the potential of these enzymes in stereoselective biotransformations.

To address these points, we have chosen to probe the active site of plant and rat liver EHs with a single substrate: *cis*-9,10-epoxystearic acid. This molecular tool, which is intrinsically a quasi *meso* molecule with regard to the binding of the epoxide moiety within the active site, was selected to circumvent the steric and/or electronic effects that are known to influence the outcome of some EH-catalysed reactions in which the nucleophilic attack, leading to the acyl–enzyme intermediate, is prevalently oriented towards the less substituted or less hindered oxirane carbon [27–29]. We also report here the purification and characterization of a recombinant soybean EH expressed in yeast, and of two other plant EHs isolated from maize seedlings and celeriac roots.

EXPERIMENTAL

Materials

Seeds from maize (*Zea mays*, var. *blizzar*), wheat (*Triticum aedivum*, var. *kamut*) and rice (*Oriza sativa*, var. *rosa marchetti*) were germinated in the dark, on four layers of cloth saturated with water, until the seedlings had reached approx. 1 cm in length. Celeriac (*Apium graveolens*, var. *rapaceum*), banana (*Musa paradisiaca*) fruit and potato (*Solanum tuberosum*) tubers were bought from a local market. Tobacco plants (*Nicotiana tabacum*, var. *samsun* N) were grown and infected with tobacco mosaic virus (TMV) essentially as described by Guo et al. [24]. Tobacco leaves were harvested, from 20 cm-height plants, 7 days after their inoculation with *Erypsiphe graminis*.

Enzyme preparation

Plant seedlings, banana fruit pulp, peeled celeriac root and potato tubers were rinsed with distilled water, rapidly chopped with a scalpel and homogenized in an Ultra-Turrax in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.2 M saccharose, 0.01 mM DETAPAC (diethylenetriaminepenta-acetic acid) and insoluble polyvinylpolypyrrolidone (20 g/l). The homogenate was squeezed through Blutex (50 μm) and the filtrate was centrifuged at 10000 ***g*** for 15 min. The pellet was discarded and the supernatant centrifuged again at 100000 ***g*** for 45 min. The resulting supernatant was used directly for measuring EH activity or as a source for further purification. Maize and celery EHs were purified to apparent homogeneity, following the experimental protocol described previously for soybean EH [4]. Tobacco leaves (2 g) were ground in a mortar with liquid nitrogen. After addition of 4 ml of 0.1 M potassium buffer (pH 7.4), the resulting slurry was vigorously shaken for 2 min, then passed through four layers of cloth and centrifuged for 5 min at 5000 ***g***. The resulting supernatant was used immediately as source of enzyme activity.

Mammalian EHs

Recombinant soluble and microsomal rat EHs were purified as previously described [18,30]. The mutant $Glu^{404}Asp$ of rat liver microsomal EH was constructed by site-directed mutagenesis according to [31].

Construction of soybean EH containing an N- or C-terminal histidine tag

PCR was performed on pBEH6, which contained the soybean EH cDNA clone [32], with primers containing 5′ *Bam*HI and 3′ *Hin*dIII restriction sites. In addition to these sites, primers contained additional bases to code for a hexahistidine tag at either the C- or N-terminal end of the EH gene. The following primers were used for the amplification of EH with a C-terminal histidine tag (designated psEH-CH): 5′-AAG GAT CCA TGT GCG AGC ACT TAC TCG-3′ as forward primer and 5′-GGA AGC TTT CAG TGG TGG TGG TGG TGG TGG AAC TTG TTG ATA AAA TCG TAT ATG TAA TTA TCG-3′ as reverse primer. Amplification of EH containing an N-terminal histidine tag (designated psEH-NH) used 5′-GGT TCA CCG CCG CAA CTT CCA GGA TCC ATG CAC CAC CAC CAC CAC CAC ATG TGC GAG CAC TTA CTC G-3′ as forward primer and 5′-GGA AGC TTT CAG AAC TTG TTG ATA AAA TCG-3′ as reverse primer. After purification on an agarose gel, the DNA fragment containing the entire EH sequence was ligated into pBluescript II KS+. *Pfu* polymerase (Stratagene) was used for high-fidelity amplification of the EH gene; in addition, the resulting amplified gene was sequenced to ensure the accuracy of the sequence. Once the sequence of the EH amplification product was established, the clone was digested with *Bam*HI and *Hin*dIII and the insert, isolated on an agarose gel, was cloned into the yeast constitutive expression vector pVT102U [33] digested with the same enzymes. Expression of recombinant soybean soluble EH was performed in *Saccharomyces cerevisae* WA6 (*ade*5 *his*7-2 *leu*2-3 *leu*2-112 *ura*3-52).

Expression in yeast of soybean soluble EH

Expression of the recombinant soybean soluble EH in transformed yeast cells was carried out as follows: 5 ml of S medium (7 g/l yeast nitrogen base, 1 g/l casamino acids, 20 g/l glucose, supplemented with 50 μg/ml histidine, 200 μg/ml adenine and 50 μg/ml leucine) was inoculated with recombinant yeast and grown for 2 days with shaking (280 rev./min; stroke 2.5 cm) at 30 °C. Then 800 μl portions were used to inoculate six 300 ml volumes of S medium. After 5 days, the resulting cultures were pelleted by centrifugation at 4000 ***g*** and the cells were washed once with 200 ml of buffer A (100 mM $NaPO_4$, 300 ml NaCl, pH 8.0), then once with 200 ml of buffer A containing 10 mM β-mercaptoethanol. The yeast culture was resuspended in a final volume of 60 ml of buffer A containing 2 mM β-mercaptoethanol and 'Complete' Protease Inhibitor without EDTA (Boehringer Mannheim). The washed yeast cultures were then disrupted by three passes in a French Pressure Cell (Aminsco) at 3000 lb/in^2 (20.7 MPa) high setting. The resulting lysate was centrifuged once at 10000 ***g*** for 15 min. The supernatant was recovered and centrifuged at 100000 ***g*** for 90 min. The final supernatant (approx. 60 ml) was further purified.

Purification of recombinant soybean soluble EH

A 7 ml (slurry volume) Ni^{2+}-nitrilotriacetate chelating column (Qiagen) was loaded overnight with 100 ml of cell lysate. The column was washed with 100 ml of buffer A or until the A_{280} had dropped below 0.02. The column was then washed with 100 ml of buffer A plus 5 mM imidazole followed by 100 ml of buffer A plus 13 mM imidazole. Elution of the EH was accomplished with 20 ml of buffer A plus 500 mM imidazole, collected in 5 ml

fractions of eluant. The enzyme activity was found to elute in a single protein peak at approx. 10 ml. Fractions containing active EH were pooled and then dialysed (cut-off 10000 Da) against 10 mM ammonium acetate, pH 7.4, concentrated on a bed of carboxymethylcellulose and dialysed a second time against 10 mM ammonium acetate, pH 7.4. To achieve a final protein concentration of > 5 mg/ml, the enzyme was applied to a Centricon (10000 Da cut-off) and centrifuged at 5000 ***g***.

Kinetic studies with EH

Determinations of the enzymic activity of EH using [1-^{14}C]*cis*-9,10-epoxystearate as substrate were carried out according to [4]. For a typical assay, the enzyme was pre-equilibrated for 1 min at 26 °C in 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 1.2 ml. The reaction was started by addition of 1.2 μM ethanolic solution of substrate. At given times, 0.1 ml aliquots were withdrawn and the reaction was stopped by addition of 50 μl of acetonitrile. The mixture was then applied to a silica Gel TLC plate (60 F_{254}; Merck). The plates were developed in diethyl ether/n-hexane/formic acid (70:30:1, by vol.) when the substrate was [1-^{14}C]9,10-epoxystearic acid, or in n-hexane/ethyl acetate (85:15, v/v) for the corresponding methyl esters.

The enantioselectivity of the different EHs was determined by studying the kinetics of hydrolysis of [1-^{14}C]*cis*-9,10-epoxystearic acid prepared chemically from the labelled fatty acid as described previously [34]. As confirmed by chiral-phase HPLC analysis (see below), this racemic form contained, as expected, equal amounts of 9*R*,10*S*- and 9*S*,10*R*-epoxide enantiomers. The selectivity of the EHs is reflected by their ability to discriminate between these two potentially competing enantiomers. According to Fersht [35], the ratio of the rates of transformation of two substrates A and B, competing for a same active site, is given by:

$$v_A/v_B = (V/K_m)_A[A]/(V/K_m)_B[B] \quad (1)$$

Since we are using a racemic substrate, with A and B being present in equimolar concentrations, this expression simplifies into:

$$v_A/v_B = (V/K_m)_A/(V/K_m)_B \quad (2)$$

Therefore the specificity of a given EH will be governed solely by the ratio of the values of V/K_m [35]. This specificity ratio, defined as $E = (V/K_m)_A/(V/K_m)_B$, where A and B denote the enantiomers that are respectively hydrolysed first and last, can be conveniently determined from analysis of progress curves using initial ^{14}C-labelled substrate concentrations of 1.2 μM, where $[S]_o \ll K_m$. Indeed, it is known that, under these experimental conditions, the integrated rate equation for irreversible single-substrate reactions simplifies into a pseudo-first-order rate equation which yields directly the ratio V/K_m [36,37]. Using a racemic epoxide as substrate for the hydrolases, the progress curves can be analysed, using a non-linear regression program (Prism; GraphPad Software), according to eqn (3):

$$P(\%) = 50(1-\exp[k_A t]) + 50(1-\exp[k_B t]) \quad (3)$$

where P represents the reaction progress, i.e. formation (in %) of the 9,10-diol product as a function of time (t), and k_A and k_B represent the observed pseudo-first-order rate constants for the hydrolysis of, respectively, the fast- and slow-reacting enantiomers. Eqn (3) also yields:

$$E = (V/K_m)_A/(V/K_m)_B = k_A/k_B$$

To validate the kinetic analysis, we have confirmed the observed selectivity E of the EHs by an independent method. Thus we have determined, at different time points during the enzyme-catalysed hydrolysis of racemic *cis*-9,10-epoxystearic acid, the *e.e.* of the unreacted epoxide by HPLC on a chiral stationary phase (see below). The *e.e.* values of the residual epoxide at different reaction times (reaction progress < 50 %) and for different runs can be used to calculate the specificity ratio according to eqn (4) [38]:

$$E = v_A/v_B = \ln\{(1-c)[1-e.e.(S)]\}/\ln\{(1-c)[1+e.e.(S)]\} \quad (4)$$

where *e.e.*(S) is the *e.e.* of the recovered substrate, c is the extent of substrate conversion, and A and B are the fast- and slow-reacting enantiomers respectively.

Analytical procedures

Radioactivity was measured on TLC plates with a Berthold TLC linear detector LB 2821, and peak integration was obtained by using the program CHROMA 1D (Packard Instrument Co.). The *erythro*- and *threo*-configurations of the products obtained by hydrolysis of this substrate by EHs was determined by GC/MS analysis after methylation of the acid function with ethereal diazomethane and silylation of the diols with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Interchim). GLC analyses of the methyl ester of 9,10-dihydroxystearate trimethylsilyl derivatives were performed on a gas-chromatograph 8000 series (Fisons) coupled to an MS Trio 2000 VG (Micro Max) apparatus with ionizing energy of 70 eV. The sample was injected directly into a DB-5-coated fused silica capillary column (30 m; 0.25 mm internal diameter; J.W. Scientific) with a temperature program of 20 °C/min to 100 °C followed by 0.5 °C/min to 250 °C. Under these conditions the retention times of the *threo*- and *erythro*-derivatives were 38.6 and 39.8 min respectively (i.e. baseline separation). Chiral-phase HPLC was performed, under isocratic conditions, on a Shimadzu instrument coupled to a radiomatic 500TR analyser (Packard Instrument Co.). Peak integration was obtained using FLO-ONE for Windows software. The enantiomeric composition of ^{14}C-labelled methyl-*cis*-9,10-epoxystearate and ^{14}C-labelled methyl-9,10-dihydroxystearate was achieved with, respectively, a Chiralcel OB and an OD (4.6 mm × 250 mm; Baker Chemical Co.) column as already described [25,26].

MS studies were performed on an electrospray ionization quadrupole mass spectrometer (VG BioQ; Micromass, Manchester, U.K.), upgraded by the manufacturer so that the source and the first quadrupole (Q1) have Quattro II performances (m/z range above 4000) and the second quadrupole (Q2) has an extended m/z range (up to 8000). For spectra acquired above m/z 4000, Q1 operates as a high-pass filter in the radiofrequency-only mode to transmit high-mass ions to the second quadrupole. For MS measurements under native conditions, the instrument was calibrated by using the multiply charged ions produced by hen egg-white lysozyme (Sigma) dissolved in water.

RESULTS AND DISCUSSION

Expression and characterization of purified recombinant soybean soluble EH

In 1995, one of us cloned a cDNA from soybean seeds encoding a putative EH [32]. In the present study we confirmed the identity of this gene by expressing it in yeast and finding enzyme activity in the extract that efficiently catalysed the hydrolysis of the oxirane ring of 9,10-epoxystearic acid. Neither extract from wild-type WA6 nor yeast transformed with an empty vector showed any catalytic activity. PCR was used to introduce hexa-histidine tags into the protein to facilitate purification. However, the addition of the tag to the N-terminus of this soybean EH (psNH-EH) was found to cause a severe decrease in its enzyme activity (20 % residual activity compared with the protein lacking the

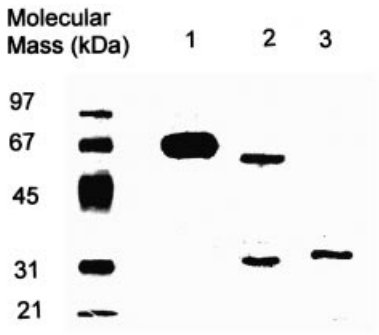

Figure 1 Analysis by PAGE of purified plant EHs

Portions of approx. 2–4 μg of purified maize (lane 1), celeriac root (lane 2) and recombinant soybean (lane 3) EHs were analysed under denaturing conditions on a 12% (w/v)-polyacrylamide gel and their molecular masses were estimated relative to those of standard proteins. The gel was silver-stained.

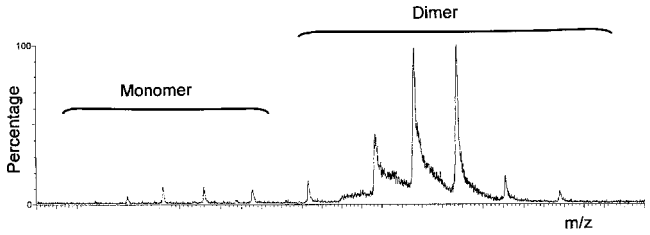

Figure 2 Analysis by electrospray ionization MS of recombinant soybean EH

The enzyme (10 pmol/μl) was prepared under near-physiological conditions (10 μM in 10 mM ammonium acetate, pH 7.0) and then desalted prior to any mass spectrometric measurements by successive dilution/concentration steps using Centricon 10MW microconcentrators. The molecular masses of the observed monomer and dimer are 36628 $\pm$ 9 Da and 73282 $\pm$ 28 Da respectively.

His tag). In contrast, psEH-CH, in which the tag was introduced at the C-terminus, retained more than 75% activity, allowing its purification on an Ni-affinity column, as described in the Experimental section. Silver nitrate staining of the purified enzyme showed on SDS/PAGE gels a single major band at 33 $\pm$ 2 kDa (Figure 1, lane 3), the purity of which was 98%, as assessed by scanning densitometry. Using 9,10-epoxystearate as substrate, kinetic analysis showed an apparent K_m and V_{max} of 25 μM and 18 μmol/min per mg respectively. These values are of the same order of magnitude as found previously for a soluble EH purified from soybean seedlings [4]. We have shown that this latter enzyme was a dimer under native conditions [4]; analysis by electron MS of the recombinant soybean EH gave a similar result (Figure 2), confirming that the soybean soluble EHs exist in solution mainly as dimers. Nevertheless, these two enzymes differ in their optimal pH values. Whereas the purified plant EH presents a pH optimum near 7.5, the recombinant enzyme has a broader maximum, ranging from pH 5.5 to pH 8. It should be noted that a soybean EH was also recently purified from mature dry seeds [39] and its cDNA was found to encode a protein that is identical to our recombinant enzyme (100% identity). This cloned soybean EH processed styrene oxide very poorly [39], a classical substrate for mammalian EHs, confirming that such plant enzymes seem to be quite specific for fatty acid epoxides [4].

Table 1 Specific activities and enantioselectivity of EHs present in soluble extracts of selected plants

Values are means $\pm$ S.D. ($n = 3$).

Plant of origin	Specific activity (pmol/min per mg)	Enantioselectivity (E)
Banana	145 $\pm$ 15	100 $\pm$ 30
Potato	441 $\pm$ 24	900 $\pm$ 200
Rice	205 $\pm$ 18	4 $\pm$ 1.7
Wheat	312 $\pm$ 22	1 $\pm$ 0.05
Maize	115 $\pm$ 12	1 $\pm$ 0.2
Celeriac	1060 $\pm$ 95	45 $\pm$ 15

Characterization of purified plant EHs

Following the same experimental procedures described for the purification of soybean fatty acid EH [4], we purified two other EHs, from maize seedlings and celeriac roots. The molecular masses of these two native proteins, determined by gel filtration, were very close to that established for soybean EH (64 kDa and 62 kDa for maize and celeriac EHs respectively; results not shown). However, SDS/PAGE analysis of maize EH under reducing conditions revealed a single band at approx. 65 $\pm$ 2 kDa (Figure 1, lane 1), whereas celeriac EH was resolved into two distinct bands: a major one at approx. 64 kDa and a minor one at approx. 31 kDa (Figure 1, lane 2). We were unable to define whether the determined molecular mass of maize EH reflects a monomeric or a dimeric enzyme.

Enantioselectivity of the hydrolysis of oleic acid epoxide catalysed by plant and mammalian EHs

Enantioselectivity of plant EHs

All EHs present in the soluble fractions of different organs (fruits, tuber, root, seedlings) of the monocotyledons and dicotyledons tested were able to hydrolyse *cis*-9,10-epoxystearic acid to the corresponding diols (Table 1). However, we found that they differed markedly in their ability to hydrate the oxirane ring of the two enantiomers (i.e. 9*R*,10*S*- and 9*S*,10*R*-epoxystearic acid) of this physiological compound. For example, while soluble EHs from rice or wheat seedlings hydrated both enantiomers at the same rate, those from potato tubers or banana fruits presented a very strong enantioselectivity in favour of 9*R*,10*S*-epoxystearic acid. Similar results were obtained with purified EHs. The soluble EH purified to apparent homogeneity from maize (a monocotyledon from the same family of Poaceae as rice or wheat) apparently lacked the ability to discriminate between the two epoxide enantiomers, i.e. the specificity ratio $E = (V/K_m)_A/(V/K_m)_B$, which reflects the relative rates of reaction with the two enantiomers in a kinetic resolution, was equal to 1. In sharp contrast, the EH purified from celeriac roots preferentially hydrated 9*R*,10*S*-epoxystearic acid ($E = 50$). We were, however, unable to establish any correlation between the enantioselectivity of a given EH and its tissue localization or its phylogenetic origin.

Figure 3 shows a representative progress curve for the hydrolysis of [1-^{14}C]*cis*-9,10-epoxystearic acid catalysed by recombinant soybean EH. Biphasic kinetics were observed, with a marked rate transition at approx. 50% reaction progress. This behaviour is typical of an enantioselective enzyme, and a specificity ratio of $E = 210 \pm 4$ ($n = 4$) was calculated, indicating a very strong preference for the transformation of one epoxide enantiomer. The residual epoxide was analysed by chiral-phase HPLC at different reaction times, and 9*R*,10*S*-epoxystearic acid

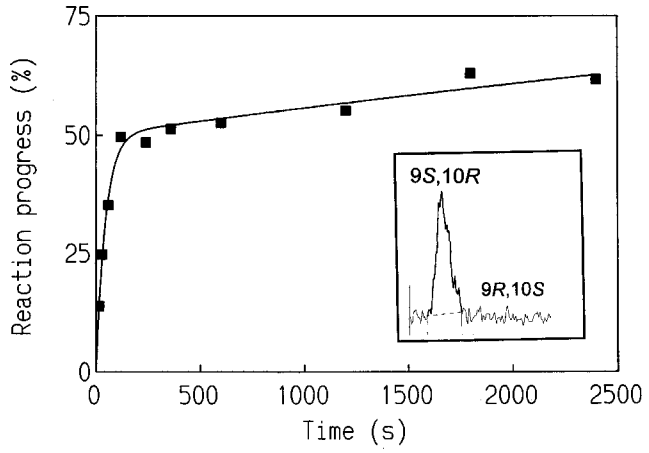

Figure 3 Representative progress curve of the hydrolysis of racemic [1-^{14}C]*cis*-9,10-epoxystearic acid catalysed by recombinant soybean EH

Substrate (1.2 μM) was incubated at 26 °C in the presence of diluted enzyme (1 μg of protein) in 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 500 μl. Aliquots were taken at the times indicated and analysed by TLC as described in the Experimental section. The solid line represents the theoretical curve obtained by fitting the data with a non-linear regression program (see [25]). Inset: chiral-phase HPLC of the unreacted epoxide after 50% reaction progress.

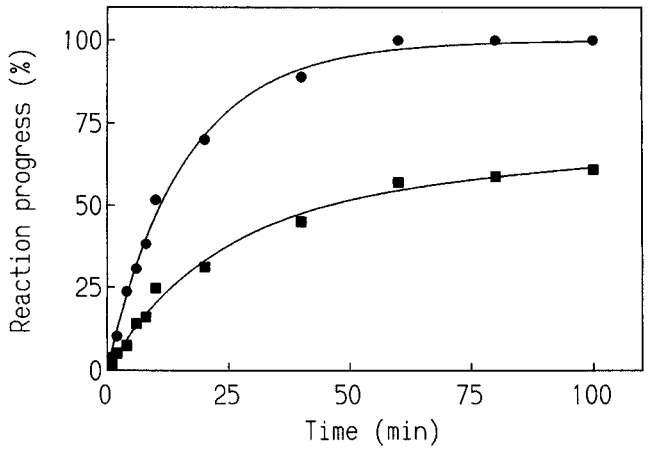

Figure 4 Progress curves of the hydrolysis of racemic [1-^{14}C]*cis*-9,10-epoxystearic acid catalysed by EHs isolated from healthy and TMV-infected tobacco leaves

The experimental conditions were the same as those of Figure 3 (600 μg of protein in 600 μl final volume). ■, Healthy leaves; ●, TMV-infected leaves.

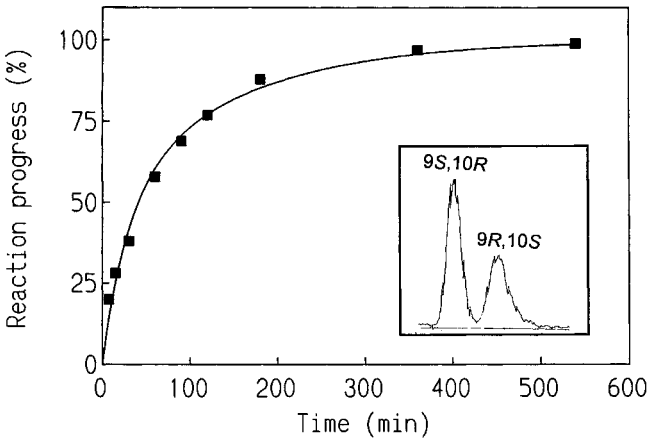

Figure 5 Progress curve of the hydrolysis of [1-^{14}C]*cis*-9,10-epoxystearic acid catalysed by rat liver soluble EH

Substrate (1.2 μM) was incubated at 37 °C in the presence of the enzyme (10 μg) in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5% BSA in a final volume of 500 μl. At given times, aliquots were taken and analysed by TLC as described in the Experimental section. Inset: chiral-phase HPLC of the unreacted epoxide after 55% reaction progress.

was found to be the preferred substrate enantiomer for the recombinant enzyme (Figure 3, inset). The measurement of the *e.e.* of the residual epoxide also allowed us to calculate the specificity ratio according to the equation given in [38] (see the Experimental section); the value $E = 180 \pm 30$ ($n = 4$) was in good agreement with that determined independently by kinetic analysis of the progress curves. The enantioselectivity was largely abolished when racemic methyl-*cis*-9,10-epoxystearate was used as substrate ($E = 8 \pm 3$; $n = 4$). Similar results have been obtained previously with soluble EH purified from soybean seedlings, although its specificity ratio for the two 9,10-epoxystearic acid enantiomers was less pronounced ($E = 20$) [25].

The differences in the extent of enantioselectivity and pH dependence of the recombinant EH (see above) compared with those exhibited by the EH we have previously purified and characterized from soybean seedlings [4,25] led us to conclude that we have probably cloned an isoform of this enzyme. Indeed, different isoforms of soluble EHs are present in soybean, since we have been able to identify at least three cytosolic EHs which fractionate differently on precipitation by $(NH_4)_2SO_4$ (E. Blée, unpublished work). Moreover, it has been reported that, in addition to constitutive EHs, EH activity could also be induced by wounding, water deprivation or plant hormones such as auxin or methyl jasmonate, or during infection by viruses [23,24]. Accordingly, we have found an approx. 3-fold increase in EH activity in TMV-infected tobacco leaves (i.e. 1200 pmol of diols formed/min per mg of protein for TMV-infected leaves, compared with 450 pmol of diols formed/min per mg for healthy control leaves). Leaves from untreated tobacco plants possess a constitutive EH activity characterized by a marked enantioselectivity against 9,10-epoxystearic acid ($E = 40$) (Figure 4). Strikingly, the EH present in TMV-infected tobacco hardly discriminated between the two enantiomers ($E = 1.5$) (Figure 4). We have observed a similar lack of enantioselectivity for the EH extracted from tobacco leaves infected by the fungus *Erysiphe graminis* ($E = 3$). Altogether, it seems that several forms of soluble EH can exist in either the constitutive or the inducible form; moreover, the EHs induced by pathogen challenges show a much lower preference for one particular enantiomer over another, relative to that of the constitutive enzymes, and this could be linked to the putative role of the induced EHs as detoxifying enzymes in plant defence mechanisms.

Enantioselectivity of rat liver EHs

The oxidized fatty acid 9,10-epoxystearic acid was found to be a substrate for both cytosolic and membrane-bound rat liver EHs [18]. However, the two enantiomers of this epoxide were hydrolysed with very different efficacies by these two enzymes. Thus the soluble mammalian EH opened the oxirane ring of both 9,10-epoxystearic acid enantiomers at rates that were independent of their configuration (Figure 5). From this point of view, the

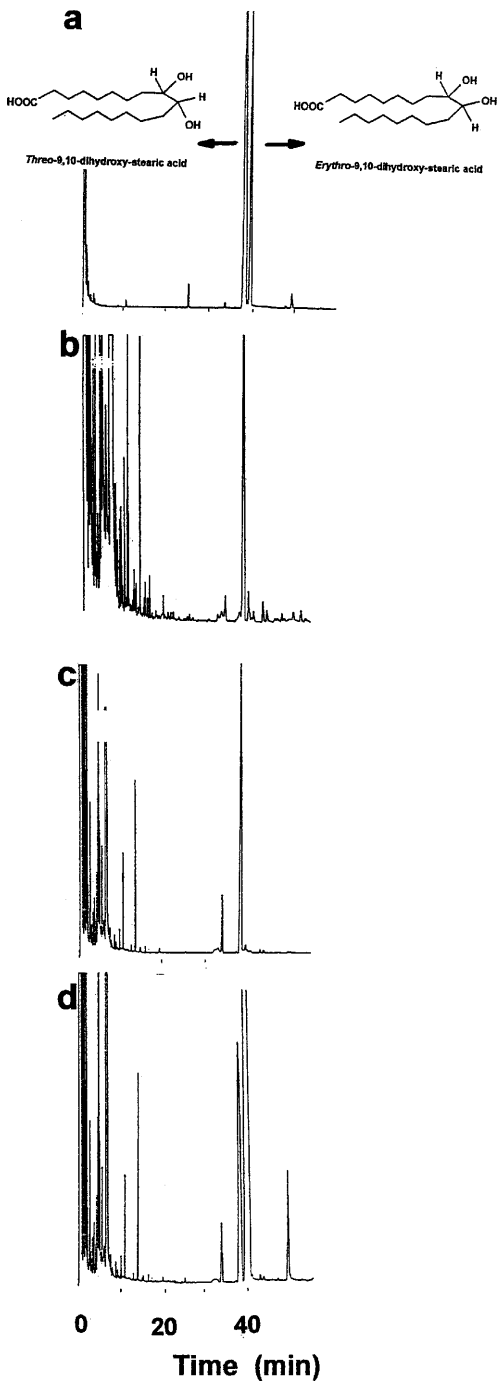

Figure 6 Molecular mechanism of the oxirane opening of 9,10-epoxystearic acid by soluble maize and rat liver EHs: analysis of the resulting 9,10-dihydroxystearates

Analysis by GC of a racemic mixture of *erythro*- and *threo*-9,10-dihydroxystearic acid (**a**) and of 9,10-dihydroxystearic acid resulting from the hydrolysis of *cis*-9,10-epoxystearic acid catalysed by maize EH (**b**) and cytosolic rat liver EH (**c**). In (**d**) the diols obtained in (**c**) were co-injected with standard *erythro*-9,10-dihydroxystearic acid. Before GC analysis, the acid functions of the 9,10-dihydroxystearic acids were methylated with ethereal diazomethane and the hydroxy groups were silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide.

kinetic profiles of the hydrolysis of racemic fatty acid epoxides catalysed by this enzyme were more akin to those observed with EHs from cereals, such as maize. In addition, we have verified that the opening of the oxirane ring was catalysed according

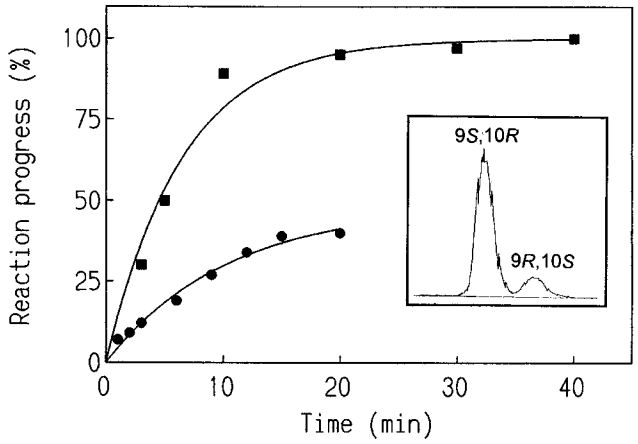

Figure 7 Progress curve of the hydrolysis of [1-^{14}C]*cis*-9,10-epoxystearic acid and its methyl ester catalysed by rat hepatic mEH

Both substrates (1.2 μM) were incubated at 37 °C in presence of the enzyme (125 μg) in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5% BSA in a final volume of 550 μl. Samples were worked up as described in the legend to Figure 3. ●, 9,10-Epoxystearic acid; ■, methyl-9,10-epoxystearate. Inset: chiral-phase HPLC of the unreacted epoxide after 40% reaction progress of the hydrolysis of 9,10-epoxystearic acid.

to similar reaction pathways by both types of enzymes. Indeed, we found that soluble rat and maize EHs catalysed the *trans*-opening of *cis*-9,10-epoxystearate, yielding in both cases exclusively *threo*-9,10-dihydroxystearate (Figure 6).

In sharp contrast with the cytosolic mammalian enzyme, 9*S*,10*R*-epoxystearic acid appeared to behave as a very poor substrate for rat mEH. In fact, mammalian mEH, which had a strong preference for 9*R*,10*S*-epoxystearic acid (Figure 7), showed a similar enantiopreference as the recombinant soybean enzyme. Moreover, for both enzymes, the enantioselectivity could be largely abolished on methylation of the carboxylic function of epoxystearic acid (Figure 7). Such a result confirms and extends to the microsomal hepatic enzyme the prevalent role of the carboxylate group in the positioning of the fatty acid epoxide within the active site of these enzymes, as suggested previously for the purified soybean enzyme [25].

All mEHs analysed so far, i.e. those from mammals and insects, have a glutamic residue as part of their catalytic triad (Asp, His, Glu) instead of the aspartate (Asp, His, Asp) that is present in soluble mammalian EHs [40]. It is assumed that in such a triad a carboxylate residue may assist the histidine in its general-base function by stabilizing the positive charge on the imidazole ring ensuing from the water molecule activation. This acid residue may also play a structural role, e.g. by influencing the positioning of the active-site histidine or by taking part in structuring of the active site. Unexpectedly, it has been shown that replacing the catalytic Glu^{404} of rat liver mEH by Asp (Glu^{404}Asp) markedly improved the turnover rate of the mutant [31]. It was proposed that native mEH has a glutamic residue in place of the aspartate because the higher hydrolytic activity of the mutant might have adverse effects for the organism. For example, the diols resulting from the hydrolysis of fatty acids epoxides were found to be more toxic and to act as signal molecules in pathological processes [41]. Interestingly, this mutation did not alter the enantioselectivity of Glu^{404}Asp against 9,10-epoxystearate, suggesting that Glu^{404} does not play a fundamental role in positioning the lateral chains of 9,10-epoxystearic acid within the active site of mEH. Moreover, it should be noted that the

Table 2 Enantioconvergence of plant EHs

Diols resulting from enzyme-catalysed hydrolysis of racemic [1-^{14}C]*cis*-9,10-epoxystearic acid were separated by chiral-phase HPLC on a Chiralcel OD column after their methylation with diazomethane. Values are expressed as means ± S.D. ($n = 5$, except for banana, rice and tobacco EHs, where $n = 3$). *e.e.* was expressed as: $([9R,10R] - [9S,10S])/([9R,10R] + [9S,10S]) \times 100$.

Plant of origin	*e.e.* (%)
Celeriac	30 ± 0.6
Maize	50 ± 1.4
Wheat	59 ± 0.5
Banana	63.5 ± 0.5
Rice	74 ± 0.6
Soybean	94 ± 0.4
Potato	96 ± 0.6
Tobacco (constitutive)	90 ± 0.5
Tobacco (induced)	86 ± 0.7

9*R*,10*S*-enantiomer, the substrate that is preferentially hydrolysed by plant EHs, also remained the best substrate for both the mutant and wild-type mEHs (Figure 7, inset).

Enantioconvergence of plant and rat EHs

Using ^{18}O-labelling experiments and chiral HPLC analysis, we have shown previously that soybean fatty acid EH catalyses the hydration of racemic 9,10-epoxystearic acid with a highly striking stereospecificity leading to the formation of a single optically active diol, i.e. *threo*-9*R*,10*R*-dihydroxystearic acid, even after complete conversion of the substrate [25]. To explain such a stereochemical outcome (i.e. deracemization), we have proposed a reaction model according to which the soybean EH docks the substrate within its active site in such a fashion that the oxirane ring moiety of this quasi *meso* substrate can adopt only a single orientation, where the attacking nucleophile (presumably the carboxylate of the catalytic triad) faces the epoxide carbon having the (*S*)-configuration [25]. This preference for the (*S*)-configured centre can be achieved by flipping either enantiomer by 180 ° within the active site, relative to the putative carboxylate and an epoxide-activating (i.e. general-acid catalysis) functionality. Recently, polarization of the epoxide was shown to be performed by a tyrosine residue in the active site of bacterial and mammalian EHs [42–44], but such a proton source which assists the initial acylation event has yet to be identified for plant enzymes.

Similar to the purified soybean enzyme, the recombinant soybean EH also catalysed a selective oxirane ring opening of racemic 9,10-epoxystearic acid at the (*S*) carbons, yielding the (*R*,*R*)-diol with a high *e.e.* (Table 2). Notably, the carboxylate group does not play a role in the stereochemistry of epoxide ring opening, since a similar *e.e.* was observed when the methyl ester of the epoxide was used as substrate. This remarkably high product enantioconvergence was not restricted to soybean enzymes, since it could also be observed for potato EH (Table 2); interestingly, these two EHs also exhibit an important enantioselectivity in favour of the 9*S*,10*R*-epoxystearate. It should be stressed that enantioconvergence is not a property that is shared by all plant EHs, and this is particularly true for the EHs that also present lower enantioselectivities, such as those found in cereals (Table 2). In the case of these latter enzymes, although the hydrolysis of racemic 9,10-epoxystearic acid resulted mainly in the formation of 9*R*,10*R*-dihydroxystearic acid, some diol with the opposite configuration was also formed. This result is consistent with an attack by the nucleophilic residue of the enzyme

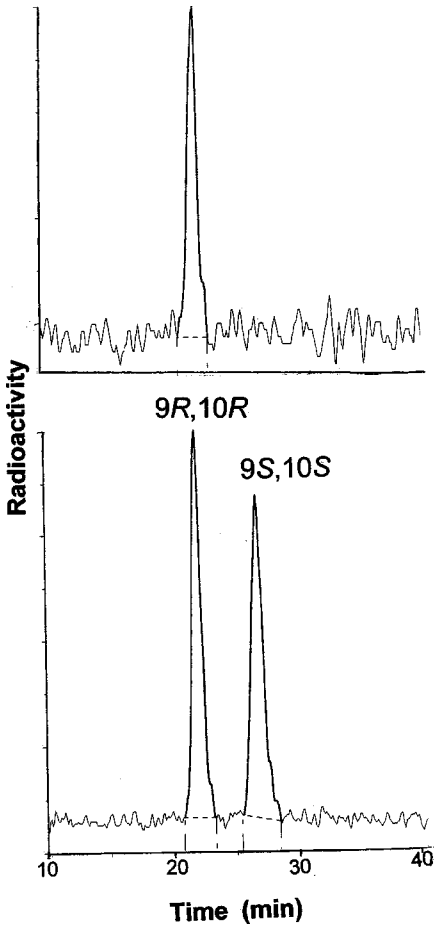

Figure 8 Chiral-phase HPLC analysis of methyl 9,10-dihydroxystearates

The 9,10-dihydroxystearates resulting from the complete hydrolysis of racemic [1-^{14}C]*cis*-9,10-epoxystearic acid catalysed by mEH (upper panel) and soluble EH (lower panel) were methylated with diazomethane. The separation of the methyl esters was achieved on a Chiralcel OD column.

also occurring on the (*R*)-configured carbon, possibly due to a looser positioning of the epoxide ring within the active site of these enzymes, resulting in a lower discrimination between the two oxirane carbons. At this point it should be underlined that a lower enantioconvergence (deracemization of the substrate) of such EHs is not inevitably correlated with a lower enantioselectivity. Indeed, the possibility of discriminating between the two epoxide enantiomers was due to the positioning of the alkyl chain extension from the oxirane ring within the subdomains of the enzyme substrate binding sites, independent of the anchoring of the epoxide group. Accordingly, we observed that celeriac and banana EHs, which exhibited a marked preference for hydrolysing 9*R*,10*S*-epoxystearic acid, did not present a strict enantioconvergence (Table 2). Conversely, constitutive and induced tobacco EHs, which diverge in their enantioselective properties, are both characterized by a high degree of enantioconvergence (Table 2).

Rat liver mEH shared a distinctive degree of stereoselectivity with the soybean enzymes. Thus only a single optically active diol, i.e. *threo*-9*R*,10*R*-dihydroxystearic acid, was observed after complete enzymic hydrolysis of the two enantiomers of 9,10-epoxystearic acid (Figure 8, upper panel). Similar results in the

Table 3 Enantioconvergence exhibited by rat soluble EH and maize EH with pure enantiomers of 9,10-epoxystearic acid and their methyl esters

Values are means ± S.D. ($n = 3$).

	Product (%)			
	Rat soluble EH		Maize EH	
Substrate	9*R*,10*R* diol	9*S*,10*S* diol	9*R*,10*R* diol	9*S*,10*S* diol
9*R*,10*S*-Epoxystearic methyl ester	79 ± 0.4	21 ± 0.4	83 ± 0.2	17 ± 0.2
9*R*,10*S*-Epoxystearic acid	80 ± 0.2	20 ± 0.2	84 ± 0.3	16 ± 0.3
9*S*,10*R*-Epoxystearic methyl ester	48 ± 0.3	52 ± 0.3	68 ± 0.2	32 ± 0.2
9*S*,10*R*-Epoxystearic acid	39 ± 0.2	61 ± 0.2	52 ± 0.4	48 ± 0.4

opening of racemic 9,10-epoxystearic acid have been reported previously for rabbit mEH [20]. It is noteworthy that the Glu^{404}Asp mutant of rat liver mEH has retained the same complete discrimination between the *R* and *S* positions of the oxirane ring as the wild-type enzyme, for which an exclusive attack on the *S* centres was observed. This result confirms that Glu^{404} does not play a major structural role in the active site of mEH. In sharp contrast with the aforementioned results, nearly racemic dihydroxystearic acid (9*R*,10*R*, 58 %; 9*S*,10*S*, 42 %) was produced after complete transformation of racemic 9,10-epoxystearic acid by cytosolic rat liver EH (Figure 8, lower panel). Two possibilities could account for this lack of stereoselectivity: either a non-regioselective attack by the catalytic aspartate on both C-9 and C-10 with inversion of configuration, or a regiospecific but non-stereoselective attack (i.e. that does not discriminate the *R* and *S* configurations). To discriminate between these two possibilities, we have carried out the hydrolysis of enantiomerically pure *cis*-9,10-epoxystearates and their methyl esters with purified recombinant EH from cytosolic rat liver. In addition, we have also determined the absolute configuration, by chiral-phase HPLC, of the diols resulting from the complete hydration of each of these enantiomers catalysed by mEH and by purified recombinant soybean and maize EHs. Microsomal mammalian and soybean EH-catalysed hydrolysis of methyl-9*R*,10*S*-epoxystearate resulted in the major formation of 9*R*,10*R*-dihydroxystearic acid with a very high *e.e.* (> 99 %). In contrast, striking differences in the stereochemical outcome of the complete conversion of the other enantiomer, 9*S*,10*R*-epoxystearic acid, were observed. As expected, recombinant soybean EH promoted the hydrolysis of this substrate via the exclusive attack of the (*S*)-configured carbon, resulting into a single diol, i.e. 9*R*,10*R*-dihydroxystearate. The same diol was also the favoured reaction product (*e.e.* = 36 %) with maize EH (Table 3). In this case, however, some *threo*-9*S*,10*S*-dihydroxystearate was also formed (approx. 30 %), which presumably resulted from an attack by the catalytic residue of this enzyme on the 10*R* carbon. Note that the orientation of the favoured carbon depends to a certain extent on the presence of a free carboxylic group in the substrate at C-1; as shown in Table 3, the presence of a methyl ester resulted in increased stereoselectivity of the maize enzyme in favour of the oxirane carbon centre of *S* chirality. A remarkable enantioconvergence was also observed in the reactions catalysed by both rat liver mEH and its mutant Glu^{404}Asp, which hydrolysed 9*S*,10*R*-epoxystearate into a single diol, i.e. *threo*-9*R*,10*R*-dihydroxystearate. In sharp contrast, using the same substrate, rat liver soluble EH was found to catalyse mainly the formation of the diol possessing the inverse configuration, i.e. 9*S*,10*S*-dihydroxystearate (Table 3). Thus our results on reactions catalysed by the rat liver soluble EH are in good agreement with a regiospecific attack on C-10 of both enantiomers of 9,10-epoxystearic acid, resulting in inversion of configuration at this carbon. Hydration of the methyl ester of this fatty acid epoxide led to some decrease in the enantioconvergence (*e.e.* = 64 % for the acid compared with 40 % for the methyl ester) (Table 3), suggesting that, in contrast with the reactions catalysed by murine EH [16], the anchoring of the carboxylic acid or ester within the active site of the rat soluble EH plays some role in the regioselective attack on C-10 of 9,10-epoxystearic acid by the nucleophile group of its active site.

Conclusions

9,10-Epoxystearic acid has proven to be a very useful substrate for discriminating among the different reaction pathways leading to the formation of the acyl–enzyme intermediate that represents the first step of the EH-catalysed hydration of epoxides. EHs can be differentiated by distinct regioselective or stereoselective attacks performed by the nucleophilic carboxylate residue of the catalytic triad that is presumably present in all their catalytic domains. The versatile tool used in the present study revealed both similarities and differences, hitherto unsuspected, in the oxirane-opening mechanism between plant and animal EHs, which indicated distinct topological properties of their active sites. For example, the docking of both enantiomers of 9,10-epoxystearate in the active site of the soybean and potato enzymes imposes a stereoselective attack prevalently at the *S*-carbons that yields a (*R*,*R*) diol with high *e.e.* Similarly, rat liver mEH and its mutant Glu^{404}Asp were also found to hydrolyse fatty acid epoxides with complete stereopecificity to give optically pure *threo*-(*R*,*R*)-diols. It seems likely, therefore, that these enzymes share similar structural determinants in their active site that allow a rather unique positioning of the epoxide moiety of 9,10-epoxystearate with respect to the catalytic residue; such a result could not be predicted solely from a comparison of the nucleotide sequences of these proteins, which show only a low degree of identity. Because of their distinctive deracemization properties, EHs such as those from soybean and potato or the Glu^{404}Asp mutant of rat mEH could be used as reagents for easy access to optically active diols; as such, their potential should be of practical interest to organic chemists. In addition, because hydrolyses catalysed by these enzymes are also characterized by a very high enantioselectivity, one could also envision the preparation of enantiomerically pure residual epoxides that could be useful as chiral synthons after 50 % reaction progress. Based on previous results, we have suggested that the preferential hydrolysis of one enantiomer of 9,10-epoxystearic acid by soybean EH,

which is largely abolished upon esterification of the carboxylic function, could be due to an orientation of the substrate in the active site that is probably governed by repulsion between the carboxylate and a residue of the enzyme [25].

In contrast with soybean EH or rat mEH, soluble EHs from rat liver and from cereals were found to catalyse a non-enantioselective hydrolysis of 9,10-epoxystearic acid. These enzymes probably lack the structural elements of the EH discussed above that influence the positioning of the alkyl chains that extend from the oxirane ring within subdomains of the enzyme substrate-binding sites and result in enantioselection. However, these non-enantioselective EHs of mammalian and plant origin differ markedly in the structural features that permit the docking of the epoxide group within their catalytic sites. These dissimilarities stipulate not only the substrate specificity of these enzymes [45], but, importantly, also the nature of the resulting products. Whereas for maize EH a nucleophilic attack on the carbons of the (*S*) configuration was still favoured, giving an optically enriched diol, regioselective formation of a covalent acyl–enzyme intermediate occurs on C-10 of 9,10-epoxystearic acid in the catalytic site of cytosolic rat liver EH, resulting primarily in the formation of racemic diols. This suggests that the epoxide group of 9,10-epoxystearic acid is probably anchored in a very different fashion in the active site of rat liver soluble EH compared with that in the soybean enzyme. Consequently, modelling of soybean EH active sites by homology with the known tridimensional structure of soluble mouse EH [46] would probably lead to somewhat biased information with regard to the stereochemical outcome of the catalysed reactions.

To continue to probe the active sites of these enzymes, several approaches could be envisaged. Ideally, three-dimensional structures of the EHs that exhibit strict enantioselectivity and enantioconvergence and of their mutants should be very helpful in identifying the residues responsible for these very interesting stereochemical features. At present, however, only three structures of EHs are known (those from mouse [46], *Agrobacterium radiobacter* [47] and *Aspergillus niger* [48]), but they are unfortunately devoid of the strict stereospecificity leading to an optically pure diol after complete hydrolysis of racemic epoxide. Therefore the analysis of such structures will probably not give pertinent clues to the mapping of the active site of soybean EH. Other alternatives are site-directed or random mutagenesis experiments which may help to elucidate the identity of the residues that anchor the epoxide group into, for example, the soybean EH active site in such a way that only strict stereochemistry of ring epoxide opening could occur. We are presently exploring these different strategies.

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