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Visualization of a covalent intermediate between microsomal epoxide hydrolase, but not cholesterol epoxide hydrolase, and their substrates

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Mammalian soluble and microsomal epoxide hydrolases have been proposed to belong to the family of α/β-hydrolase-fold enzymes. These enzymes hydrolyse their substrates by a catalytic triad, with the first step of the enzymatic reaction being the formation of a covalent enzyme-substrate ester. In the present paper, we describe the direct visualization of the ester formation between rat microsomal epoxide hydrolase and its substrate. Microsomal epoxide hydrolase was precipitated with acetone after brief incubation with [1-^{14}C]epoxystearic acid. After denaturing SDS gel electrophoresis the protein-bound radioactivity was detected by fluorography. Pure epoxide hydrolase and crude microsomes showed a single radioactive signal of the expected molecular mass that could be suppressed by inclusion of the competitive inhibitor 1,1,1-trichloropropene oxide in the incubation mixture. In a similar manner, 4-fluorochalcone-oxide-sensitive binding of epoxystearic acid to rat soluble epoxide hydrolase could be demonstrated in rat liver cytosol. Under similar conditions, no covalent binding of [26-^{14}C]cholesterol-5α,6α-epoxide to microsomal proteins or solubilized fractions tenfold enriched in cholesterol epoxide hydrolase activity could be observed. Our data provide definitive proof for the formation of an enzyme-substrate-ester intermediate formed in the course of epoxide hydrolysis by microsomal epoxide hydrolase, show no formation of a covalent intermediate between cholesterol epoxide hydrolase and its substrate under the same conditions as those under which an intermediate was shown for both microsomal and soluble epoxide hydrolases and therefore indicate that the cholesterol epoxide hydrolase apparently does not act by a similar mechanism and is probably not structurally related to microsomal and soluble epoxide hydrolases.

Keywords: epoxide hydrolase; mechanism; α/β hydrolase fold; cholesterol; fatty acid metabolism.

Epoxide hydrolases (EH) represent a group of ubiquitous enzymes with important functions in the detoxification of reactive intermediates, namely epoxides, that arise from a large variety of compounds during their metabolism. The two mammalian enzymes implicated in the metabolism of foreign compounds are microsomal EH (Oesch, 1973) and soluble EH (Ota and Hammock, 1980). Both enzymes have been cloned from a variety of species (Beetham et al., 1993; Grant et al., 1993; Jackson et al., 1987; Knehr et al., 1993; Porter et al., 1986; Wojtasek and Prestwich, 1996). A third enzyme, cholesterol EH, which is membrane bound similarly to microsomal EH but otherwise distinct from the latter (Oesch et al., 1984), is less well investigated. Its physiological function appears to be the conversion of 5α,6α-epoxycholestane-3β-ol and 5β,6β-epoxycholestane-3β-ol, the two epoxides arising from cholesterol during, e.g. lipid peroxidation, to give the single product cholestane-3β,5α,6β-triol (Watabe et al., 1981).

For a long time it was believed that EH convert their substrates by direct hydrolysis. The pioneering work of Hanzlik et al. (1976) and DuBois et al. (1978) seemed to indicate a general base-catalysis mechanism, in that a specific histidine residue of the enzyme was proposed to activate a water molecule by proton abstraction, which in turn would hydrolyze the epoxide by nucleophilic attack. Armstrong et al. (1980) however, suggested a possible alternative mechanism. They proposed that a nucleophilic attack of an acidic amino acid residue at the epoxide ring could lead to the transient formation of an ester bond between substrate and enzyme that could be hydrolyzed by a water molecule, activated as described above, to yield the respective vicinal diol and the regenerated enzyme (Fig. 1). The authors themselves disfavoured this mechanism for a long time because of their inability to isolate the postulated covalent intermediate formed between enzyme and substrate (Armstrong, 1987; Armstrong et al., 1980). Eventually, they presented strong support for the two-step mechanism by showing ^{18}O transfer from microsomal EH to the substrate 1,10-phenanthroline 5,6-oxide in single-turnover experiments (Lacourciere and Armstrong, 1993), an observation that is best explained by the intermediate formation of an enzyme-substrate ester.

At the same time, we gathered strong support for the mechanism involving the covalent intermediate by a different approach. Analysis of the phylogenetic relationship between microsomal EH and soluble EH revealed their common origin and indicated that they belong to a large superfamily of hydrolytic enzymes of diverse function related by amino-acid-se-

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Abbreviation. EH, epoxide hydrolase.
Enzyme. Epoxide hydrolase (EC 3.3.2.3).

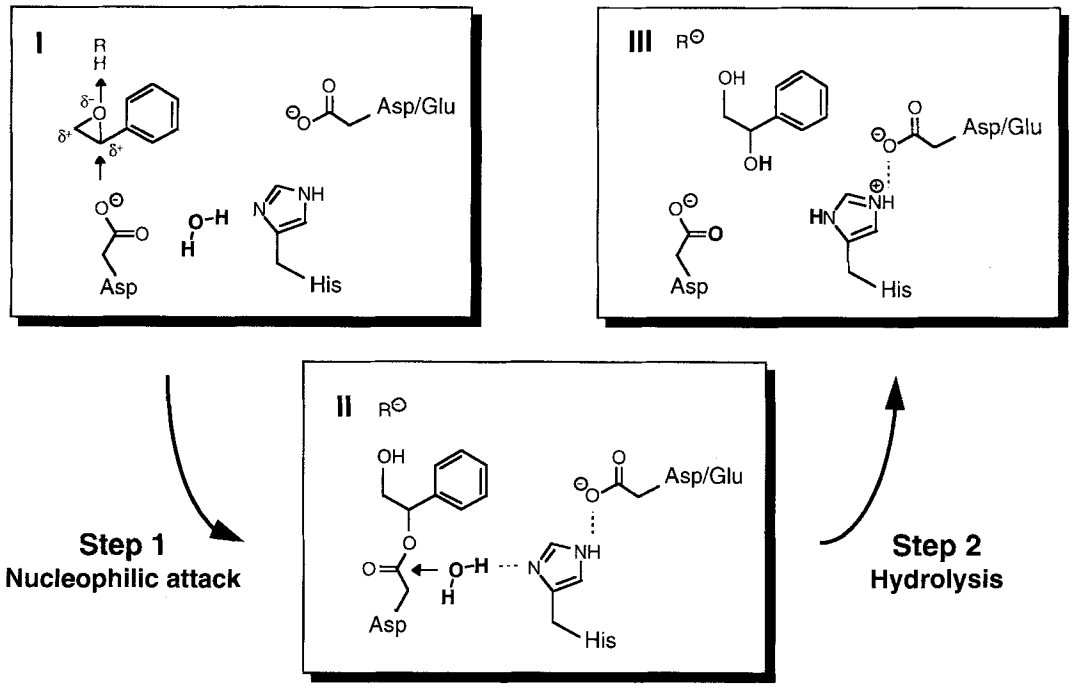

Fig. 1. Enzymatic mechanism of epoxide hydrolysis. The presentation is adapted from Arand et al. (1994).

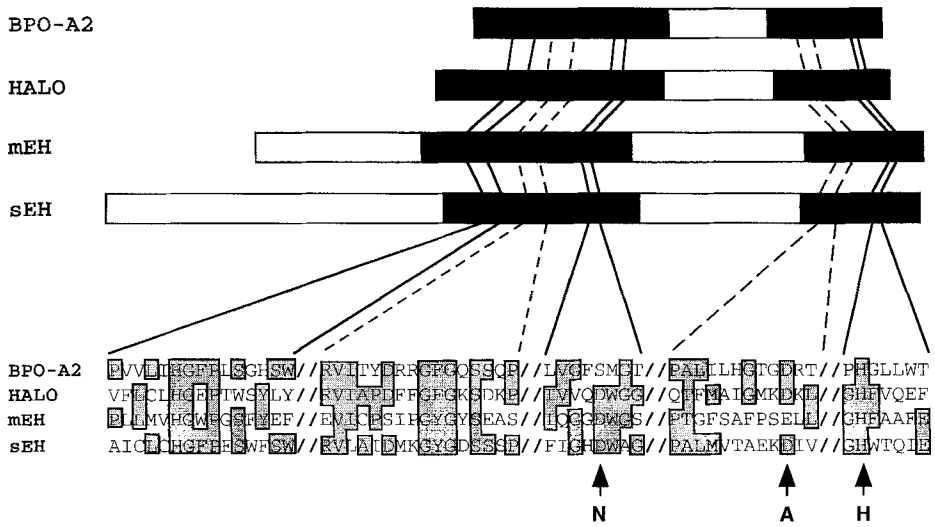

Fig. 2. Amino-acid-sequence alignment of microsomal EH and soluble EH with bacterial α/β-hydrolase-fold enzymes. The distant sequence similarity of epoxide hydrolases to two bacterial α/β-hydrolase-fold enzymes of resolved three-dimensional structure is represented by comparing the regions of highest similarity, including those around the residues forming the catalytic triad (indicated by arrows; N, catalytic nucleophile, i.e. Asp or Ser; A, acidic residue, i.e. Asp or Glu; H, His). The horizontal bars in the upper part of the diagram represent the coding regions of the cDNAs, with the black boxes representing the parts that form the α/β-hydrolase fold. Small parts of the sequences representing regions of high similarity and/or known important function are aligned in the lower part of the diagram. Residues are boxed when at least 50% of the sequences have the identical residue at the respective position. BPO-A2, bromoperoxidase A2 from *Streptomyces aureofaciens* (Hecht et al., 1994); HALO, haloalkane dehalogenase from *Xanthobacter autotrophicus* (Franken et al., 1991); mEH, microsomal EH; sEH, soluble EH.

quence similarity (Arand et al., 1994; Beetham et al., 1995; for a representative sequence alignment of a small subset of these related sequences see Fig. 2). Among these, the haloalkane dehalogenase has been studied in most detail (Janssen et al., 1989; Verschueren et al., 1993). On the basis of its three-dimensional structure it has been grouped into the superfamily of α/β-hydrolase-fold enzymes, the most prominent member of which is acetylcholine esterase (Franken et al., 1991; Ollis et al., 1992). These enzymes have a catalytic triad with similarity to that of different protease families (Ollis et al., 1992). They form covalent intermediates with their substrates by means of nucleophilic attack. These intermediates are subsequently hydrolyzed by a histidine-activated water molecule. The inferred membership of epoxide hydrolases in this group of enzymes lent strong support to the two-step mechanism for the enzymatic hydrolysis of epoxides proposed by Armstrong et al. (1980). We substantiated this hypothesis for soluble EH by co-precipitating radioactive substrate during denaturing precipitation of the enzyme and subsequent chemical analysis of the nature of its observed association with the substrate (Hammock et al., 1994). Subsequently,

we were able to identify the members of the soluble EH catalytic triad by site-directed mutagenesis (Arand et al., 1996; Pinot et al., 1995).

In the present study, we extend our work to microsomal EH and cholesterol EH. We present definitive proof for the formation of a covalent intermediate during epoxide hydrolysis by microsomal EH by directly visualizing the resulting enzyme-substrate ester. Furthermore, we provide evidence that cholesterol EH probably does not work by the same mechanism because we do not find any evidence for the formation of an ester intermediate between cholesterol EH and cholesterol epoxide by means of the same experimental approach.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Oleic acid (50 μCi/μmol) and [26-^{14}C]cholesterol (52 μCi/μmol) were purchased from NEN Dupont. [26-^{14}C]Cholesterol-5α,6α-epoxide (Watabe et al., 1981), [^{3}H]styrene oxide (Oesch et al., 1971a), [^{3}H]trans-stilbene oxide (Oesch et al., 1980), and 4-fluorochalcone oxide (Turner et al., 1971) were synthesized essentially as described earlier. 1,1,1-Trichloro-2,3-propene oxide was purchased from EGA-Chemie (but is no longer commercially available).

Synthesis of *cis*-9,10-epoxystearic acid and 9,10-dihydroxy stearic acid. Synthesis of *cis*-9,10-epoxystearic acid was performed with dimethyl dioxirane (Murray and Jeyaraman, 1985), similar to the procedure recently described by Borhan et al. (1995). Oleic acid was dissolved at 100 mM in acetone. 5 vol. 100 mM dimethyl dioxirane in acetone, prepared as described by Murray and Jeyaraman (1985), were added and the mixture was kept at room temperature for 1 h. The solvent and any excess oxidant were evaporated under a gentle stream of nitrogen to afford the product as a colourless solid. High-field-proton-NMR analysis of the product confirmed quantitative formation of *cis*-9,10-epoxystearic acid and the absence of any detectable by-products {400-MHz ^{1}H NMR ($CDCl_3$) δ 2.88 (bs, 2H, H9, H10) 2.33 [*t*, 2H, -CH_2- (C2), J = 7.4 Hz] 1.63−1.25 [*m*, 26H, -CH_2- (C3−C8 and C11−C17) 0.86 (*t*, 3H, CH_3, J = 6.65 Hz}. For storage, the isolated compound was dissolved in acetonitrile at 1 mM and kept frozen in aliquots at −80 °C until use. Under otherwise identical experimental conditions, [1-^{14}C]oleic acid, 50 μCi/μmol, was used to synthesize ^{14}C-labelled *cis*-epoxystearic acid. The identity and purity of the labelled product were assessed by thin-layer chromatography on silica gel 60 plates with *n*-hexane/diethyl ether/formic acid (30:70:2, by vol.) as solvent system, as described by Blée and Schuber (1992). The labelled material was detected by autoradiography of the chromatogram. The unlabelled *cis*-9,10-epoxystearic acid, analyzed on the same plate as reference material, was visualized by developing the plate in a chamber containing iodine vapour. The R_f values of the labelled and the unlabelled products (R_f = 0.5) were identical. 9,10-Dihydroxy stearic acid was prepared by incubating 10 μmol *cis*-9,10-epoxystearic acid with 200 μg rat soluble EH in 75 mM sodium phosphate, pH 7.4, overnight at ambient temperature in 0.5 ml. After extraction with 1 ml of ethyl acetate, the only product in the organic phase detectable by thin-layer chromatography performed under the conditions described above showed a significantly lower R_f value compared with that of the epoxide (R_f = 0.2 vs R_f = 0.5), indicating the quantitative formation of the desired product.

Purification of enzymes. Microsomal EH was purified to homogeneity from the livers of *trans*-stilbene-oxide−treated male Sprague Dawley rats essentially as described earlier (Bentley and Oesch, 1975). Rat soluble EH was expressed in Sf21 insect cells after infection with a recombinant baculovirus harbouring the epoxide hydrolase cDNA (Knehr et al., 1993) under the control of the p10 promoter similar to that described for the human soluble EH by Beetham et al. (1993). The recombinant enzyme was purified from the 100000×*g* supernatant of the cell homogenate by the method of Wixtrom et al. (1988).

Cholesterol EH was enriched by hydrophobic-interaction chromatography on phenyl-Sepharose as described in the following. Rat liver microsomes, prepared by standard procedures (Friedberg et al., 1992), were suspended in buffer A (10 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, pH 7.4) to yield a protein concentration of 10 mg/ml in 50 ml.

Solubilization was performed by addition of solid sodium chloride and Chaps to 2 M and 1%, respectively. The sample was kept on ice for 1 h and centrifuged at 100000×*g* and 4 °C for 1 h. The resulting supernatant was dialyzed overnight against 100 vol. buffer A containing 2 M sodium chloride to yield a theoretical concentration of 0.01% Chaps. After centrifugation at 100000×*g* for 1 h the sample was loaded on a phenyl-Sepharose CL-4B column (12 cm×2.5 cm), previously equilibrated with dialysis buffer containing 0.01% Chaps, at a flow rate of 1 ml/min. Afterwards, the column was washed with 5 column vol. equilibration buffer, followed by a linear gradient of sodium chloride in the same buffer from 2 M to 0 M. Under these conditions, at least 50% of the cholesterol EH activity could be recovered with the flow-through fraction, while more than 95% of the total protein loaded on the column showed significant retention (Fig. 3).

Enzyme assays. The enzymatic activity of microsomal EH was assessed with styrene oxide as described (Oesch, 1974). The enzymatic activity of soluble EH was determined according to the procedure of Schladt et al. (1986). Epoxystearic-acid-hydrolase activity of microsomal EH and soluble EH was measured by the following procedure. [1-^{14}C]Epoxystearic acid, prepared as described above, was added in 3 μl acetonitrile (at a final concentration of 3% acetonitrile did not significantly affect the enzymatic activity of microsomal EH or soluble EH towards their standard substrates styrene oxide and *trans*-stilbene oxide) to either 200−500 ng soluble EH or 2−5 μg microsomal EH in 97 μl buffer A stabilized with 100 μg/ml gelatin and was incubated at 37 °C for 5 min unless stated differently. The reaction was terminated by extraction of the mixture with 200 μl ethyl acetate. After vigorous shaking and subsequent centrifugation, 50 μl of the upper organic phase were spotted on a silica-gel 60 F_{254} plate, which was developed with *n*-hexane/diethyl ether/formic acid (30:70:2, by vol.) as the solvent system. The spots corresponding to the product were identified by chromatography with excess unlabelled 9,10-dihydroxystearic acid that was detected by iodine-vapour exposure. They were cut out of the chromatography plate, added to 6 ml Rotiszint 22, and radioactivity was quantified by means of a Packard Tri-carb scintillation counter. Under these conditions, the recovery of radioactive signal was 80% compared with direct addition of a solute sample to the scintillation cocktail. This was taken into account on calculating the amount of product formation.

Cholesterol EH activity was measured essentially as described (Levin et al., 1983; Nashed et al., 1985), with the exception that tetrahydrofurane/dichloromethane (1:1, by vol.) was used as the solvent system for the chromatographic resolution of the cholesterol epoxide (R_f = 0.5) and the cholestane-3β,5α,6β-triol (R_f = 0.2) on silica-gel 60 F_{254} plates. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Fluorographic visualization of the covalent enzyme-substrate complex. 1 μg pure microsomal EH, 1 μg pure soluble EH, 100 μg liver microsomal protein from *trans*-stilbene-ox-

ide-treated rats (Schmassmann and Oesch, 1978) or 100 µg liver cytosolic protein from fenofibrate-treated rats (Gebel et al., 1992) were incubated in 50 µl buffer A with 1 nmol [1-^{14}C]-epoxystearic acid at 37 °C for 15 s. Alternatively, 100 µg rat liver microsomal protein or 100 µg protein from the cholesterol-EH-enriched fraction eluting from the phenyl-Sepharose column were incubated under otherwise identical conditions with 1 nmol [26-^{14}C]cholesterol-5α,6α-epoxide. Inhibitors were added 3 min before the addition of substrate. In each case, the reaction was stopped by adding the sample to 450 µl chilled acetone (−20 °C) containing 1 mM hydrochloric acid. The mixture was kept at −20 °C for 3 h, and the precipitated protein was recovered thereafter by centrifugation at 13000×*g* and 4 °C for 10 min. The resulting pellet was washed twice with chilled acidic acetone, dried in a desiccator for 10 min and dissolved in 25 µl 10 mM Tris/HCl, 1 mM EDTA, 20 mM dithiothreitol, 2% SDS, 10% glycerol, 0.01% bromophenol blue, pH 8, by incubation at 37 °C for 5 min. Samples prepared this way were subjected to discontinuous SDS/PAGE on a 10% gel according to the method of Laemmli (1970). After completion of electrophoresis, the gel was fixed for 30 min in a mixture of isopropanol/water/acetic acid of 25:65:10 (by vol.) and incubated for 30 min in Amplify solution (Amersham) according to the instructions of the manufacturer. Thereafter, the gel was dried on a gel slab dryer, model 224 (Bio-Rad Laboratories) and exposed to an X-ray film (Kodak X-omat) at -80 °C for up to 3 months.

RESULTS

Purification of enzymes. The purification of microsomal EH and soluble EH by standard methods resulted in the isolation of electrophoretically pure proteins. Specific enzymatic activities obtained were 390 nmol styrene oxide converted · mg protein^{-1} · min^{-1} for the microsomal EH and 290 nmol *trans*-stilbene oxide converted · mg protein^{-1} · min^{-1} for soluble EH. No purification procedure is available for cholesterol EH. We were able to isolate a fraction of microsomal enzymes with increased specific enzymatic activity for cholesterol EH by means of hydrophobic-interaction chromatography on phenyl-Sepharose. In the presence of a low concentration of Chaps as the solubilizing detergent, cholesterol EH did not bind to phenyl-Sepharose, while most of the other proteins of the membrane extract did, resulting in a significant enrichment (about tenfold) of the enzyme in the flow-through fraction (Fig. 3). However, further attempts by a variety of additional procedures, including ion-exchange chromatography, preparative isoelectric focusing, size-exclusion chromatography and affinity chromatography, to purify cholesterol EH to homogeneity failed.

Substrate characteristics of epoxystearic acid with microsomal EH and soluble EH. By means of a thin-layer-chromatography-based assay, kinetic analyses of epoxystearic acid hydrolysis by microsomal EH and soluble EH were performed. With both enzymes, a linear relationship was observed over a suitable range between substrate conversion and either incubation time or the amount of enzyme protein employed in the reaction (data not shown). While the K_m of both enzymes with the substrate was in the low micromolar range, soluble EH displayed an almost 20-fold higher V_{max} towards the epoxystearic acid than did microsomal EH (see Table 1).

Covalent binding of epoxystearic acid to microsomal EH and soluble EH. Acid/acetone precipitation was used to trap the postulated covalent intermediate formed between [1-^{14}C]epoxystearic acid and either microsomal EH or soluble EH during

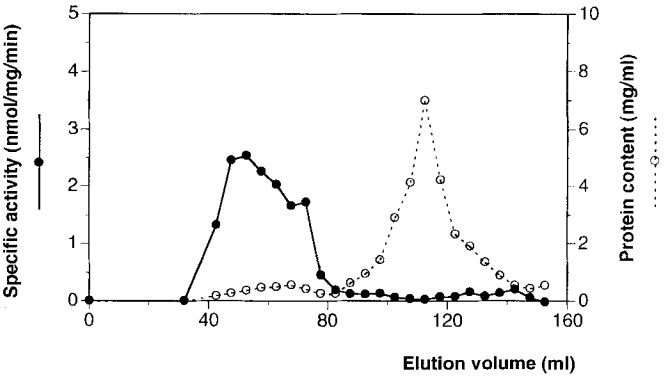

Fig. 3. Enrichment of cholesterol EH by chromatography on phenyl-Sepharose. Rat liver microsomes were solubilized and chromatographed on phenyl-Sepharose as described under Experimental Procedures. The graph shows the cholesterol EH activity (●) and protein content (○) eluting from the column during sample application (0–40 ml) and washing (40–160 ml), prior to the start of the decreasing salt gradient.

Table 1. Kinetic constants of the different epoxide hydrolases with the substrates used for the covalent-binding analysis. Analyses were carried out as described in Experimental Procedures.

Enzyme	Substrate	K_m	V_{max}
		µM	nmol · mg^{-1} · min^{-1}
Microsomal EH	*cis*-9,10-epoxystearic acid	4.5	10
Soluble EH	*cis*-9,10-epoxystearic acid	3	190
Cholesterol EH-enriched fraction after phenyl-Sepharose chromatography	cholesterol-5α,6α-epoxide	5.0	2.4[a]

[a] Approximate value.

enzymatic conversion of this substrate. Detection of the covalent intermediate was readily achieved, after incubation of either of the pure enzymes with the compound, by autoradiography of the precipitated complex after SDS-gel electrophoresis (Fig. 4). The specificity of the formation of the covalent intermediate was assessed by means of more-complex protein mixtures, i.e. microsomes for microsomal EH and cytosol for soluble EH, and by including varying amounts of competitive inhibitors in the assays. With microsomes, only one prominent radioactive signal was obtained, which migrated with purified microsomal EH treated in the same way. The intensity of this signal decreased in a dose-dependent manner when increasing amounts of 1,1,1-trichloro-2,3-propene oxide were included in the incubation with the substrate. At 625 µM, trichloropropene oxide completely abolished any detectable binding of radioactivity to microsomal EH in rat liver microsomes. Two major signals for protein-bound radioactivity were obtained on analysis of epoxystearic acid binding to cytosolic proteins. The slower migrating band displayed the same electrophoretic mobility as pure soluble EH. The signal intensity of this band gradually diminished when increasing amounts of 4-fluorochalcone oxide were included in the incubation. The somewhat stronger signal of higher electrophoretic mobility was apparently less affected by the addition of 4-fluorochalcone oxide to the assay. With respect to its estimated molecular mass, the corresponding protein appears to be similar to microsomal EH. This signal, like that obtained with microsomal EH, could be completely eradicated by the inclusion of

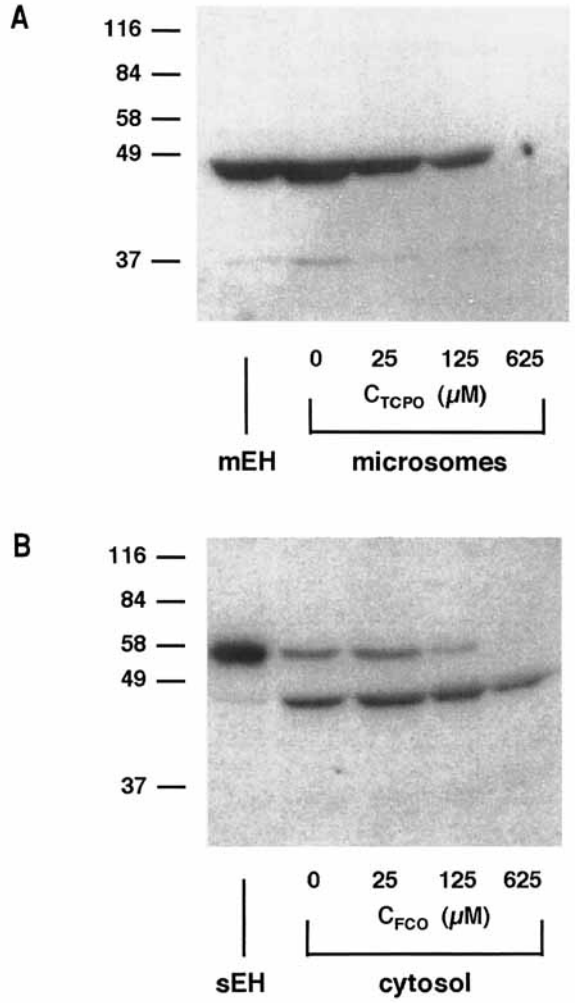

Fig. 4. Covalent binding of epoxystearic acid to microsomal EH and soluble EH. Analyses were performed as described under Experimental Procedures. (A) The almost exclusive binding of epoxystearic acid to microsomal EH (mEH) in rat liver microsomes and the competitive inhibition of this labelling by 1,1,1-trichloro-2,3-propene oxide (TCPO). The presented X-ray film was exposed to the sample for 5 days. (B) Binding of epoxystearic acid to soluble EH (sEH) in rat liver cytosol that can be diminished by increasing amounts of 4-fluorochalcone oxide (FCO) in the incubation. The lower band probably represents binding to microsomal EH that is present in low amounts in rat liver cytosol (Gill et al., 1982). The film was exposed to the sample for 6 weeks. The lanes designated with mEH (A) and sEH (B) show the results obtained with the purified enzymes. The molecular-mass markers were prestained proteins (Sigma). The sizes indicated to the left of each autoradiogram refer to the molecular masses of the unstained proteins (kDa). The staining somewhat reduces the electrophoretic mobility of the proteins, which leads to a slight underestimation of the molecular masses of the proteins, and the values are therefore only used for orientation purposes and not as an exact measure.

trichloropropene oxide in the incubation mixture (data not shown).

Covalent binding of cholesterol epoxide to rat liver microsomal proteins. Attempts were made to detect covalent binding of cholesterol epoxide to cholesterol EH. Crude rat liver microsomes or the cholesterol-EH-enriched fraction from the phenyl-Sepharose chromatography were used as the source of enzyme. Under the conditions used, the microsomes and phenyl-Sepharose fraction displayed specific enzymatic activities of 0.234 ± 0.009 and 2.42 ± 0.10 nmol cholesterol-5α,6α-epoxide converted · mg protein^{-1} · min, with an apparent K_m of 5 μM (Table 1). The specific radioactivity of the [26-^{14}C]cholesterol-5α,6α-epoxide used for the detection of covalent binding was similar to that of the [1-^{14}C]epoxystearic acid used for the labelling of soluble EH and microsomal EH. Despite the use of the maximum tolerable amount of protein in the covalent binding analyses (the SDS-gel electrophoresis for analysis of the covalent nature of the enzyme-substrate interaction limits the amount of protein to 100 μg/lane; otherwise, the resolution on the gel is poor due to sample overloading, which may significantly impair the signal intensity obtained by autoradiography), no detectable signal was obtained on the corresponding fluorography, even after extended exposure of the X-ray film for up to 3 months.

DISCUSSION

In recent years, a growing body of evidence has led to the realization that EH probably belong to the family of α/β-fold hydrolases (Arand et al., 1994; Beetham et al., 1995; Lacourciere and Armstrong, 1994; Pries et al., 1994). Theoretical considerations and experimental data suggest that the enzymatic hydrolysis of epoxides proceeds via intermediate formation of a covalent bond between enzyme and substrate. While the complete catalytic triad of soluble EH has been resolved (Arand et al., 1996; Pinot et al., 1995), only the water-activating histidine of the microsomal EH has been experimentally identified (Bell and Kasper, 1993). In an elegant study, Lacourciere and Armstrong (1993) demonstrated transfer of ^{18}O from microsomal EH to its substrate 1,10-phenanthroline 5,6-oxide under single-turnover conditions with a stoichiometry that is best explained by the formation of the postulated enzyme-substrate ester. However, with this experimental set-up, the possibility of a water molecule tightly fixed in the active center of the enzyme and refractory to removal by dialysis cannot be eliminated. In the present paper, we present unequivocal proof for the formation of the covalent bond between microsomal EH and its substrate by directly visualizing this complex.

For the visualization of the covalent intermediate formed during the EH reaction we chose 9,10-epoxystearic acid as the substrate for several reasons: it can readily be synthesized from [1-^{14}C]oleic acid by a one-step procedure; it is a substrate for microsomal EH and soluble EH; and compared with the standard substrates of microsomal EH it has a low K_m with this enzyme (4.5 μM compared with, e.g., 150 μM with styrene oxide), which makes it possible to use the radioactive compound labelled to high specific activity while keeping the amount of radioactive material required for each sample at a moderate level. Under our experimental conditions, one single protein displayed binding to radioactive *cis*-9,10-epoxystearic acid in rat liver microsomes. The covalent nature of this interaction is evident from the stability of the complex under the strongly denaturing conditions of the SDS-gel electrophoresis (2% SDS in the sample buffer). The detected protein was unequivocally identified as microsomal EH since pure microsomal EH also covalently bound epoxystearic acid, the complex formed with pure microsomal EH showed the same elecrophoretic mobility as the complex formed with microsomes, and the covalent binding of the substrate could be suppressed by addition of 1,1,1-trichloro-2,3-propene oxide, a potent inhibitor of microsomal EH (Oesch et al., 1971b). In contrast, the same analysis performed with rat liver cytosol indicated the presence of two proteins capable of binding epoxystearic acid. As expected, one of these was soluble EH, as demonstrated by the same methods detailed above for the microsomal EH, with the exception that

4-fluorochalcone oxide was used as the soluble-EH-specific inhibitor. The other protein closely resembled microsomal EH in all aspects under investigation. This observation is in agreement with earlier reports that a microsomal-EH-like protein is detectable in cytosols from rat and human liver (Gill et al., 1982, 1983).

While we were able to show the covalent binding of epoxystearic acid to microsomal EH and soluble EH, the same experimental approach could not reveal covalent binding of cholesterol epoxide to cholesterol EH, neither in microsomes nor in solubilized samples enriched for cholesterol EH activity. In principle, several explanations are compatible with this observation. First, despite several attempts to isolate it, cholesterol EH has not been obtained in pure form (Watabe et al., 1986; this report). Therefore, its abundance in rat liver microsomes is not known. Its comparatively low specific activity in microsomes towards its natural substrate cholesterol-5,6-oxide suggests that it may be present in rather low amounts. However, soluble EH is also of low abundance in rat liver cytosol, even after induction with specific inducers (<0.1% of cytosolic protein), but we were able to detect the covalent intermediate formed with epoxystearic acid in rat liver cytosol in the present study (Fig. 4B). An alternative explanation for the failure to detect cholesterol epoxide binding to microsomal proteins is that the formation of the covalent intermediate (step 1, Fig. 1) and not the hydrolysis of the covalent intermediate (step 2, Fig. 1) is the rate-limiting step in the enzymatic cholesterol epoxide hydrolysis. While this possibility cannot be eliminated, the positive results obtained with microsomal EH and soluble EH in the present work by means of an aliphatic substrate may serve as an argument for a third explanation, namely that cholesterol EH does not act via covalent binding to its substrate but rather by a fundamentally different mechanism. This idea is supported by the finding that cholesterol EH converts both diastereomeric cholesterol epoxides, the α-epoxide and the β-epoxide, to the same enantiomerically pure product, namely the cholestane-3β,5α,6β-triol (Watabe et al., 1981). *Trans*-opening of the two epoxides can only lead to the same product if the nucleophilic attack at the oxirane ring comes from opposite sides for the different diastereomers, namely from above the ring plane with the α-epoxide at the 6-position and from below the ring plane with the β-epoxide at the 5-position. This scenario appears unlikely because effective binding of the rather asymmetric substrate cholesterol epoxide in different orientations would imply a high flexibility of the enzyme in accomodating substrates and thus a broad substrate specificity, which has not been reported. We therefore conclude that cholesterol EH is probably mechanistically and thus structurally unrelated to microsomal EH and soluble EH and belongs to a separate class of hydrolytic enzymes. Further insight into its structure and/or mechanism has to be obtained to elucidate its possible relationship to other enzymes, such as the leukotriene-A4 hydrolase (Medina et al., 1991).

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