Microsomal epoxide hydrolase of rat liver

Purification and characterization of enzyme fractions with different chromatographic characteristics

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Microsomal epoxide hydrolase was purified from rat liver, and different fractions of the purified enzyme, which varied in their contents of phospholipid, were obtained by ion-exchange chromatography. One fraction (A), which did not bind to CM-cellulose, had a high phospholipid content, and a second fraction (B), which was eluted from CM-cellulose at high ionic strength, had a low phospholipid content. Removal of most of the phospholipid from fraction A altered its chromatographic behaviour. When the delipidated material was re-applied to CM-cellulose, most of the enzyme bound to the cation-exchanger. The specific activities of all the fractions described (with styrene epoxide [(1,2-epoxyethyl)benzene] as substrate) were altered by adding the non-ionic detergent Lubrol PX or phospholipid. Lubrol PX inhibited enzyme activity, and phospholipid reversed this inhibition. The various enzyme fractions isolated appeared to be different forms of the same protein, as judged by their minimum M_r values and immunochemical properties. These results indicate that different fractions of epoxide hydrolase isolated by ion-exchange chromatography probably are not different isoenzyme forms.

INTRODUCTION

Microsomal epoxide hydrolase (EC 3.3.2.3) is localized in the endoplasmic reticulum of various tissues. It catalyses the hydration of arene epoxides to yield chemically less reactive *trans*-dihydrodiols (Oesch *et al.*, 1970; Oesch & Daly, 1971). As some of these arene epoxides have toxic, carcinogenic and mutagenic properties, the ability of epoxide hydrolase to transform them has been recognized as an important step in detoxification. It has been shown that certain *trans*-dihydrodiols of polycyclic aromatic hydrocarbons that contain a 'bay-region' double bond can be metabolized further by the cytochrome *P*-450 mixed-function oxidase system, to yield highly reactive vicinal diol epoxides (Wood *et al.*, 1976). These compounds have mutagenic and carcinogenic properties (Wood *et al.*, 1976; Yang *et al.*, 1977), and they are, in general, poor substrates for epoxide hydrolase (Jerina *et al.*, 1977; Levin *et al.*, 1978). Thus epoxide hydrolase plays a central role in both the detoxification and activation of polycyclic aromatic hydrocarbons.

Rat liver microsomal epoxide hydrolase has been purified to apparent homogeneity in several laboratories (Lu *et al.*, 1975; Bentley & Oesch, 1975; Knowles & Burchell, 1977). More recently, various apparently different fractions of epoxide hydrolase have been purified (Guengerich *et al.*, 1979a), and evidence suggesting that these fractions are different isoenzyme forms of epoxide hydrolase has been published. These fractions had different chromatographic, electrophoretic and immunochemical properties, and they varied in their relative substrate specificities (Guengerich *et al.*, 1979a, b). Microsomal membranes were dispersed with the use of Lubrol PX, and the detergent-solubilized material was chromatographed on DEAE-cellulose (Guengerich *et al.*,

1979a). The enzyme fraction that was eluted directly with loading buffer was applied to CM-cellulose. One fraction (A) was eluted directly, and a second fraction (B) then was eluted with a linear gradient of potassium phosphate. Both fractions subsequently were purified to apparent homogeneity by chromatography on hydroxyapatite. These different fractions of epoxide hydrolase showed slight variations in their amino acid compositions, immunochemical properties, relative substrate specificities and sensitivities towards activators and inhibitors.

Recently, however, it has been shown that different fractions of detergent-solubilized microsomal UDP-glucuronyltransferase (EC 2.4.1.17) varying in their contents of phospholipid can be isolated by ion-exchange chromatography (Wood *et al.*, 1985), and in the present paper we show that the phospholipid content of the fractions of epoxide hydrolase, isolated by the method of Guengerich *et al.* (1979a), determines their different chromatographic and catalytic properties. Fraction A had a high phospholipid content, whereas that of fraction B was very low. Also, we have characterized these fractions by immunochemical and electrophoretic methods, and we have studied the alterations of their catalytic properties caused by adding detergents and phospholipid.

EXPERIMENTAL

Materials

[7-³H]Styrene 7,8-epoxide {(1,2-epoxy[1-³H]ethyl)benzene} was obtained from Amersham International (Amersham, Bucks., U.K.). Allylbenzene epoxide [(2,3-epoxypropyl)benzene] was prepared by treating allylbenzene with *m*-chloroperbenzoic acid (Schwartz & Blumbers, 1964). Styrene 7,8-glycol [(1,2-dihydroxy-

Scheme 1. Purification of the various epoxide hydrolase fractions from rat liver microsomal fraction

ethyl)benzene] and allylbenzene glycol [(2,3-dihydroxypropyl)benzene] were prepared by treating their epoxides with 0.1 M-H₂SO₄ in aq. 40% (v/v) tetrahydrofuran. Styrene epoxide and allylbenzene were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Ion-exchange DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were obtained from Whatman (Maidstone, Kent, U.K.). Lubrol PX was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Hydroxyapatite was prepared by the method of Mazin *et al.* (1974). Materials for electrophoresis were from BDH Chemicals (Poole, Dorset, U.K.).

Methods

Liver microsomal fraction from male Wistar rats (200–260 g) was prepared as described previously (Bulleid & Craft, 1984). Epoxide hydrolase activity was measured with styrene 7,8-epoxide as substrate (Bulleid & Craft, 1984). The rates of hydrolysis of allylbenzene epoxide were measured by the method of Hammock & Hasagawa (1983). The initial stages of the purification of epoxide hydrolase (solubilization and chromatography on DEAE-cellulose) were identical with those described by Knowles & Burchell (1977). The subsequent stages of enzyme purification are outlined in Scheme 1. Fractions A and B were eluted from CM-cellulose as described by Guengerich *et al.* (1979a). Fraction B was submitted to chromatography on hydroxyapatite (Guengerich *et al.*, 1979a); the purified material obtained is designated CMB1. In order to remove phospholipid, fraction A was adjusted by dialysis to 1% (w/v) Lubrol/0.2 M-potassium phosphate buffer, pH 8.25, and fractionated with (NH₄)₂SO₄ (see Wood *et al.*, 1985). The enzyme was precipitated from the solution between 30 and 60% saturation with (NH₄)₂SO₄, and the precipitate was dissolved in 0.01% Lubrol PX/5 mM-potassium phosphate buffer, pH 6.5, and dialysed against 250 vol. of this buffer. The dialysed sample was applied to a column (2.5 cm × 40 cm) of CM-cellulose equilibrated with dialysis buffer. One fraction of the enzyme was eluted directly with loading buffer (fraction CMA2); a second fraction was desorbed with a similar buffer containing 200 mM-potassium phosphate (fraction CMB2). Both

fractions were dialysed against 250 vol. of 0.01% Lubrol PX/5 mM-potassium phosphate buffer, pH 7.4.

Protein concentrations were measured by a modification of the Lowry method (Wang & Smith, 1975), with bovine serum albumin as standard. Phospholipid was determined as inorganic phosphorus after extractions of phospholipid with chloroform/methanol (2:1, v/v) and drying (Graham *et al.*, 1974). Contents of detergent of the various fractions were measured by the method of Garewal (1973). Microsomal phospholipids were extracted by the method of Bligh & Dyer (1959) and were dispersed in 15 mM-EDTA/20 mM-Tris/HCl buffer, pH 8.0, by sonication.

Antisera to the epoxide hydrolase fractions were raised in New Zealand White rabbits by injecting 300 µg of protein in Freund's complete adjuvant. After 3 weeks, booster injections of 300 µg of protein in Freund's incomplete adjuvant were administered. After 6 weeks the animals were bled and the antisera were prepared. Immunodiffusion on 1.5 mm-thick agarose gels containing 0.1% Lubrol was carried out in a moist chamber at room temperature for 48 h (Ouchterlony, 1967).

SDS/polyacrylamide-gel electrophoresis was by the method of Laemmli (1970). Peptide mapping of trypsin digests with the use of SDS/polyacrylamide-gel electrophoresis was as described by Cleveland *et al.* (1977).

RESULTS

Purification of epoxide hydrolase

Table 1 shows data for a typical purification of epoxide hydrolase from rat liver and the ranges of results from eight such purifications. The purification scheme is shown in Scheme 1. As reported by Guengerich *et al.* (1979a), two fractions of epoxide hydrolase were separated on CM-cellulose. Fraction A had a high content of phospholipid, whereas fraction B had a low phospholipid content (Table 1). By assuming values of 800 and 50000 for the relative molecular masses of phospholipid and epoxide hydrolases, the phospholipid content was approx. 200 mol/mol for fraction A and 17 mol/mol for fraction B. When these fractions were re-applied to CM-cellulose, they retained their original chromatographic behaviour. Most of the phospholipid associated with fraction A (> 90%) was removed (Table 1) by precipitating the protein with (NH₄)₂SO₄ (30–60% saturation), and, when this partially delipidated material was applied to CM-cellulose, two fractions of enzyme activity were isolated. Fraction CMA2 was not absorbed, whereas fraction CMB2 bound and was desorbed at higher ionic strength (200 mM-potassium phosphate). Most of the activity recovered (75%) was associated with fraction CMB2. Fraction CMA2 had a higher content of detergent than did fraction CMB2. By assuming the relative molecular masses of Lubrol PX and epoxide hydrolase to be 600 and 50000 respectively, the detergent content of fraction CMA2 was near 500 mol/mol and that of fraction CMB2 was about 40 mol/mol.

When the activities of fractions CMB1 and CMB2 were measured with styrene 7,8-epoxide as substrate in the presence of various concentrations of Lubrol PX (Fig. 1) it was found that increasing detergent concentrations progressively inhibited the enzyme; little or no activity was observed in any of the fractions at a Lubrol PX concentration of 0.02% (Fig. 1).

The specific activities of the fractions were also

Table 1. Purification of microsomal epoxide hydrolase from rat liver

Epoxide hydrolase was purified from rat liver microsomal fraction as described in the Experimental section. Enzyme activity, protein concentration and phospholipid content were determined as described in the Experimental section. One unit of epoxide hydrolase activity is defined as the formation of 1 nmol of styrene glycol/min. Values are from one typical purification; data in parentheses are the ranges of values from eight purifications.

Purification stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Relative purification (fold)	Recovery (%)	Phospholipid content (μg of P/mg of protein)
Lubrol-soluble microsomal protein	1032	17266	17.1 (13.8–27.1)	1	100	—
DEAE-cellulose eluate	97.58	16076	164.8 (85.0–314)	9.6	90.9	—
Fraction A	25.315	10860	429.0 (190–661)	25.1	61.4	129.2 (69.6–328)
Fraction B	11.4	2025	177.7 (23.2–177)	10.4	11.4	11.05 (0–14.6)
Fraction CMB1 after hydroxyapatite chromatography	4.13	1054	255.0 (255–303)	14.9	6.1	—
30–60% -satn. - $(\text{NH}_4)_2\text{SO}_4$ precipitate (dialysed)	8.32	4650	559.0 (280–559)	32.7	26.3	6.9 (0–6.9)
Fraction CMA2	1.6	414	256.3 (123.0–468.5)	14.9	2.3	9.5 (0–9.5)
Fraction CMB2	3.8	2837	735.5 (489–1285)	43	16	7.02 (0–7.02)

Fig. 1. Inhibition by Lubrol PX of the activities of the epoxide hydrolase fractions

The rates of hydrolysis of styrene 7,8-epoxide by the various epoxide hydrolase fractions were determined in the presence of the concentrations of the detergent indicated. The values shown are means of duplicate determinations obtained with fractions CMB1 (●), CMA2 (△) and CMB2 (▲).

determined with allylbenzene epoxide as substrate. As with styrene epoxide, increasing concentrations of Lubrol PX progressively inhibited enzyme activity (results not shown). The inhibition of the catalytic activity by detergent was reversed by adding microsomal phospholipid (Table 2). When microsomal phospholipid was included in reaction mixtures containing high detergent concentrations, the specific activity was approximately doubled.

The antigenic properties of the different enzyme fractions obtained were investigated by Ouchterlony double diffusion with antisera raised against fraction CMB2 (Fig. 2). A single continuous precipitin band was formed against all the enzyme fractions, and identical results were obtained with antisera raised against fraction CMA2 (results not shown).

SDS/polyacrylamide-gel electrophoresis of the various epoxide hydrolase fractions (Fig. 3) indicated that all are highly purified. The subunit M_r of the major band of each fraction was close to 50000, and they could not be distinguished from one another.

All the fractions showed identical peptide maps after digestion with trypsin (results not shown).

DISCUSSION

Various fractions of liver microsomal epoxide hydrolase have been separated chromatographically (Guengerich *et al.*, 1979a), and it is widely accepted that these fractions are different isoenzyme forms of epoxide hydrolase. Evidence to support this claim is based on the slightly different electrophoretic and immunological properties of the fractions and on the differences in their relative substrate specificities (Guengerich *et al.*, 1979a,b).

Evidence now presented here shows that such differences in chromatographic and catalytic properties may be due to reasons other than the existence of isoenzyme variants of the enzyme. Solubilization of microsomes with Lubrol PX gives rise to at least two populations of epoxide hydrolase. One population contains significant quantities of phospholipid and is not bound by CM-cellulose, whereas the other population contains little phospholipid and is bound (Table 1). The two populations are partially interconvertible in that delipidation of the lipid-rich fractions alters its properties so that most of the enzyme activity now binds to CM-cellulose. Enzyme fractions obtained from a second

Fig. 2. Ouchterlony double-diffusion analysis of the purified epoxide hydrolase fractions

Immunodiffusion was carried out as described in the Experimental section. The centre well contained 25 μ l of antiserum to fraction CMA2. The surrounding wells contained: 1 and 4, fraction CMA1, 3 μ g of protein; 2 and 6, fraction CMB2, 5 μ g of protein; 3, fraction CMB1, 2 μ g of protein; 5, fraction CMA2, 3 μ g of protein.

CM-cellulose chromatography step indicated a further possible cause of artifacts. Thus the two fractions obtained contained markedly different quantities of Lubrol PX. It could be argued that Lubrol PX may also alter the chromatographic behaviour of epoxide hydrolase. Although this is a non-ionic detergent, its binding to epoxide hydrolase could alter the pattern of charges in contact with CM-cellulose. Thus, although the net charge of the enzyme is unaltered, the binding characteristic of the complex may change (Peterson, 1975). Evidence to support this argument comes from the ion-exchange behaviour of another detergent-solubilized integral

Fig. 3. SDS/polyacrylamide-gel electrophoresis of Lubrol PX-solubilized microsomal fraction and of the purified epoxide hydrolase fractions

The purified fractions were prepared as described in the Experimental section. Lane A, Lubrol PX-solubilized microsomal material, 20 μ g of protein; lane B, fraction CMA1, 5 μ g of protein; lane C, fraction CMB1, 2 μ g of protein; lane D, fraction CMA2, 3 μ g of protein; lane E, fraction CMB2, 5 μ g of protein.

Table 2. Effects of added microsomal phospholipid on the activities of the purified epoxide hydrolase fractions in the presence of added Lubrol PX

Epoxide hydrolase was assayed with styrene 7,8-epoxide as substrate by the method described in the text. Lubrol PX was added to the reaction mixture to the concentration indicated. Enzyme activity was measured in the absence and in the presence of added phospholipid at a concentration of 7 μ g of P/mg of protein.

Epoxide hydrolase fraction	Concn. of Lubrol PX in reaction mixture (% , w/v)	Specific activity (nmol/min per mg of protein)	
		Without added phospholipid	With phospholipid
CMB1	0.013	82.0 $\pm$ 2.0	159.0 $\pm$ 28.0
CMA2	0.012	100.0 $\pm$ 50.2	243.4 $\pm$ 50.7
CMB2	0.012	198.3 $\pm$ 11.0	483.2 $\pm$ 8.5

enzyme. UDP-glucuronosyltransferase fractions with high phospholipid contents were not retained by DEAE-cellulose; fractions with much lower lipid contents were bound and were desorbed by eluent of increased ionic strength. Moreover, the chromatographic behaviour of this enzyme was modified after delipidation by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Wood *et al.*, 1985).

Microsomal epoxide hydrolase activity is affected by detergents and phospholipid. Detergent causes either an activation (Burchell *et al.*, 1976) or an inhibition (Bulleid & Craft, 1984) of styrene epoxide hydrolase activity in rat liver microsomal fractions. Phospholipid altered the activity of the purified enzyme towards a variety of substrates, possibly by causing substrate partitioning into phospholipid micelles (Lu *et al.*, 1977). The activity towards styrene epoxide of the purified fractions isolated in the present work was inhibited by Lubrol PX (Fig. 1). Addition of phospholipid reversed this inhibition (Table 2). Although a mechanism for this phenomenon is unclear, the implications of these findings are quite obvious. If small quantities of detergent or phospholipid alter the specific activity of the purified enzyme, any comparison of the specific activities of fractions with different contents of phospholipids and detergent, or of differential effects of activators or inhibitors upon these enzyme fractions, must be viewed with caution.

The apparent different structural and functional properties of the enzyme fractions generated by CM-cellulose chromatography are probably artifacts due to lipid and/or detergent binding. Further evidence of the similarity of the enzyme fractions isolated here includes the immunochemical properties (Fig. 2), electrophoretic mobilities (Fig. 3) and the identical peptide maps after digestion with trypsin.

Although the present paper demonstrates that apparent isoenzymes of epoxide hydrolase can be generated as artifacts, this does not mean that isoenzymes of epoxide hydrolase do not exist. Indeed, work by Levin *et al.* (1983) indicates that distinct microsomal epoxide hydrolase isoforms catalyse the hydration of 5,6-epoxycholesterol and certain xenobiotic alkene and arene epoxides. Also Guenther & Oesch (1983) have identified from rat liver microsomal fractions an epoxide hydrolase fraction with completely different immunochemical properties, substrate specificity and pH optimum from those of the enzyme fractions studied in the present work. However, Dubois *et al.* (1982) have suggested that rat epoxide hydrolase exists as a single enzyme species in the endoplasmic reticulum, and this conclusion was reached from the highly conserved nature of the terminal sequences and amino acid composition of purified epoxide hydrolase from rat liver.

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