Fractionation and purification of hepatic microsomal cytochrome *P*-450 isoenzymes from phenobarbital-pretreated rats by h.p.l.c.

A convenient tool for screening of isoenzymes inactivated by allylisopropylacetamide

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A procedure incorporating the salient features of ion-exchange column chromatography with ion-exchange h.p.l.c. is described for the fractionation and purification to homogeneity of several membrane-bound rat hepatic phenobarbital (PB)-inducible cytochrome *P*-450 isoenzymes, including the major PB-inducible species. The resolving power of this technique makes it a highly promising tool for the isolation and purification of closely related cytochrome *P*-450 isoenzymes. In addition, it may also be used for screening of individual isoenzymes either selectively induced or repressed by a variety of endobiotics or xenobiotics. Accordingly, we have exploited this particular feature to identify not only the PB-inducible cytochrome *P*-450 isoenzymes destroyed *in vivo* by allylisopropylacetamide, a suicide inactivator of cytochrome *P*-450, but also to distinguish those that are reparable by exogenous haemin from those that are irreparably damaged.

INTRODUCTION

Cytochrome *P*-450 consists of a family of closely related isoenzymes that catalyse the oxidative metabolism of a wide variety of endogenous and exogenous substrates. These isoenzymes are regulated not only by hormonal and dietary factors (Levin *et al.*, 1975; Waxman *et al.*, 1985), but also by exposure to xenobiotics that can induce and/or suppress specific cytochrome *P*-450 isoenzymes. The most extensively studied of such inducers is phenobarbital (PB), and as many as eight distinct hepatic isoenzymes have been purified from PB-pretreated rats (Waxman, 1984). However, it is possible that additional inducible and/or constitutive hepatic isoenzymes are present, but that the conventional column-chromatographic approaches lack the necessary resolving power to reveal their individual existence. Such a shortcoming, however, may now be remedied with introduction of h.p.l.c. for the separation of proteins, including the membrane-bound cytochrome *P*-450 isoenzymes (Kotake & Funae, 1980; Kusunose *et al.*, 1981; Regnier, 1983; Bansal *et al.*, 1985; Bornheim *et al.*, 1985; Fujita *et al.*, 1985; Holm & Kupfer, 1985; Iversen & Franklin, 1985; Ueng & Alvares, 1985). The dual features of speed and a resolution capacity superior to that of the conventional techniques indicate that h.p.l.c. would be a promising tool for protein separations. Accordingly, the pioneering studies by Kotake & Funae (1980) and the more recent reports from several laboratories including our own have indicated that ion-exchange h.p.l.c. may be utilized not only for separation and/or purification of multiple cytochrome *P*-450 isoenzymes, but also for screening the inductive/suppressive effects of xenobiotics on particular isoenzyme species (Kusunose *et al.*, 1981; Bansal *et al.*, 1985; Bornheim *et al.*, 1985; Holm & Kupfer, 1985; Iversen & Franklin, 1985). However, reports that h.p.l.c.-isolated/purified cytochrome *P*-450 isoenzymes either lacked

functional viability (Bansal *et al.*, 1985) or exhibited low specific content (Holm & Kupfer, 1985) as a result of possible haem-stripping during chromatography may have discouraged serious consideration of this technique for the isolation of multiple cytochrome *P*-450 isoenzymes. Since the original submission of this manuscript, we became aware of the report by Funae & Imaoka (1985), which should allay any reservations to the use of this technique for the isolation and purification of mammalian cytochrome *P*-450 isoenzymes.

We became specifically interested in this technique because its protein-resolving power would allow us not only to resolve systematically the entire complement *in vivo* of hepatic microsomal cytochrome *P*-450 isoenzymes in PB-pretreated rats, but also to screen those that were susceptible to destruction *in vivo* by allylisopropylacetamide (AIA), a prototype cytochrome *P*-450 suicide inactivator (De Matteis, 1970; Ortiz de Montellano *et al.*, 1978). Such screening would allow us to confirm whether the 70% loss of hepatic cytochrome *P*-450 content induced by AIA entails loss of several isoenzymes in addition to that of cytochrome *P*-450b (PB-4), the major PB-inducible isoenzyme. Although this isoenzyme is believed to be AIA-selective by assessments made *in vitro* (Ortiz de Montellano *et al.*, 1981; Waxman & Walsh, 1982), it represents at best only 30% of the hepatic microsomal cytochrome *P*-450 complement of PB-pretreated rats (Guengerich *et al.*, 1982). In fact, from findings obtained with isoenzyme-specific substrates, we and others have inferred that multiple cytochrome *P*-450 isoenzymes are indeed inactivated *in vivo* by AIA treatment but that only a few of these are amenable to repair by exogenous haem (Bonkowsky *et al.*, 1984; Bornheim *et al.*, 1985). Furthermore, we believe that inactivation of some of these isoenzymes might involve mechanisms other than the prosthetic haem *N*-alkylation, an intrinsic feature of AIA-mediated inactivation of

Abbreviations used: PB, phenobarbital; AIA, allylisopropylacetamide.

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cytochrome *P*-450b. Examination of such mechanistic possibilities, however, requires that the individual isoenzymes not only be first identified but also be isolated and purified in a functionally competent form. With this ultimate goal in mind we now report the isolation and purification by h.p.l.c. in tandem with conventional chromatography of certain PB-inducible cytochrome *P*-450 isoenzymes in a catalytically viable form. We also demonstrate that, because of its relatively greater resolving power, h.p.l.c. may be a useful tool for preliminary screening of cytochrome *P*-450 isoenzymes of interest. Thus in this study we have employed h.p.l.c. to identify certain isoenzyme populations susceptible to AIA inactivation and to distinguish those isoenzymes that are haemin-reparable from those that are not.

METHODS

Preparation of rat liver microsomal fraction

Male Sprague-Dawley rats (200–250 g) were given food and water *ad libitum*, and were pretreated with PB (80 mg/kg, intraperitoneally) daily for 5 days. In some cases PB-pretreated animals were killed 1 h after administration of AIA (200 mg/kg, intraperitoneally). A liver homogenate (50%, w/v) was prepared in 0.25 M-sucrose containing GSH (10 mM), pH 7.4. Homogenates were incubated with or without haemin (50 μ M) at 37 °C for 15 min. Microsomal fraction (referred to below simply as 'microsomes') was prepared as previously described (Bornheim *et al.*, 1985). Cytochrome *P*-450 content was determined by the method of Estabrook *et al.* (1972), and protein concentration was determined by the method of Lowry *et al.* (1951), with human serum albumin as the standard.

Initial fractionation by DEAE-cellulose DE-52 column chromatography

Microsomes were solubilized as described previously (Warner *et al.*, 1978) with sodium cholate (0.5% w/v), Emulgen 911 (0.2%, v/v), EDTA (0.1 mM) and glycerol (20%, v/v) in 10 mM-sodium phosphate buffer, pH 7.4, and then applied to a DEAE-cellulose DE-52 column (2.5 cm $\times$ 35 cm) previously equilibrated with the solubilizing buffer (West *et al.*, 1979). Fraction I was eluted in the void volume when 100 ml of the solubilizing buffer was passed through the column at a flow rate of 1 ml/min (Fig. 1a). Fractions II and III were eluted when the column was washed with 600 ml of the solubilizing buffer containing 20 mM-KCl. This procedure caused fraction IV to move midway down the column and thus resolve itself from the band containing cytochrome *b*₅ and NADPH-cytochrome *P*-450 reductase. The column packing was gently extruded from the support, and the band containing fraction IV (30% of the original cytochrome *P*-450 content and presumably enriched in cytochrome *P*-450b) was physically excised and eluted with the solubilizing buffer containing 150 mM-KCl, desalted, concentrated, and either applied to a second DEAE-cellulose DE-52 column (Fig. 1b) or chromatographed by h.p.l.c. (Fig. 1d).

Rechromatography of fraction IV on DEAE-cellulose DE-52 with subsequent h.p.l.c. on Sphergel-TSK DEAE-3SW

After extrusion, excision and subsequent desalting and concentration of fraction IV from the first DEAE-

cellulose DE-52 column, portions were applied to a second DEAE-cellulose DE-52 column (2.5 cm $\times$ 35 cm) that was then developed with a linear 0–150 mM-KCl gradient in solubilizing buffer to yield a chromatographic profile very similar to that previously reported (West *et al.*, 1979; Waxman & Walsh, 1982). Arbitrarily pooled peak fractions, denoted A, B, C and D, (Fig. 1b), were collected, desalted, concentrated and individually subjected to h.p.l.c. on an analytical Beckman Sphergel-TSK DEAE-3SW (10 μ m particle size) column (7.5 mm $\times$ 7.5 cm) equilibrated with buffer T [20 mM-Tris/HCl buffer, pH 7.2, containing EDTA (0.1 mM), glycerol (20%, v/v) and Emulgen 911 (0.2%, v/v)] at a flow rate of 1 ml/min. A stepwise gradient of increasing salt concentration was developed by introducing buffer T containing 0.5 M-NaCl. The resultant h.p.l.c. profiles are indicated in Fig. 1(c).

H.p.l.c. chromatography of DEAE-cellulose DE-52 fraction IV

For this study, portions of fraction IV (0.6 mg of protein) obtained from the initial DEAE-cellulose DE-52 column were directly applied to the analytical h.p.l.c. column described above and eluted with the same stepwise gradient as in Fig. 1(c) to yield the chromatographic profile depicted in Fig. 1(d).

Purification of cytochrome *P*-450 isoenzymes by h.p.l.c.

To obtain sufficient quantities of isoenzymes for purification, portions of fraction IV were chromatographed on a preparative Beckman Sphergel-TSK DEAE-3SW (10 μ m particle size) column (21.5 mm $\times$ 15 cm) with a similar gradient to that described above, which yielded a comparable profile (Fig. 2). The fractions eluted at 60–80 min under peak B, and representing the major PB-inducible cytochrome *P*-450 in fraction IV, were collected, pooled, desalted, concentrated and applied to a Beckman Sphergel-TSK CM-3SW (10 μ m particle size) cation-exchange column (7.5 mm $\times$ 7.5 cm). Cytochrome *P*-450 isoenzymes were eluted with buffer T, pH 7.2, at 1 ml/min with a salt gradient (Fig. 3). The fractions eluted in the void peak 1 and in the peak fractions 3 and 4 with retention times of 50 and 64 min respectively were collected, individually concentrated, desalted and rechromatographed on the analytical DEAE-3SW (10 μ m particle size) column (7.5 mm $\times$ 7.5 cm) with buffer T, pH 7.2, and a gradually increasing salt gradient (Fig. 4).

The individual fractions were collected and further purified and freed from Emulgen 911 by detergent exchange on a Bio-Rad HPHT (h.p.l.c.) column (100 mm $\times$ 7.8 mm) or conventional hydroxyapatite column chromatography (Bio-Gel HTP; Bio-Rad Laboratories) at 0.5 ml/min with the following buffer sequence: (a) initially, buffer A, consisting of sodium phosphate (10 mM), CaCl₂ (0.01 mM) and glycerol (20%, v/v), pH 7.4, was used, (b) followed by buffer B, in which sodium phosphate and sodium cholate concentrations were increased to 25 mM and 0.5% respectively, and finally (c) buffer C, in which sodium phosphate and sodium cholate concentrations were increased to 150 mM and 1.5% respectively.

Sodium cholate was initially decreased to 0.005% by repetitive concentration and dilution of sample with 10 mM-sodium phosphate buffer, pH 7.4, containing glycerol (20%, v/v) by the use of an Immersible CX-30

Ultrafiltration unit (Millipore Corp.). However, a considerable loss of material was observed, this being due to irreversible binding to the ultrafiltration membrane when the sodium cholate concentration was decreased to below 0.15%. In subsequent studies cholate was therefore removed with a Centricon-30 Microconcentrator (Amicon) or a Micro-Pro Di Con concentrator (Pierce), which was found quite satisfactory.

SDS/polyacrylamide-gel electrophoresis

Discontinuous polyacrylamide-gel electrophoresis was carried out in the presence of 0.1% SDS as described by Laemmli (1970). A 20 µg portion of protein was loaded on to a 1.5 mm gel and electrophoresed at 20 and 30 mA through the stacking and running gels respectively. Gels were stained with 0.05% Coomassie Brilliant Blue and destained by the procedure of Fairbanks *et al.* (1971).

Peptide mapping

Purified isoenzymes (6 µg of protein each) were subjected to limited proteolysis by the procedure of Cleveland *et al.* (1977) in the presence of *Staphylococcus aureus* V8 proteinase (Cooper Biomedical) (18 µg) or chymotrypsin (Worthington) (4.5 µg). Samples were incubated for 15 min at 37 °C, denatured with 3% (w/v) SDS and 10% (v/v) 2-mercaptoethanol, and boiled for 5 min before electrophoresis on a 12% polyacrylamide gel. Gels were then stained with Bio-Rad Silver Stain (Bio-Rad Bulletin 1989).

Enzyme assays

For assessment of mixed-function oxidase activity, the purified cytochrome *P*-450 isoenzymes (0.1 nmol) were reconstituted in the presence of saturating amounts of NADPH (2 mM), substrate [benzphetamine (2 mM), ethylmorphine (2 mM) or 7-ethoxycoumarin (0.2 mM)], lipid [50 µg of dimyristoyl phosphatidylcholine (Sigma Chemical Co.)/ml], cytochrome *b*₅ (0.1 nmol), partially purified rat liver microsomal NADPH-cytochrome *P*-450 reductase and 50 mM-sodium phosphate buffer, pH 7.4. The reductase was prepared by the method of Yasukochi & Masters (1976) as modified by Chung & Chao (1980), and had a specific activity of 16.7 µmol of cytochrome *c* reduced/min per mg of protein at 25 °C in 0.1 M-sodium phosphate buffer, pH 7.4. Cytochrome *b*₅ was purified by the method of Waxman & Walsh (1982) and was more than 95% pure as judged by SDS/polyacrylamide-gel electrophoresis. When the effect of exogenous haemin on the activity of these isoenzymes was tested, haemin (5 µM) dissolved in 0.1 M-NaOH and neutralized to pH 7.4 was included in the above incubation system. The amount of formaldehyde generated was quantified by the method of Nash (1953). 7-Ethoxycoumarin *O*-de-ethylase activity was determined by the direct fluorimetric assay method of Burke & Mayer (1974).

Effect of AIA on hepatic cytochrome *P*-450 isoenzyme profiles

Liver homogenates obtained from AIA-treated PB-pretreated rats were incubated with or without haemin (50 µM) as previously described (Bornheim *et al.*, 1985). Microsomes were prepared, washed and then subjected to DEAE-cellulose DE-52 chromatography as described above. Fraction IV (0.6 mg of protein) was directly applied to the analytical Spherogel-TSK DEAE-3SW

column and eluted under conditions identical with those for fraction IV from the PB controls (Fig. 7). In addition, fraction I (1.36 mg of protein) of rat liver microsomes from PB (control), AIA-treated, AIA-treated and haemin-incubated rat liver homogenates was also subjected to h.p.l.c. on the analytical Spherogel-TSK CM-3SW column (Fig. 8).

RESULTS AND DISCUSSION

Consistent with previous reports (Warner *et al.*, 1978; West *et al.*, 1979; Waxman & Walsh, 1982), DEAE-cellulose DE-52 anion-exchange chromatography of solubilized hepatic microsomes from PB-pretreated rats yields four fractions containing cytochrome *P*-450 isoenzymes (Fig. 1*a*). The non-anionic cytochrome *P*-450 species are eluted in the void peak (fraction I), whereas fractions II and III are eluted with a salt concentration of 20 mM-KCl. Under these conditions, fraction IV, containing mainly cytochrome *P*-450b, remains bound to the column.

When fraction IV was extracted from this band (see the Methods section) and portions were rechromatographed on a second DEAE-cellulose DE-52 column under the conditions detailed in the Methods section, the profile obtained (Fig. 1*b*) is similar to that previously reported by others (West *et al.*, 1979; Waxman & Walsh, 1982). Subfractions of DEAE-cellulose-DE-52-rechromatographed fraction IV, denoted A, B, C and D, were subjected to h.p.l.c. on an analytical Spherogel-TSK DEAE-3SW column under the conditions detailed in the Methods section, the corresponding profiles depicted in Fig. 1*c* were obtained. In parallel, when portions of fraction IV obtained from the initial DEAE-cellulose DE-52 column were directly subjected to h.p.l.c. under conditions identical with those described for its subfractions A–D, three fully resolved major peaks were distinctly observed (Fig. 1*d*), with retention times of 15, 28 and 36 min for peaks 1, 2 and 3 respectively.

Comparison of the h.p.l.c. profiles in Figs. 1*c* and 1*d* shows that fraction A contained primarily cytochrome *P*-450 isoenzymes eluted in peak 1 with traces of those in peak 3. Fraction B consisted of equal amounts of the cytochrome *P*-450 isoenzymes in peaks 1 and 3. On the other hand, fraction C contained cytochrome *P*-450 isoenzymes eluted in all three peaks, whereas fraction D contained predominantly cytochrome *P*-450 isoenzymes eluted in peaks 2 and 3, with traces of those in peak 1. These findings thus indicate that not only is fraction IV composed of at least three populations of cytochrome *P*-450 isoenzymes of distinct anionic characteristics, but also that the cytochrome(s) eluted in peak 2 was/were more sharply eluted from the DEAE-cellulose DE-52 than those in either peak 1 or 3, which appeared to exhibit a more widespread distribution. These findings indicated that not only was the resolution obtained by this initial h.p.l.c. step far superior to that afforded by conventional column chromatography, but also it entailed considerably less time. Moreover, because this h.p.l.c. step can provide proportionately larger amounts of relatively well-resolved isoenzymes for subsequent purification steps, it would obviate the customary wasteful procedure of obtaining cuts enriched in a given cytochrome *P*-450 isoenzyme. Furthermore, it is also evident that, because no DEAE-cellulose DE-52 cut contains a single isoenzyme, further separation of

Fig. 1. Comparison of profiles of cytochrome P-450 isoenzymes separated by conventional column chromatography with that by h.p.l.c.

(a) Elution scheme for DEAE-cellulose DE-52 column chromatography of solubilized microsomes from PB-induced rats. Solubilized microsomes were loaded on to the DEAE-cellulose DE-52 column, and fraction I was eluted with solubilizing buffer (100 ml) (see the Methods section). Fractions II and III were eluted with solubilizing buffer containing 20 mM-KCl (600 ml). Fraction IV, which remained bound, was physically excised from the column packing and eluted with solubilizing buffer containing 150 mM-KCl. After desalting and concentration, fraction IV was rechromatographed either on a second DEAE-cellulose DE-52 column (b) and/or by h.p.l.c. (c and d). (b) Elution profile of fraction IV after rechromatography on a second DEAE-cellulose DE-52 column. After fraction IV had been loaded on to a second DEAE-cellulose DE-52 column, cytochrome P-450 isoenzymes were eluted with a linear gradient of 0–150 mM-KCl in solubilizing buffer (1 litre). Cytochrome P-450 content of individual fractions was determined and several fractions of each peak were pooled to yield fractions A–D. (c) Elution profile of fractions A–D (obtained from DEAE-cellulose DE-52 rechromatography) after anion-exchange h.p.l.c. Fractions A–D were applied to a Beckman Spherogel-TSK DEAE-3SW anion-exchange column and eluted at a flow rate of 1 ml/min by increasing salt concentration in buffer T (see the Methods section) according to the following stepwise gradient conditions: 0–15 min, 25 mM-NaCl; 15–25 min, 50 mM-NaCl; 25–35 min, 100 mM-NaCl; 35–45 min, 500 mM-NaCl [---- in (d)]. The column was then re-equilibrated with buffer T containing 25 mM-NaCl for 20 min before subsequent injections. (d) Elution profile of fraction IV (obtained from the first DEAE-cellulose DE-52 chromatography step) after anion-exchange h.p.l.c.. Fraction IV was chromatographed by h.p.l.c. exactly as described in (c).

isoenzymes is imperative when conventional DEAE-cellulose DE-52 column chromatography is employed.

Further purification of the h.p.l.c.-resolved isoenzymes, however, necessitated larger quantities than those obtainable by analytical h.p.l.c. For this purpose, 100 nmol of cytochrome P-450 (fraction IV) was chromatographed on a preparative Spherogel-TSK

DEAE-3SW anion-exchange column. A representative h.p.l.c. chromatogram of three such separate injections is illustrated (Fig. 2) in order to document the reproducibility of the method. The major peak fraction B (eluted at 60–80 min; Fig. 2) was selected for further purification. After pooling, desalting and concentration, portions of this fraction were applied to an analytical Spherogel-TSK

Fig. 2. Elution profile of fraction IV (obtained from the first DEAE-cellulose DE-52 chromatography step) after preparative anion-exchange h.p.l.c.

Fraction IV (100 nmol of cytochrome *P*-450) was applied to a preparative Beckman Spherogel-TSK DEAE-3SW anion-exchange column and eluted at a flow rate of 3 ml/min with buffer T containing a progressive stepwise salt gradient over 115 min as follows: 0–40 min, 25 mM-NaCl; 40–70 min, 50 mM-NaCl; 70–100 min, 100 mM-NaCl; 100–115 min, 500 mM-NaCl (----). The column was then re-equilibrated with buffer T containing 25 mM-NaCl for 40 min before subsequent injections. Included are the chromatograms of two additional injections (— and ·····) to illustrate the reproducibility of the method.

Fig. 3. Cation-exchange h.p.l.c. elution profile of cytochrome *P*-450 isoenzymes in peak B obtained by preparative anion-exchange h.p.l.c.

Cytochrome *P*-450 isoenzymes eluted in peak B fraction from the preparative anion-exchange h.p.l.c. (Fig. 2) were applied to a Beckman Spherogel-TSK CM-3SW cation-exchange column and eluted at a flow rate of 1 ml/min with buffer T containing an increasing salt concentration according to the following gradient conditions: 0–15 min, 0–50 mM-NaCl; 15–25 min, 50 mM-NaCl; 25–55 min, 50–100 mM-NaCl; 55–65 min, 100 mM-NaCl; 65–75 min, 500 mM-NaCl (----). The column was re-equilibrated with buffer T for 30 min before subsequent injections.

Fig. 4. Elution profile of cytochrome *P*-450 isoenzymes in peaks 1, 3 and 4 (obtained by cation-exchange h.p.l.c.) after a second anion-exchange h.p.l.c. step

Cytochrome *P*-450 isoenzymes eluted in peaks 1, 3 and 4 from the cation-exchange h.p.l.c. column (Fig. 3) were subjected to an analytical anion-exchange h.p.l.c. step and eluted at a flow rate of 1 ml/min with buffer T containing an increasing salt concentration according to the following gradient conditions: 0–30 min, 0–150 mM-NaCl; 30–35 min, 150–500 mM-NaCl; 35–40 min, 500 mM-NaCl (----). The column was re-equilibrated with buffer T for 20 min before subsequent injections. In order to obtain a better resolution of the isoenzymes in peak 1a from those in peak 1b, gradient conditions were altered as follows (inset): 0–40 min, 0–100 mM-NaCl; 40–45 min, 100–500 mM-NaCl; 45–50 min, 500 mM-NaCl (----). The column was re-equilibrated with buffer T for 20 min before additional injections.

Fig. 5. SDS/polyacrylamide-gel electrophoretogram of various chromatographic fractions and purified isoenzymes

Fractions (20 μ g of protein per well) were loaded and electrophoresed at 20 and 30 mA through the stacking and running gels respectively. (a) Gel electrophoresis of various chromatographic fractions. Lane 1, solubilized microsomes; lane 2, fraction IV from the first DEAE-cellulose DE-52 step; lane 3, peak B from the preparative anion-exchange h.p.l.c. (Fig. 2); lanes 4, 5 and 6, peaks 1, 3 and 4 from the cation-exchange h.p.l.c. respectively (Fig. 3). (b) Gel electrophoresis of purified isoenzymes obtained as in Fig. 4. Lane 2, purified isoenzyme 1a; lane 3, purified isoenzyme 1b; lane 4, purified isoenzyme 3; lane 5, purified isoenzyme 4; lanes 1 and 6, molecular-mass protein standards (21.5, 31, 45, 66.2 and 92.5 kDa). (c) Gel electrophoresis of an isoenzyme purified from peak B subfraction eluted between 75 and 80 min (Fig. 2) by sequential cation-exchange and anion-exchange h.p.l.c. and conventional hydroxyapatite chromatography. Lanes 1 and 5, molecular-mass protein standards (21.5, 31, 45, 66.2 and 92.5 kDa); lane 2, isoenzyme (1 μ g); lane 3, isoenzyme (2 μ g); lane 4, isoenzyme (4 μ g). The apparent molecular mass of this isoenzyme is 51.9 kDa and its specific content is 13 nmol/mg of protein.

CM-3SW cation-exchange column (Fig. 3). The major peak fractions (1, 3 and 4) were individually collected and rechromatographed on an analytical Spherogel-TSK DEAE-3SW anion-exchange column (Fig. 4), which resolved peak 1 into two peaks (1a and 1b) as well as further purifying peaks 3 and 4. Additional purification and removal of Emulgen 911 was accomplished by hydroxyapatite h.p.l.c. as described in the Methods section. The above procedures permitted purification of at least four isoenzymes from the major peak B eluted from the preparative column (Fig. 2), with specific contents of 11.0, 9.4, 9.4 and 13.9 nmol per mg of protein for isoenzymes in peaks 1a, 1b, 3 and 4 respectively.

Comparison of the SDS/polyacrylamide-gel-electrophoresis profiles of the various chromatographic peak

fractions (Fig. 5a) with those of the four purified isoenzyme fractions (Fig. 5b) reveals the progressive course of the purification of the isolated isoenzymes and permits assessment of their relative purity and molecular masses. Thus isoenzymes 1a, 1b and 4 were found to have near-identical molecular masses (approx. 51900 Da) whereas isoenzyme 3 was found to exhibit a slightly higher molecular mass (52500 Da). By this SDS/polyacrylamide-gel-electrophoretic criterion, isoenzyme 1a appears to be relatively pure as evidenced by a single band at the 51900 Da region, whereas isoenzymes 1b, 3 and 4 exhibit minor degrees of cross-contamination. However, higher purification of any single cytochrome P-450 isoenzyme may be achieved by taking narrower cuts from the preparative anion-exchange h.p.l.c. subfraction. For instance, a single isoenzyme with no

Fig. 6. Peptide maps of purified isoenzymes proteolysed with *Staphylococcus aureus* V8 proteinase and α -chymotrypsin

(a) Purified isoenzymes (6 μ g of protein each) were incubated with *Staphylococcus aureus* V8 proteinase (18 μ g) for 15 min at 37 °C before electrophoresis on a 12% polyacrylamide gel. Lane 1, proteinase only; lane 2, isoenzyme 1a; lane 3, isoenzyme 1b; lane 4, isoenzyme 3; lane 5, isoenzyme 4. Arrows denote peptide differences between isoenzymes. (b) As in (a) except that α -chymotrypsin (4.5 μ g) was used as the proteinase. Lane 1, isoenzyme 1a; lane 2, isoenzyme 1b; lane 3, isoenzyme 3; lane 4, isoenzyme 4; lane 5, proteinase only.

Table 1. Mono-oxygenase activities of h.p.l.c.-purified cytochrome *P*-450 isoenzymes

Purified cytochrome *P*-450 isoenzymes (0.1 nmol) were reconstituted in the presence of saturating amounts of partially purified rat liver microsomal NADPH-cytochrome *P*-450 reductase, cytochrome *b*₅ (0.1 nmol), NADPH (2 mM), substrate [benzphetamine (BZP, 2 mM), ethylmorphine (EM, 2 mM) or 7-ethoxycoumarin (EC, 0.2 mM), dimyristoyl phosphatidylcholine (50 μ g/ml) and 50 mM-sodium phosphate buffer, pH 7.4. Enzyme activity was monitored as described in the Methods section. No significant changes in benzphetamine *N*-demethylase activity were observed when haemin (5 μ M) was included in the reconstituted system.

Isoenzyme	Cytochrome <i>P</i> -450		<i>N</i> -Demethylase activity (nmol of formaldehyde formed/min per nmol of cytochrome <i>P</i> -450)			<i>O</i> -De-ethylase activity (nmol of 7-hydroxycoumarin formed/min per nmol of cytochrome <i>P</i> -450)
	Specific content (nmol/mg of protein)	λ_{max} of ferrocyclochrome <i>P</i> -450-CO complex (nm)	With BZP	With EM	BZP/EM ratio	
1a	11.0	450	43	15	2.9	0.43
1b	9.4	450	26	9	2.9	0.35
3	9.4	451	9	14	0.6	< 0.10
4	13.9	450	51	16	3.2	0.36

contaminating bands (Fig. 5c) and a specific content of 13.0 nmol/mg of protein was obtained when the 75–80 min ‘window’ of peak fraction B (Fig. 2) was taken as the starting material. Although apparently pure, the specific content of this isoenzyme is somewhat lower than those reported by others for such isoenzymes. This low value might be due to some haem-stripping during h.p.l.c., although addition of exogenous haem did not appear to improve it.

Limited peptide proteolysis of the four isoenzymes revealed almost identical peptide maps for isoenzymes 1a, 1b and 4 after partial digestion with either of the two proteinases employed (Figs. 6a and 6b), but several peptide map differences were observed after digestion of isoenzyme 3 with either proteinase (arrows).

No significant differences were observed in the CO-induced spectral characteristics of these isoenzymes (Table 1). However, upon reconstitution of catalytic enzyme activity, isoenzymes 1a, 1b and 4 were found to exhibit markedly similar specific activities as well as comparable ratios of benzphetamine *N*-demethylase to ethylmorphine *N*-demethylase activity (Table 1). In contrast, isoenzyme 3 was found to exhibit relatively poor benzphetamine *N*-demethylase activity and 7-ethoxycoumarin *O*-de-ethylase activity.

In summary, isoenzyme 3 was found to exhibit clearly different molecular mass, peptide maps, cationic chromatographic properties and functional activity from those of isoenzymes 1a, 1b and 4. Isoenzyme 1a may be distinguished from isoenzymes 1b and 4 by distinct

Fig. 7. Anion-exchange h.p.l.c. profiles of cytochrome P-450 isoenzymes in fraction IV of liver microsomes

(a) Elution profile of fraction IV (0.6 mg of protein obtained from liver microsomes of a PB-pretreated rat fractionated on DEAE-cellulose DE-52) subjected to anion-exchange h.p.l.c. exactly as detailed in the legend to Fig. 1(c). (b) Similar profile to that in (a) except that the liver microsomes were obtained from PB-pretreated rats given AIA (200 mg/kg, intraperitoneally) for 1 h. (c) As in (b) except that microsomes were prepared from liver homogenates incubated with haemin (50 μ M) for 15 min at 37 °C (see the Methods section). (d) Composite profiles of (a)–(c). The area bounded by the continuous line represents the chromatogram in (a). The stippled area represents the chromatogram in (b). The area bounded by the broken line represents the chromatogram in (c).

anionic chromatographic properties (Fig. 4), and from isoenzyme 4 by its different cationic properties (Fig. 3), whereas isoenzyme 1b may be distinguished from isoenzyme 4 only by its cationic properties (Fig. 3). Since, by and large, these isoenzymes appear to be structurally and functionally similar, their differential chromatographic behaviour may reflect either structural microheterogeneities, or more probably variable residual lipid/detergent content, as indeed noted for cytochrome P-450 isoenzymes (Liu *et al.*, 1986) and for microsomal epoxide hydratases (Bulleid *et al.*, 1986). The above findings thus indicate that ion-exchange h.p.l.c. may be used not only to resolve different ionic species of hepatic cytochrome P-450, but also to isolate and purify them in a functionally competent form.

In this initial approach, we deliberately focused on h.p.l.c. characterization of a single rat liver microsomal fraction containing the major PB-inducible cytochrome P-450 isoenzyme. However, since *in vivo* AIA apparently inactivates several additional enzymes, we have used

Fig. 8. Composite cation-exchange h.p.l.c. profiles of cytochrome P-450 isoenzymes in fraction I of liver microsomes from PB-pretreated rats given AIA

DE-52 fraction I (1.36 mg of protein) was subjected to cation-exchange h.p.l.c. and eluted at a flow rate of 1 ml/min with buffer T containing a progressive salt gradient as follows: 0–3 min, 0 mM-NaCl; 7–10 min, 25 mM-NaCl; 10–25 min, 25–50 mM-NaCl; 25–30 min, 50 mM-NaCl; 30–45 min, 50–100 mM-NaCl; 45–55 min, 100–200 mM-NaCl; 55–65 min, 500 mM-NaCl. The column was then re-equilibrated with buffer T for 20 min before subsequent injection. The areas bounded by the continuous line, stippled area and broken line represent chromatograms from fraction I of liver microsomes from PB-pretreated rats, AIA-treated rats and haemin-incubated homogenates from AIA-treated rats respectively.

h.p.l.c. in tandem with conventional anion-exchange column chromatography to identify cytochrome P-450 isoenzyme populations from PB-pretreated rats that are susceptible to AIA-mediated inactivation, and to distinguish those that are repairable by exogenous haem from those that are irreparably damaged. For this purpose, DEAE-cellulose DE-52 fractions I and IV of solubilized microsomes obtained from liver homogenates of PB/AIA-treated rats incubated in the presence and in the absence of haem were subjected to ion-exchange h.p.l.c. and the profiles compared with those obtained with corresponding liver microsomal fractions from PB-pretreated rats not given AIA (Fig. 7). Anion-exchange h.p.l.c. of DEAE-cellulose DE-52 fraction IV obtained from AIA-treated rat liver indicated the loss of at least three cytochrome P-450 isoenzyme populations (Fig. 7b), all of which could be restored by incubation of the rat liver homogenates *in vitro* with haemin (Figs. 7c and 7d). Similarly, cation-exchange h.p.l.c. of DEAE-cellulose DE-52 fraction I revealed the loss of a single isoenzyme population (Fig. 8, peak 2), which, however, could not be similarly restored by incubation *in vitro* with haemin. We believe this fraction might contain cytochrome P-450_p (PB-2a), which appears to be inexorably

inactivated by AIA treatment (Underwood *et al.*, 1985). Thus the two fractions of rat hepatic microsomes examined reveal AIA-mediated loss of at least four different cytochrome *P*-450 isoenzymes, one of which remains refractory to haemin-mediated repair.

No significant restoration of any of these isoenzyme populations was observed if before h.p.l.c. haemin was simply incubated with the DEAE-cellulose DE-52 fraction of solubilized hepatic microsomes from AIA-treated rats. Haemin so added was, however, eluted in the peak fraction emerging at 45 min (Fig. 7c), thereby accounting for the relative increase in the A_{417} of this peak fraction (Fig. 7c). It should be noted, however, that this peak is devoid of any spectrally detectable cytochrome *P*-450, but contains proteins that loosely bind haem. This feature, coupled with the huge A_{280} of these proteins, contributes to the apparent increase in the A_{417} absorbance of the eluate at 45 min. It may also be noted that, with the exception of the 45 min peak fraction, the haem content spectrophotometrically determined by pyridine haemochromogen is stoichiometric to the spectrally determinable cytochrome *P*-450 content of the various peak fractions irrespective of whether they were derived from haemin-reconstituted or non-reconstituted preparations.

Our findings indicate that h.p.l.c. may not only be an efficient and expedient tool for isolation and characterization of several functionally viable cytochrome *P*-450 isoenzymes, but also may be used for screening of individual forms that might be selectively affected by xenobiotics, nutrients, hormones or toxicants.

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