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Purification of Human Liver Cytosolic Epoxide Hydrolase and Comparison to the Microsomal Enzyme†

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ABSTRACT: Epoxide hydrolase (EC 3.3.2.3) was purifed to electrophoretic homogeneity from human liver cytosol by using hydrolytic activity toward *trans*-8-ethylstyrene 7,8-oxide (TESO) as an assay. The overall purification was 400-fold. The purified enzyme has an apparent monomeric molecular weight of 58 000, significantly greater than the 50 000 found for human (or rat) liver microsomal epoxide hydrolase or for another TESO-hydrolyzing enzyme also isolated from human liver cytosol. Purified cytosolic TESO hydrolase catalyzes the hydrolysis of *cis*-8-ethylstyrene 7,8-oxide 10 times more rapidly than does the microsomal enzyme, catalyzes the hydrolysis of TESO and *trans*-stilbene oxide as rapidly as the microsomal enzyme, but catalyzes the hydrolysis of styrene 7,8-oxide, *p*-nitrostyrene 7,8-oxide, and naphthalene 1,2-oxide much less effectively than does the microsomal enzyme. Purified cytosolic TESO hydrolase does not hydrolyze benzo[*a*]pyrene 4,5-oxide, a substrate for the microsomal enzyme. The activities of the purified enzymes can explain the specific activities observed with subcellular fractions. Anti-human liver microsomal epoxide hydrolase did not recognize cytosolic TESO hydrolase in purified form or in cytosol, as judged by double-diffusion immunoprecipitin analysis, precipitation of enzymatic activity, and immunoelectrophoretic techniques. Cytosolic TESO hydrolase and microsomal epoxide hydrolase were also distinguished by peptide mapping. The results provide evidence that physically different forms of epoxide hydrolase exist in different subcellular fractions and can have markedly different substrate specificities.

Epoxide hydrolase (EC 3.3.2.3) is an enzyme found in a variety of mammalian tissues as well as in lower species (Guengerich, 1982; Lu & Miwa, 1980; Oesch, 1980). Activity has been demonstrated toward a wide variety of epoxides (Guengerich, 1982). Such activity is of importance because many epoxides are capable of irreversibly binding to nucleophilic sites present in tissue macromolecules and may initiate toxic or carcinogenic conditions. The action of epoxide hydrolase presumably serves to protect against the effects of epoxides which can enter the body or be produced from the action of monooxygenases on olefins and aromatic compounds (El-Tantawy & Hammock, 1980).

Early studies indicated that this activity is localized in microsomal fractions of tissues of experimental animals (Oesch, 1973; Oesch et al., 1971). More recently, activity has also been demonstrated in nuclear (Bornstein et al., 1979; Mukhtar et al., 1979), mitochondrial (Gill & Hammock, 1981), and cytoplasmic fractions (Gill & Hammock, 1979, 1980; Ota & Hammock, 1980). The current literature suggests that the epoxide hydrolases in nuclear and microsomal fractions are similar (Thomas et al., 1979a). However, Hammock and his associates have reported that the epoxide hydrolase activity in the cytosolic and mitochondrial fractions differs from that found in microsomal fractions in terms of pH optima and substrate specificity (Gill & Hammock, 1979–1981; Gill et al., 1974; Ota & Hammock, 1980). The cytosolic epoxide hydrolase has been reported to act on TESO[1] but not SO or BP-4,5-oxide, in contrast to the specificity of microsomal fractions (Gill & Hammock, 1981; Mullin & Hammock, 1980; Oesch & Golan, 1980). Antibodies raised against rat liver microsomal epoxide hydrolase precipitated microsomal BP-4,5-oxide hydrolase activity but not cytosolic TESO hydrolase activity in mouse and rat liver samples (Guenthner et al., 1981).

While microsomal epoxide hydrolase has been purified to electrophoretic homogeneity from rat and human liver in

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[1] Abbreviations: TESO, *trans*-8-ethylstyrene 7,8-oxide; CESO, *cis*-8-ethylstyrene 7,8-oxide; PNSO, *p*-nitrostyrene 7,8-oxide; SO, styrene 7,8-oxide; BP, benzo[*a*]pyrene; $NaDodSO_4$, sodium dodecyl sulfate; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid.

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several laboratories (Bentley & Oesch, 1975; Guengerich et al., 1979b; Knowles & Burchell, 1977; Lu et al., 1975), the cytosolic enzyme has not been purified previously. Considerable species variation in the substrate specificity of cytosolic epoxide hydrolase exists (Gill & Hammock, 1980; Ota & Hammock, 1980). In order to better understand the relationship of the microsomal and cytosolic forms of epoxide hydrolase and the importance of these enzymes during the exposure of humans to potential toxins and carcinogens, we have purified cytosolic and microsomal epoxide hydrolases from human liver and used electrophoretic and immunochemical techniques, peptide mapping, and catalytic activities to compare these enzymes.

Experimental Procedures

Chemicals. 8-Ethyl[7-^{3}H]styrene 7,8-oxide was synthesized, and the cis and trans isomers were separated by high-pressure liquid chromatography using a preparative Lichrosorb column as described by Mullin & Hammock (1980). TESO was prepared from *trans*-4-phenyl-2-butene by using *m*-chloroperoxybenzoic acid and purified by vacuum distillation (Mullin & Hammock, 1980). [7-^{3}H]TESO was diluted with carrier TESO to give a material with a specific radioactivity of 4.2 mCi mmol^{-1} for routine assays. PNSO and [7-^{3}H]PNSO were prepared from *p*-nitrophenacyl bromide by treatment with $NaBH_4$ or NaB^3H_4 in the general manner described for TESO (Mullin & Hammock, 1980). The specific radioactivity of the material was 1.25 mCi mmol^{-1}. All of the above radiolabeled epoxides showed only a single radioactive zone when chromatographed on layers of silica gel G (for TESO, toluene/1-propanol, 10:1 v/v, and ethyl acetate/$CHCl_3$, 2:1 v/v; for PNSO, ethyl acetate/$CHCl_3$, 2:1 and 4:1 v/v). The corresponding dihydrodiols were obtained for reference purposes by treatment of the epoxides with acid (Mullin & Hammock, 1980).

trans-[^{3}H]Stilbene oxide and its dihydrodiol derivative were gifts of Dr. J. W. DePierre, University of Stockholm. [2-^{3}H]Naphthalene 1,2-oxide was synthesized as described elsewhere (Guengerich & Davidson, 1982; Oesch et al., 1972). [G-^{3}H]BP-4,5-oxide and the corresponding dihydrodiol were obtained through the National Cancer Institute Chemical Repository.

DEAE-cellulose (DE-52 and DE-53) and CM-cellulose (CM-52) were obtained from Whatman, Inc., Clifton, NJ. Hydroxylapatite was obtained from Clarkson Chemical Co., Williamsport, PA. Chromatofocusing materials were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Sources of materials used for $NaDodSO_4$–polyacrylamide gel electrophoresis and immunochemical staining were as described elsewhere (Guengerich et al., 1982).

Enzyme Sources, Enzymes, and Antibodies. Human liver autopsy samples were collected from organ donors within 30 min after cessation of life support in the cases of patients 22 and 23, perfused with cold 0.9% NaCl (w/v), and frozen at −70 °C. Other liver autopsy samples were obtained within 4 h of death, washed, and frozen. Samples from the following patients were used in these studies: patient 6, female, 32 years of age, death due to gunshot wound; patient 15, male, 37 years of age, death due to gunshot wound; patient 18, female, 35 years of age, death due to gunshot wound; patient 22, female, 22 years of age, death due to intracranial hemorrhage; patient 23, female, 17 years of age, death due to head injury. Microsomal and cytosolic fractions were prepared by differential centrifugation (Guengerich, 1977).

Rat liver microsomal epoxide hydrolase was prepared from phenobarbital-treated animals as described elsewhere (fraction "A") (Guengerich et al., 1979b). Human liver microsomal epoxide hydrolase (fraction "DE") was purified from patient 22 for use in the comparative studies, and the microsomal "DE" preparation isolated from patient 6 was used to raise antibodies in rabbits as described elsewhere (Guengerich et al., 1979a).

Assays. Enzymatic hydrolyses of *cis*- and *trans*-8-ethyl-[7-^{3}H]styrene 7,8-oxides, [7-^{3}H]SO, [7-^{3}H]PNSO, *trans*-[G-^{3}H]stilbene oxide, [2-^{3}H]naphthalene 1,2-oxide, and [G-^{3}H]BP-4,5-oxide were monitored by thin-layer chromatography by using the general procedure of Jerina et al. (1977). Protein concentrations were estimated by using the method of Lowry et al. (1951). Double-diffusion immunoprecipitin analysis was carried out as described elsewhere (Guengerich et al., 1981a). Peptide mapping was carried out by using the general procedure of Cleveland et al. (1977). $NaDodSO_4$–polyacrylamide gel electrophoresis [7.5% (w/v) acrylamide unless noted otherwise] was carried out as described by Laemmli (1970), and in some cases the resolved polypeptides were electrophoretically transferred to sheets of nitrocellulose paper and stained with antibodies raised to human liver microsomal epoxide hydrolase. Briefly, the procedure involves subsequent treatment of the sheets with rabbit anti-epoxide hydrolase, goat anti-rabbit IgG, horseradish peroxidase/rabbit anti-peroxidase complex, and 3,3′-diaminobenzidine/H_2O_2 to give stains corresponding to antigenic sites recognized by the antibodies (Guengerich et al., 1982).

Purification of PNSO Hydrolase from Human Liver Cytosol. All purification steps were performed at 4 °C. A volume of 2 L of cytosol was brought to 20% saturation by addition of solid ammonium sulfate. The resulting precipitate was removed by centrifugation at 10^4g for 30 min. Ammonium sulfate was added to the supernatant to yield a final saturation of 60%. The resulting precipitate was dissolved in a minimum volume of buffer A [5 mM potassium phosphate (pH 7.7) containing 0.1 mM EDTA] and dialyzed against three changes of 20 volumes of buffer A over 24 h.

The dialyzed 20–60% ammonium sulfate fraction was applied to a DEAE-cellulose (DE-52) column (5.0 × 80 cm) that had been equilibrated with buffer A. The column was washed with 4 L of buffer A and then eluted with a 6-L linear gradient of 0–0.3 M NaCl in the same buffer. Fractions with PNSO hydrolase activity were pooled.

The pooled DE-52 fraction was applied to a 2.6 × 50 cm *n*-octylamino-Sepharose 4B column (Guengerich et al., 1979a) which had been previously equilibrated with 50 mM potassium phosphate buffer containing 0.1 mM EDTA. The column was washed with 1 L of the same buffer and eluted in a stepwise manner with 1 L of 500 mM potassium phosphate buffer (pH 7.7), 2 L of 50 mM potassium phosphate buffer (pH 7.7), and 1 L of 50 mM potassium phosphate buffer (pH 7.7) containing 0.5% (w/v) Lubrol PX. All buffers also contained 0.1 mM EDTA and 0.5% (w/v) sodium cholate. PNSO hydrolase was monitored by activity measurements or by immunodiffusion using antibodies raised against human microsomal epoxide hydrolase. The pooled PNSO hydrolase fraction was stirred with Bio-Beads SM-2, filtered, centrifuged, and dialyzed against 5 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA.

The dialyzed PNSO hydrolase was applied to a 1.5 × 15 cm hydroxylapatite column which had been equilibrated with 5 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA. The column was washed with 250 mL of the equilibration buffer. An 800-mL linear gradient of 5–200 mM potassium phosphate buffer (pH 7.7) was applied. PNSO

Table I: Purification of Human Liver Cytosolic TESO Hydrolase[a]

fraction	protein (mg)	sp act. [nmol hydrolyzed min^{-1} (mg of protein)$^{-1}$]		total act. (nmol hydrolyzed min^{-1})		TESO hydrolase		ratio of TESO/PNSO hydrolase act.
		TESO	PNSO	TESO	PNSO	fold purification	yield (%)	
cytosol	27300	0.12	0.61	3330	16600	(1)	(100)	0.20
ammonium sulfate, 40–60%	12400	0.09	0.25	1100	3060	0.75	33	0.36
DEAE-cellulose (DE-53)	277	5.6	1.3	1550	353	47	46	4.4
CM-cellulose	163	7.2	1.7	1170	275	60	35	4.3
hydroxylapatite	59	10	1.8	584	105	83	18	5.6
DEAE-cellulose (DE-53), second column	0.31	51	7.8	15.6	2.4	425	0.5	6.6

[a] The liver sample was obtained from patient 23. Fractionation procedures and assays are described under Experimental Procedures. Similar results were obtained with the sample derived from patient 22: the purified enzyme had the same apparent monomeric molecular weight (M_r 58 000), the final TESO specific activity was 22 nmol of TESO hydrolyzed min^{-1} (mg of protein)$^{-1}$ (390-fold purification), the final PNSO specific activity was 1.1 nmol of PNSO hydrolyzed min^{-1} (mg of protein)$^{-1}$, and the yield was 2%.

hydrolase was recovered in the void volume and subsequently found to be homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis (vide infra).

Purification of TESO Hydrolase from Human Liver Cytosol. All purification steps were performed at 4 °C. Two liters of cytosol was sequentially brought to 40% and then 60% ammonium sulfate saturation. Precipitates were collected by centrifugation; the 40–60% ammonium sulfate pellet was dissolved in buffer A and dialyzed 3 times against 20 volumes of buffer A.

The dialyzed 40–60% ammonium sulfate fraction was applied to a DEAE-cellulose (DE-53) column (5.0 × 80 cm) that had been equilibrated with buffer A. The column was washed and eluted with a linear NaCl gradient (0–0.3 M) as described for PNSO hydrolase. Fractions with TESO hydrolase activity were pooled. (PNSO hydrolase could be further purified by *n*-octylamino-Sepharose 4B and hydroxylapatite chromatography as described above.)

The pooled TESO hydrolase fraction was dialyzed 3 times against 20 volumes of buffer A over a period of 24 h. The pH of the dialyzed fraction was adjusted to 6.5, and the sample was immediately applied to a CM-cellulose (CM-52) column (5.0 × 30 cm), which had been equilibrated with 5 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA, at a flow rate of greater than 3 mL min^{-1}. Fractions containing protein (assayed at 280 nm) that did not bind to CM-cellulose were pooled, and the pH was immediately readjusted to 7.25. Subsequently, the CM-cellulose column was eluted with 2 L of the equilibrating buffer and a 3-L linear NaCl gradient of 0–0.3 M in the same buffer.

A 5.0 × 25 cm column of hydroxylapatite was equilibrated with 5 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA. The void volume fraction from CM-cellulose chromatography was applied to this column immediately after adjustment of the pH. The column was eluted with a stepwise gradient consisting of 750 mL volumes of potassium phosphate buffer (pH 7.25) containing 20, 40, 60, 80, and 120 mM phosphate and 0.1 mM EDTA. Fractions containing TESO hydrolase activity were pooled and dialyzed against buffer A.

DEAE-cellulose (DE-53) was treated with enzyme-grade ammonium sulfate as described elsewhere (Elshourbagy & Guzelian, 1980). A 2.6 × 40 cm column of DEAE-cellulose was equilibrated with buffer A. The dialyzed fraction from the previous step was applied to this column, and the column was washed with 800 mL of buffer A. An 800-mL linear NaCl gradient (0–75 mM) was applied. The column was further eluted with 500 mL of buffer A containing 75 mM NaCl. The TESO hydrolase fraction from this step was judged to be electrophoretically homogeneous (vide infra).

Results

Purification of Cytosolic PNSO Hydrolase. In a series of preliminary experiments we consistently observed the presence of SO hydrolase activity in human liver cytosolic fractions. The percentage of total SO hydrolase activity recovered in the 10^5g supernatant varied from 9 to 25%. A pathologically normal human liver biopsy sample was processed immediately, and 24% of the activity was found in the cytosol. During the course of the experiments we began routinely using PNSO instead of SO as a substrate because of an increased turnover number (Guengerich, 1982). Cytosolic PNSO hydrolase was purified to electrophoretic homogeneity (vide infra) from liver autopsy samples obtained from three different individuals, using methods adapted from our purification scheme for human liver microsomal epoxide hydrolase (Guengerich et al., 1979b). However, the purified enzyme contained little TESO hydrolase activity. Other experiments indicated that antibodies raised to human liver microsomal epoxide hydrolase could inhibit and precipitate most of the cytosolic SO hydrolase activity but not cytoslic TESO hydrolase activity (vide infra).

Purification of Cytosolic TESO Hydrolase. The above experiments suggested that much of the TESO hydrolase activity was catalyzed by an enzyme other than the cytosolic PNSO hydrolase. Thus, activity toward TESO was monitored during fractionation of cytosol. TESO and PNSO hydrolase activities could be separated when ammonium sulfate fractions were chromatographed on columns of DEAE-cellulose (Figure 1). Cytosolic TESO hydrolase was purified to electrophoretic homogeneity by using a combination of techniques, as outlined in Table I.

Several points should be made about the procedure. The overall purification of cytosolic TESO hydrolase activity was 400-fold, and the yield was 1–2%. The ratio of TESO to PNSO activity increased from 0.2 to 6.6 during purification. The enzyme precipitates at pH 6.5; thus, the pH was adjusted to 6.5 immediately prior to the CM-cellulose step, and the chromatography was carried out rapidly. The fractions that did not bind to CM-cellulose were pooled, and the pH was immediately readjusted to 7.25 without assaying activity. The recovery of activity of TESO activity from the second DEAE-cellulose chromatography step was poor, but this step was necessary for removal of four to six residual contaminating polypeptides. No other activity peaks were recovered from the column. The reason for the low yield is not apparent, as high recovery was obtained in the first DEAE-cellulose procedure. Some purification could be obtained by using a

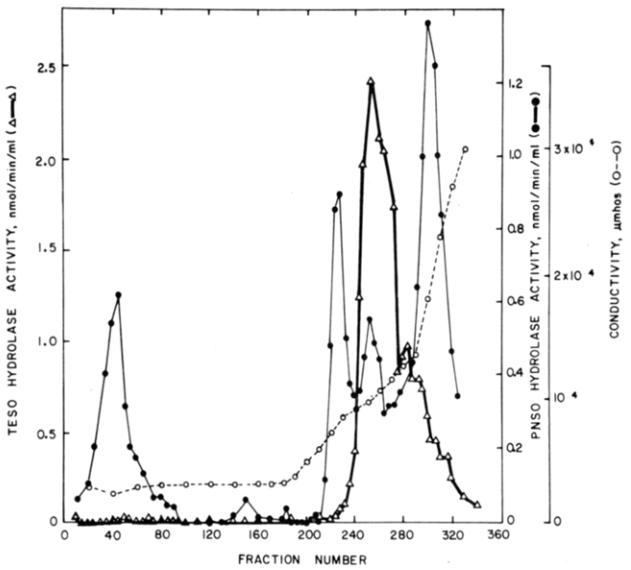

FIGURE 1: Chromatography of human liver cytosol ammonium sulfate fraction on DEAE-cellulose. The fraction precipitating between 40 and 60% ammonium sulfate saturation was dialyzed and applied to a 5 × 100 cm column of Whatman DE-53 DEAE-cellulose; the column was washed with phosphate buffer and eluted with a NaCl gradient as described under Experimental Procedures. Aliquots of fractions were assayed for hydrolase activity toward TESO and PNSO.

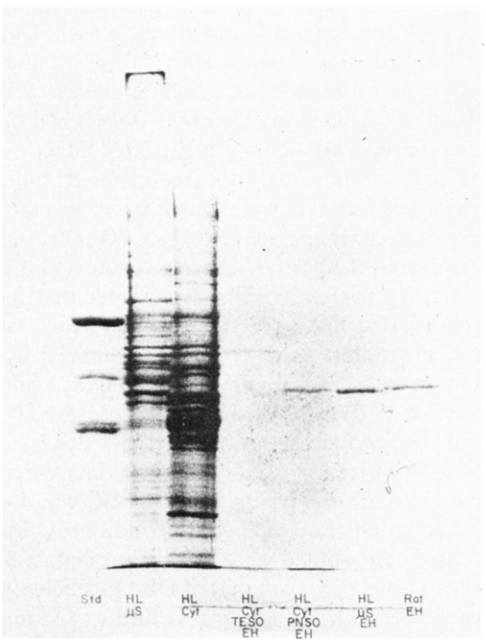

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of epoxide hydrolase fractions. Samples were electrophoresed according to Laemmli (1970) in 0.075 × 0.3 × 10 cm lanes of a slab gel. Gels were stained with Coomassie Brilliant Blue R-250 by using the schedule of Fairbanks et al. (1971). The anode was at the bottom of the figure. Samples included the following: well 1, 1 μg each of standard bovine serum albumin (accepted M_r 68 000), bovine liver catalase (M_r 58 000), *Escherichia coli* L-glutamate dehydrogenase (M_r 53 000), equine liver alcohol dehydrogenase (M_r 43 000), and rabbit muscle aldolase (M_r 40 000); well 2, human liver microsomes (patient 23), 50 μg; well 3, human liver cytosol (patient 23), 50 μg; well 4, human liver cytosolic TESO hydrolase (patient 23), 0.15 μg; well 5, human liver cytosolic PNSO hydrolase (patient 18), 0.3 μg; well 6, human liver microsomal epoxide hydrolase (patient 22), 0.3 μg; and well 7, rat liver microsomal epoxide hydrolase, 0.3 μg.

Pharmacia chromatofocusing system (pH range 6.5–5.0) after the hydroxylapatite step, but the degree of purification was not reproducible. However, these experiments suggest that the p*I* of human liver cytosolic TESO hydrolase is between

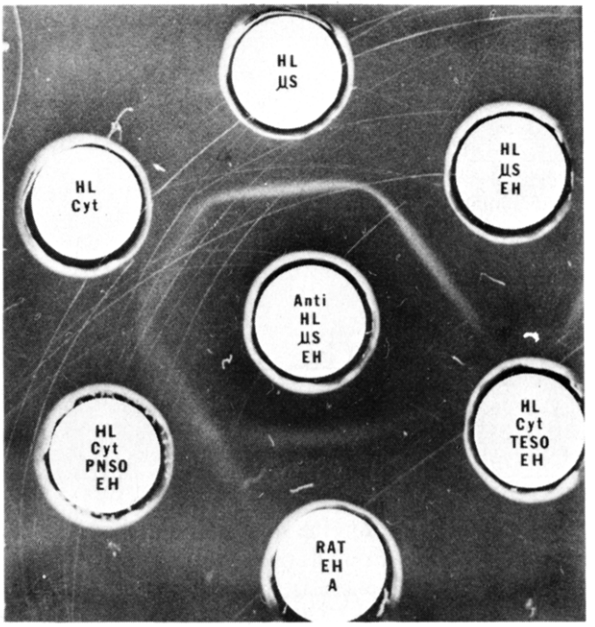

FIGURE 3: Double-diffusion immunoprecipitin analysis of human liver epoxide hydrolase fractions using anti-human liver microsomal epoxide hydrolase. The center well contained the IgG fraction of the antibody at a concentration of 50 mg of protein mL^{-1}. The other wells contained the following: rat liver microsomal epoxide hydrolase (RAT EH), 0.5 mg mL^{-1}; purified human liver cytosolic PNSO hydrolase (HL CYT PNSO EH) from patient 18, 0.4 mg mL^{-1}; human liver cytosol (CYT), from patient 22, 11.5 mg of protein mL^{-1}; human liver microsomes (μS), from patient 23, 4.0 mg of protein mL^{-1} with 1.0% Lubrol PX present; purified human liver microsomal epoxide hydrolase (HL μS EH) from patient 22, 0.1 mg mL^{-1}; and purified human liver cytosolic TESO hydrolase (HL CYT TESO EH) from patient 23, 0.7 mg mL^{-1}.

5.1 and 6.1. Gel filtration using columns of Bio-Gel A-0.5m and Pharmacia S-200 was attempted at various stages but did not yield significant purification.

Molecular Weight. $NaDodSO_4$–polyacrylamide gel electrophoresis of human cytosolic TESO hydrolase showed a single band with an apparent monomeric molecular weight of 58 000 (Figure 2). The monomeric molecular weights of human microsomal epoxide hydrolase, human cytosolic PNSO hydrolase, and rat microsomal epoxide hydrolase were all 50 000 under these conditions.

Purified TESO hydrolase was chromatographed on a Sephadex S-300 column at 4 °C in the presence of 0.1 M potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA. A molecular weight of 140 000 was estimated from TESO hydrolase activity measurements with blue dextran, equine spleen ferritin (accepted M_r 440 000), bovine liver catalase (M_r 240 000), porcine muscle lactate dehydrogenase (M_r 140 000), equine liver alcohol dehydrogenase (M_r 80 000), and $K_3Fe(CN)_6$ as standards. Thus, TESO hydrolase appears to exist as a dimer under these conditions, as suggested by Gill & Hammock (1980) on the basis of studies with crude mouse liver cytosol. This behavior is in contrast to that of purified rat liver microsomal epoxide hydrolase, which aggregates to form a 700 000–800 000-dalton species (Guengerich & Davidson, 1982). The aggregation behavior of human liver microsomal epoxide hydrolase has not been carefully studied yet, but gel filtration studies suggest a behavior similar to that of the rat liver microsomal epoxide hydrolase.

Immunochemical Studies. Antibodies to purified human liver epoxide hydrolase were used to examine the similarity between human and rat liver epoxide hydrolases.

Double-diffusion immunoprecipitin analysis showed a single

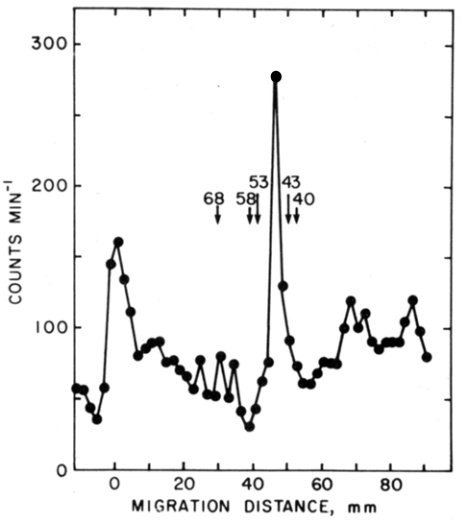

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of ^{125}I-labeled human liver cytosolic EH isolated by immunoaffinity chromatography. An aliquot of cytosol containing 250 μg of protein, derived from patient 15, was labeled with ^{125}I as described elsewhere (Guengerich et al., 1981b) with the exception that the specific activity of the $Na^{125}I$ was diluted 10-fold and the concentrations of the other reagents were increased 5-fold. The labeled preparation was passed through Sephadex G-25 as described (Guengerich et al., 1981a). Sepharose 4B gel was derivatized with either rabbit anti-human EH (IgG) or goat preimmune IgG at a concentration of 10 mg of IgG mL^{-1} of packed gel essentially as described by Thomas et al. (1979b). Washing of the gel was carried out as described in that reference. The ^{125}I-labeled sample was applied to a 2.5-mL column of the preimmune IgG–Sepharose 4B gel and a 2.5-mL column of the anti-human EH IgG–Sepharose 4B gel connected in tandem. The pair of columns was washed with about 100 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 0.9% (w/v) NaCl, 0.02% (w/v) NaN_3, 0.02% (w/v) bovine serum albumin, and 0.1% (w/v) Lubrol PX to remove unbound radioactivity, which was monitored in fractions that were collected. The columns were disconnected, and the latter column was washed with 100 mL of the same buffer devoid of albumin and Lubrol. This column was then eluted with 50 mM potassium phosphate buffer (pH 8.0) containing 0.9% (w/v) NaCl, 0.02% (w/v) NaN_3, and 4 M KSCN. The eluted fractions containing ^{125}I were pooled and dialyzed vs. 50 volumes of distilled water for 16 h. (All of the preceding steps were carried out at 23 °C, and the column flow rate was 20 mL h^{-1}.) The dialyzed fraction was concentrated by lyophilization, and aliquots were electrophoresed in $NaDodSO_4$–polyacrylamide gels as described by Laemmli (1970) in 1.0 × 0.075 × 15 cm lanes of a slab gel. The stained and dried gels were sliced into 2-mm segments, and radioactivity in each slice was determined by using a γ counter. The anode was at the right side of the figure, and the migration distance starts at the top of the separating gel. The standard proteins described under Figure 2 were electrophoresed in the same gel. The positions of the standard proteins are indicated by arrows, with accepted molecular mass values in kilodaltons. Background counts were not subtracted before plotting.

precipitin line formed with human microsomal epoxide hydrolase (Figure 3). The line formed a pattern of fusion with the single precipitin lines resulting from the interaction of human cytosolic PNSO hydrolase and crude human microsomes and cytosol. A spur formed with the line precipitated in the reaction with rat liver microsomal epoxide hydrolase, consistent with previous results with human microsomes and anti-rat liver epoxide hydrolase (Guengerich et al., 1979a). No line was formed with purified human liver cytosolic TESO hydrolase.

Human liver cytosol was labeled with ^{125}I and solubilized with detergent. The material was applied to a column of anti-human liver microsomal epoxide hydrolase bound to Sepharose, and unbound material was washed from the column. Tightly bound material was eluted from the column with KSCN, dialyzed, lyophilized, and electrophoresed on $NaDodSO_4$–polyacrylamide gels (Figure 4). The radioactivity

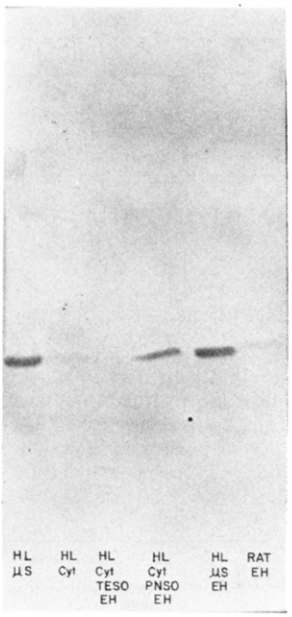

FIGURE 5: Immunoelectrophoresis of epoxide hydrolase samples. The various samples were electrophoresed in $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970). The anode was at the bottom of the gel. Proteins were transferred to nitrocellulose sheets and stained with rabbit anti-human liver microsomal epoxide hydrolase (diluted to 0.2 mg of IgG mL^{-1}) as described under Experimental Procedures. The individual wells included the same samples used in Figure 2.

corresponded to some very high molecular weight material which did not enter the gel and primarily to a protein with an apparent molecular weight of 48 000–50 000 corresponding to cytosolic PNSO hydrolase but not TESO hydrolase.

When the various epoxide hydrolase preparations or human liver cytosol or microsomes were electrophoresed on $NaDodSO_4$–polyacrylamide gels and the resolved proteins were transferred to nitrocellulose sheets, anti-human liver microsomal epoxide hydrolase did not bind to purified human liver cytosolic TESO hydrolase, but proteins corresponding to M_r 50 000 were stained in human liver microsomal epoxide hydrolase, human liver cytosolic PNSO hydrolase, and rat liver microsomal epoxide hydrolase preparations and human liver cytosol and microsomes (Figure 5).

Some of the antibody preparations raised to human liver microsomal epoxide hydrolase in rabbits were capable of inhibiting as much as 60% of the activity of purified human liver microsomal epoxide hydrolase, but others were not inhibitory. However, all antibody preparations were found to precipitate more than 90% of the activity. Thus, we examined the ability of the antibody to precipitate epoxide hydrolase activity from human liver cytosol. The antibody preparation was found to precipitate PNSO hydrolase activity from human liver cytosol but did not precipitate TESO hydrolase activity (Figure 6).

Peptide Mapping. The various purified epoxide hydrolase preparations were subjected to partial proteolytic digestion with *Staphylococcus aureus* V8 protease, α-chymotrypsin (Figure 7), or papain (results not shown). Some peptides may be common, but the data show that different maps were produced for the cytosolic TESO and microsomal hydrolases, indicative of differences in primary structure. Some homology was observed between the human and rat liver microsomal hydrolases, with some peptides not in common. Although the same amount of protein was used in all samples, human cytosolic PNSO hydrolase was degraded more extensively than the microsomal hydrolases. Most of the peptides visible in the cytosolic PNSO hydrolase digest appeared to be common to the microsomal hydrolases.

Table II: Hydrolase Activities of Various Crude and Purified Human Liver Preparations toward Various Epoxides[a]

substrate	sp act. [nmol hydrolyzed min^{-1} (mg of protein)$^{-1}$] human liver: cytosolic TESO hydrolase	cytosolic PNSO hydrolase	microsomal hydrolase	cytosol	microsomes	rat liver microsomal hydrolase
SO	22 ± 7	44 ± 7	970 ± 110	2.3 ± 0.2	52 ± 11	530 ± 190
PNSO	1.1 ± 0.1	41 ± 2	1510 ± 200	2.1 ± 1.0	105 ± 11	1010 ± 80
TESO	22 ± 4	1.5 ± 0.2	19 ± 2	0.056 ± 0.001	1.03 ± 0.16	2.2 ± 0.6
CESO	0.63 ± 0.06	0.016 ± 0.009	0.069 ± 0.040	0.040 ± 0.010	0.056 ± 0.001	0.19 ± 0.01
trans-stilbene oxide	150 ± 28	14 ± 3	230 ± 83	6.6 ± 2.0	50 ± 10	190 ± 18
naphthalene 1,2-oxide	330 ± 43	160 ± 90	1650 ± 250	53 ± 22	290 ± 170	1540 ± 260
BP-4,5-oxide	<0.1	6.6 ± 0.9	68 ± 1	0.28 ± 0.14	8.7 ± 0.9	*b*

[a] Human liver cytosolic epoxide (TESO) hydrolase was purified from patient 22. Microsomal epoxide (PNSO) hydrolase was purified from patient 22. Cytosolic PNSO hydrolase was purified from patient 18. Cytosolic and microsomal samples used directly in assays were obtained from patient 22. In all assays, either 1–5 μg of purified epoxide hydrolase or 50–100 μg of microsomal or cytosolic protein was used. Substrate concentrations and incubation times were as follow: SO, 1.0 mM, 5 min; PNSO, 0.9 mM, 2 min; TESO, 1.0 mM, 30 min; CESO, 1.0 mM, 30 min; *trans*-stilbene oxide, 1.25 mM, 10 min; naphthalene 1,2-oxide, 2.5 mM, 5 min; and BP-4,5-oxide, 0.2 mM, 10 min. Rates of nonenzymatic hydrolysis were substracted in all cases, and all values presented are means of triplicate assays ± SD. [b] Not determined.

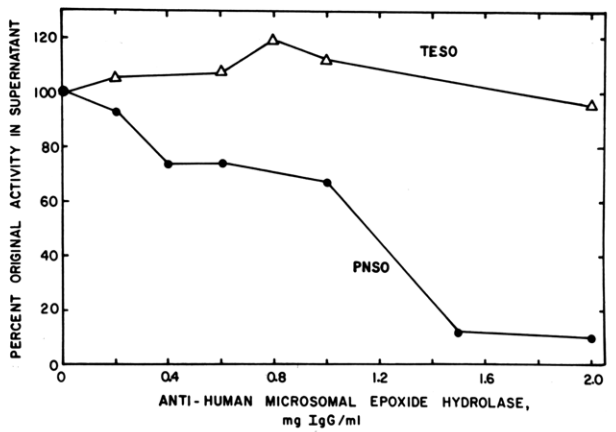

FIGURE 6: Immunoprecipitation of TESO and PNSO hydrolase activities from human liver cytosol with anti-human liver microsomal epoxide hydrolase. The cytosolic fraction from patient 23 was diluted 2-fold in 10 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, and the indicated amounts of the IgG fraction of the antibody were added to 1.0 mL of the diluted material. The samples were mixed and incubated for 30 min at 37 °C and then at 4 °C for 16 h. Immunoprecipitates were collected by centrifugation at 10^4g for 30 min, and activities toward PNSO and TESO were assayed in the remaining supernatants. In this experiment, the initial specific TESO hydrolase activity was 0.14 nmol min^{-1} (mg of protein)$^{-1}$ and the initial specific PNSO hydrolase activity was 0.84 nmol min^{-1} (mg of protein)$^{-1}$.

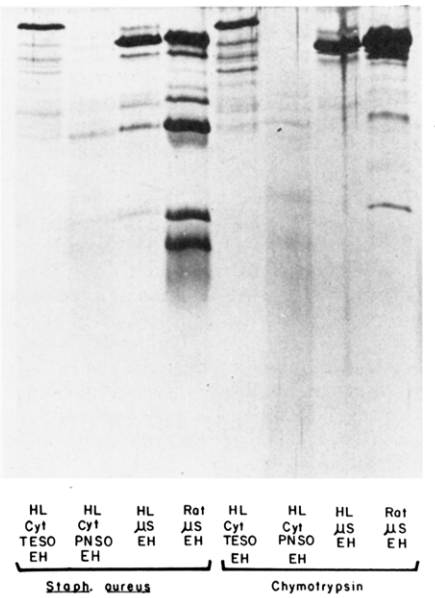

FIGURE 7: Peptide mapping of human and rat liver epoxide hydrolases using partial proteolytic digestion and $NaDodSO_4$–polyacrylamide gel electrophoresis. Either 0.5 μg of *Staphylococcus aureus* V8 protease or α-chymotrypsin was incubated with 25 μg of each epoxide hydrolase in 0.25 mL of a buffer system described by Cleveland et al. (1977) and electrophoresed as described in that same reference. Gels were stained with alkaline silver by using the procedure described by Wray et al. (1981). Individual wells included human liver cytosolic TESO hydrolase from patient 23 (HL CYT TESO EH), human liver cytosolic PNSO hydrolase from patient 18 (HL CYT PNSO EH), human liver microsomal epoxide hydrolase from patient 18 (HL CYT PNSO EH), and rat liver microsomal epoxide hydrolase (RAT EH).

Catalytic Activities. The activities of the purified human liver epoxide hydrolases are shown for several substrates in Table II, and data are also presented for human liver microsomes and cytosol and purified rat microsomal epoxide hydrolase.

Purified cytosolic TESO hydrolase catalyzed the hydrolysis of SO, PNSO, TESO, CESO, *trans*-stilbene oxide, and naphthalene 1,2-oxide at significant rates. However, the rates of hydrolysis of SO and PNSO were quite low when compared to the rates found with the purified human and rat liver microsomal epoxide hydrolases. The rates of hydrolysis of TESO and *trans*-stilbene oxide were similar for the human cytosolic TESO and microsomal epoxide hydrolases. Cytosolic TESO hydrolase hydrolyzed CESO at a rate 10 times faster than did human liver microsomal epoxide hydrolase.

The specificity pattern of purified human cytosolic PNSO hydrolase was similar to that of the microsomal hydrolase, although in every case the rates were an order of magnitude lower.

Comparison of the rates of hydrolysis observed with the purified hydrolase and crude cytosolic and microsomal fractions was also revealing. The conclusion could be reached that all of the hydrolase activities are mainly localized in microsomal fractions, with the exception of CESO hydrolase. The basis of the observed activities in crude subcellular fractions is the substantial rates with the microsomal hydrolase found toward most of the substrates, even those which are metabolized by cytosolic TESO hydrolase. On the other hand, the specific CESO hydrolase activities of cytosolic and microsomal fractions were similar, for purified cytosolic TESO hydrolase was an order of magnitude more active than the microsomal enzyme toward this substrate. We feel that these data are consistent with the view that human liver contains roughly 10

times more microsomal epoxide hydrolase than cytosolic TESO hydrolase.

Discussion

Early studies with SO and other substrates strongly suggested that epoxide hydrolase is strictly a microsomal enzyme (Oesch, 1973; Oesch et al., 1971), and this view was strengthened when the enzyme was purified from rat liver microsomes. The first suggestion that a soluble epoxide hydrolase might exist was given by Gill et al. (1974) with a juvenile hormone analogue. Since that first report, Hammock and his associates have developed several substrates that appear to be preferentially hydrolyzed by cytosolic fractions; differences in pH optima for hydrolyses catalyzed by microsomal and cytosolic fractions have also been identified (Gill & Hammock, 1979–1981; Ota & Hammock, 1980). In addition, an antibody raised to rat liver microsomal epoxide hydrolase was reported not to precipitate rat or mouse liver cytosolic TESO hydrolase activity (Guenthner et al., 1981). TESO hydrolase and similar activities have been reported to vary considerably with regard to animal species, tissue, and sex (Gill & Hammock, 1980; Ota & Hammock, 1980). To date, activity of this type in humans has not been considered. Questions also existed about the identity of the cytosolic TESO hydrolase and its similarity to the more extensively studied microsomal epoxide hydrolase.

The isolation of the cytosolic TESO hydrolase now provides the most definitive proof for its existence. The cytosolic enzyme clearly is physically different from the microsomal protein as judged by a number of criteria, including monomeric molecular weight, tendency to aggregate in the absence of detergents, peptide maps, and several immunological tests. These data are in agreement with some of the conclusions about differences reached only on the basis of substrate specificity in crude samples and provide a physical basis for this specificity, although the details of the relationship are not yet obvious.

Other studies have suggested that microsomal epoxide hydrolase can catalyze the hydrolysis of a wide variety of epoxides ranging from propylene oxide to oxides of large polycyclic aromatic hydrocarbons or steroids (Guengerich, 1982; Guengerich & Mason, 1980; Lu & Miwa, 1980; Oesch, 1973, 1980). Thus the specificity of the microsomal and cytosolic enzymes for the 8-alkyl-substituted styrene oxides is surprising. Nevertheless, substrate specificity is not complete, and the different hydrolases can all be considered to hydrolyze a variety of epoxides. The relatively high rate of hydrolysis of naphthalene 1,2-oxide by cytosolic TESO hydrolase is of interest. In this regard, TESO hydrolase activity does not appear to be a very specific marker of the cytosolic TESO hydrolase, particularly when the large amount of microsomal hydrolase present in the tissue is considered (Table II). CESO hydrolase, despite a low absolute activity, might be more useful as a marker.

Several questions about the PNSO hydrolase activity found in human cytosolic fractions have not been resolved. The activity is always found in cytosol, but biochemical tests do not indicate major differences with regard to the microsomal enzyme. Rat liver microsomal epoxide hydrolase is found in cytosol during development of preneoplastic lesions (Levin et al., 1978). Two hypotheses can be considered: (1) the enzyme is identical with the microsomal protein, but no longer bound to the membrane for some reason attributed to the membrane itself, or (2) the enzyme is slightly modified (with regard to the microsomal protein) in some way such that it no longer binds tightly to the membrane. In either case, the resulting effect on substrate specificity does not seem significant. However, the catalytic activity of the sample considered in Table II was much lower than that of the microsomal enzyme. We have isolated three other preparations of cytosolic PNSO hydrolase (in electrophoretically homogeneous form) from different patients, and the specific SO hydrolase activity has not exceeded 100 nmol min^{-1} (mg of protein)$^{-1}$. Extensive proteolysis could not have occurred (Figure 2), and the reason for the lower catalytic activity remains unclear. Deglycosylation can be considered, although rat liver microsomal epoxide hydrolase does not appear to be a glycoprotein (Guengerich & Davidson, 1982).

Questions about multiplicity of epoxide hydrolase within subcellular locations also need to be considered. Evidence has been presented that variants of microsomal epoxide hydrolase can be distinguished in rats and mice on the basis of genetic segregation (Lyman et al., 1980) and the catalytic, immunological, and physical properties of purified epoxide hydrolase fractions (Guengerich et al., 1979a,b). Others have obtained data to support a similar view using preferential inhibition of different activities (Watabe et al., 1981). Other data on immunoprecipitation of activities (Oesch, 1980) and partial sequencing of a purified epoxide hydrolase preparation (DuBois et al., 1979) have been interpreted as evidence for the existence of only one form of the enzyme. The data presented on the cytosolic TESO hydrolase suggest that this enzyme differs from microsomal epoxide hydrolase more than any of the isolated microsomal epoxide hydrolases differ from each other (Guengerich et al., 1979a,b; Lyman et al., 1980). However, the question of multiplicity of the cytosolic TESO hydrolase can also be raised. Samples obtained from some of the patients behaved somewhat differently in our purification scheme than did those described here. Even in Figure 1, a trailing shoulder of TESO hydrolase activity was found. (Three peaks of PNSO hydrolase activity were also eluted.) Further studies will be needed to determine if the enzyme isolated here is the only variant of epoxide hydrolase found in the cytosol or if other forms exist which have similar activites.

The importance of cytosolic epoxide hydrolase remains unclear. The microsomal enzyme is situated in close proximity to the enzymes (i.e., cytochromes P-450) which form epoxides and may even be coupled to those enzymes (Guengerich & Davidson, 1982; Kaminsky et al., 1981) to detoxicate these epoxides as they are formed. On the other hand, the view can be taken that a significant amount of epoxides can leave the endoplasmic reticulum and come into contact with cytosolic enzymes. In this regard, experiments with isolated hepatocytes have shown that the bulk of adducts formed from reactive metabolites of benzo[*a*]pyrene (Shen et al., 1980), vinyl chloride (Guengerich et al., 1981b), trichloroethylene,[2] and other compounds can be trapped with DNA located outside of the intact cells. Other work suggests that epoxides of arachidonic acid serve as substrates for both cytosolic and microsomal epoxide hydrolases (Oliw et al., 1982), and finite levels of such materials might be expected to be present under physiological conditions. The degree of purification needed to obtain a homogeneous preparation of human liver cytosolic TESO hydrolase (400-fold; cf. Guengerich et al., 1979b) and the comparisons of catalytic activities of crude and purified human liver preparations suggest that the level of this protein is roughly an order of magnitude less than that of microsomal epoxide hydrolase. The use of immunological procedures may be of help in ascertaining the levels of the enzyme found in various tissues and cell types, as well as in distinguishing

[2] R. E. Miller and F. P. Guengerich, unpublished results.

between roles for microsomal and cytosolic epoxide hydrolases and in the selection of an appropriate animal model. At this time, the available data do not point to a major role for the cytosolic epoxide hydrolases in the detoxication of most epoxides in human liver, when the higher concentrations of microsomal epoxide hydrolase and cytosolic glutathione *S*-transferase (Jakoby & Habig, 1980) are considered. However, the preferential action of cytosolic epoxide hydrolase on some potentially dangerous epoxides should not be ruled out.

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