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High Pressure Liquid Chromatographic Separation of Multiple Forms of Cytochrome P-450

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The major form of cytochrome P-450 isolated and purified from the hepatic microsomes of phenobarbital pretreated rats by sequential chromatography on n-octylamino-Sepharose 4B and DEAE-cellulose columns was found to be homogeneous by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. However, this cytochrome P-450 was resolved into three bands by high pressure liquid chromatography on an Anpac ion-exchange column. High pressure liquid chromatography isolated forms had similar molecular weights of 55,000 with λ_{max} of the CO-reduced difference spectrum at 450 nm and were found to be in the low spin state. The results demonstrate the effectiveness of high pressure liquid chromatography in the resolution of cytochrome P-450s of similar molecular weights but different net charges.

Cytochrome P-450s, terminal oxidases of the MFO system, are pivotal enzymes in the metabolism of a wide variety of endogeneous and exogeneous compounds (1,2). During the recent years, different forms of cytochrome P-450 isozymes have been reported (2-10). Resolution of MFO complex into its components, and isolation, characterization and identification of cytochrome P-450 isozymes is essential to the understanding of chemical carcinogenesis, mutagenesis, teratogenesis, chemical toxicology and pharmacological activity of most drugs, including some anticancer drugs.

The number and form(s) of cytochrome P-450 existing in a species is known to vary with induction by a xenobiotic (2-3). This has facilitated purification of several forms of cytochrome P-450. Several laboratories have isolated a major form of cytochrome P-450 from phenobarbital-induced Sprague-

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Abbreviations: MFO, microsomal mixed function oxidase; HPLC, High pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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Dawley rat liver microsomes (hereafter known as major PB-inducible cytochrome P-450) (6-8). This form of cytochrome P-450 has been shown to be homogeneous by SDS-PAGE (6-8). However, SDS-PAGE is capable of resolving cytochrome P-450s of different molecular weights but not cytochrome P-450s which may differ only in their net charges. Indeed, the presence of more than one species in the major PB-inducible cytochrome P-450 has been reported (2,6,9,11). In this report, with the use of HPLC, three forms of cytochrome P-450 have been isolated from the major PB-inducible cytochrome P-450 and physical properties of these three forms have been described.

Materials and Methods

Materials. Sepharose 4B and DEAE-cellulose were purchased from Pharmacia Fine Chemicals, Piscataway, NJ and n-octylamino Sepharose 4B was prepared as described previously (6,12). Cholic acid, coomassie brilliant blue R, butylated hydroxytoluene and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, Missouri. Emulgen 911 was obtained from Kao-Altas Co., Tokyo, Japan.

Isolation of Hepatic Microsomes. Male Sprague-Dawley rats (200-300 g) obtained from Harlan Sprague-Dawley Industries, Indianapolis, IN, were used for the isolation of hepatic microsomes. Rats received commercially available rat chow and water ad libitum and were treated with phenobarbital by inclusion of the drug (0.1%, w/v) in drinking water for 6 days prior to sacrifice. Rats were decapitated and livers were removed and perfused with ice-cold saline. The perfused livers were minced into small pieces and homogenized in a Potter-Elvehjem tissue grinder with teflon pestle for 90 sec at 1000 rpm in 3 volumes of 0.154 M KCl. The homogenate was centrifuged at 15,000 g for 15 min and the resulting supernatant was centrifuged at 105,000 g for 60 min. The resulting microsomal pellet was suspended in 0.154 M KCl containing 1 mM EDTA and 20 μM butylated hydroxytoluene and reisolated by centrifugation at 105,000 g for 90 min. The final pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4. Isolation of microsomes was carried out at 4°C. Protein in the microsomal suspension was determined by the method of Lowry et al. (13) using bovine serum albumin as reference standard.

Isolation of the Major PB-Inducible Cytochrome P450. The major PB-inducible cytochrome P-450, "Peak B_2", was isolated as described by Guengerich and Martin (6). Briefly, the hepatic microsomes from phenobarbital pretreated rats were solubilized in potassium phosphate buffer, pH 7.25 with recrystallized sodium cholate (0.6%, w/v) in the presence of 20% (v/v) glycerol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 25 μM butylated hydroxytoluene. The 105,000 g supernatant of the solubilized microsomes was subjected to n-octylamino-Sepharose 4B chromatography and the resulting cytochrome P-450-containing band, after concentration and dialysis, was subjected to DEAE cellulose chromatography. SDS-PAGE was performed on individual fractions under peak B_2 (Figure 2 in Reference 6) and the fractions exhibiting a single band on the gel corresponding to cytochrome P-450 were pooled. The pooled fractions were treated with Bio-beads SM-2 (Bio-Rad Labs., Richmond, CA) to partially remove the detergent, concentrated and dialyzed against 10 mM tris-acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol.

The purified major PB-inducible cytochrome P-450, thus obtained, had a specific content of 14.5 nmol cytochrome per mg protein.

High Pressure Liquid Chromatography. The HPLC separation of cytochrome P-450s was carried out according to the procedure of Kotake and Funae (14) on a Waters HPLC system consisting of two 6000A pumps, System Controller, Data Module and a model 440 Absorbance Detector fitted with a 405 nm wavelength kit. The separation was performed on a 4.6 x 250 mm Anpac anion-exchange column (Anspec, Warrenville, IL). Upto 1 mg of protein was applied to the column equilibrated with buffer A [20 mM tris-acetate buffer, pH 7.2 containing 0.2% (v/v) Emulgen 911 and 20% (v/v) glycerol]. The elution was performed with a linear gradient of buffer B (0.8 M sodium acetate in buffer A) incorporating 50% buffer B in buffer A linearly in 20 min at a flow rate of 1.6 ml/min. At the end of the gradient, the column was eluted with 100% buffer B for 10 min and subsequently washed with buffer A for 10 min at a flow rate of 1.6 ml/min before application of the next sample.

Other Procedures. Difference and absolute spectra were recorded at ambient temperature on an Aminco DW-2 spectrophotometer calibrated with holmium oxide filter. The cytochrome P-450 content was determined from the CO-reduced difference spectrum according to the method of Omura and Sato (15) using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. SDS-PAGE was carried out in a Hoeffer (Bio-Rad Labs.) slab gel apparatus (10 cm length, 1.5 mm gel thickness) at ambient temperature, according to Laemmli (16). Concentrations of the stacking and separating gels were 3 and 10% acrylamide, respectively, and the gel was subjected to a constant current of 25 mA. Gels were stained in 0.25% Coomassie blue in water:methanol:acetic acid (45:45:10) for 40 min and destained in water:methanol:acetic acid (45:45:10) overnight with gentle shaking.

Results

HPLC Analysis of Solubilized Rat Hepatic Microsomes. The hepatic microsomes of Sprague-Dawley rats suspended at a concentration of 10 mg/ml protein were solubilized with sodium cholate (0.5%, w/v) and Emulgen 911 (0.2%, v/v) in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol and 1 mM EDTA. The solubilization was performed by gently stirring the suspension at 4°C for 30 min. The solubilized microsomes were spun in a microfuge for 30 sec and 100 µl of the supernatant was applied to the HPLC. The HPLC profile (Figure 1A) contained several peaks at elution times ranging from 1 to 26.23 min. The peaks at 9.16, 11.50, 13.43 and 26.23 min were collected individually and the peaks between 1 and 3 min, and between 4 and 8 min were collected as pools. The CO-reduced difference spectra of these collected peaks were recorded to determine the presence of cytochrome P-450. The peak at 26.23 min did not contain any cytochrome P-450 and was probably due to cytochrome b5 (14). The spectra of all other peaks indicated the presence of varying amounts of cytochrome P-450.

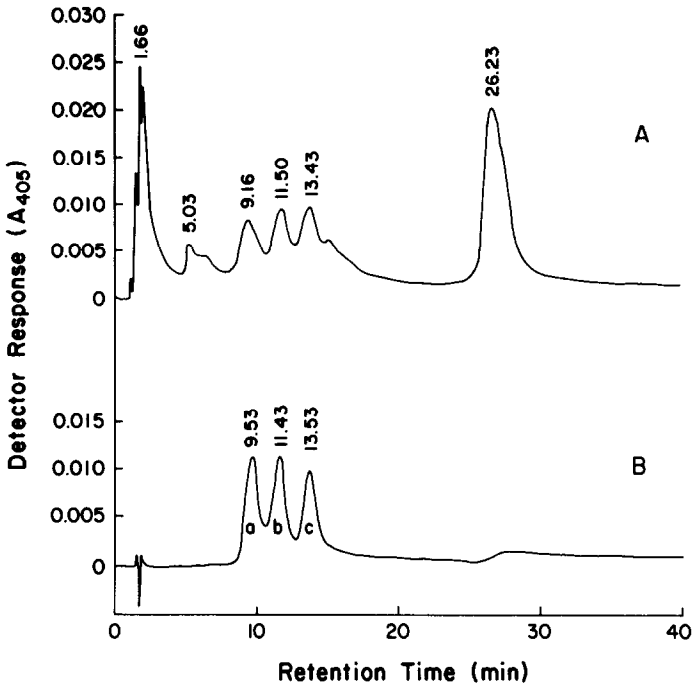

Figure 1: High pressure liquid chromatographic separation of cytochrome P450s. (A) 1 mg solubilized hepatic microsomal protein from phenobarbital pretreated rats. (B) 1.2 nmol major PB-inducible cytochrome P-450. The retention times of major peaks have been indicated. Experimental details are given under Materials and Methods.

HPLC Analysis of the Major Cytochrome P450 Purified from the Hepatic Microsomes. The major form of cytochrome P-450 was isolated and purified from the hepatic microsomes of PB-induced rats by sequential chromatography on n-octylamino-Sepharose 4B and DEAE cellulose columns as described under Materials and Methods. The purified cytochrome P-450 exhibited a single band by SDS-PAGE (Figure 2). An aliquot of the purified cytochrome P-450 was analyzed by HPLC and the resulting profile is shown in Figure 1B. Three bands were obtained at retention times of 9.53, 11.43 and 13.53 min. These three bands correspond to the three bands present in the HPLC profile of solubilized microsomes at retention times of 9.16, 11.50 and 13.43 min (Figure 1A). The three HPLC bands obtained from the major PB-inducible cytochrome P-450 were collected from several HPLC runs (the retention times of the three bands in different runs varied less than 0.5 min). The collected peaks a, b, and c (Figure 1B) were concentrated and then dialyzed

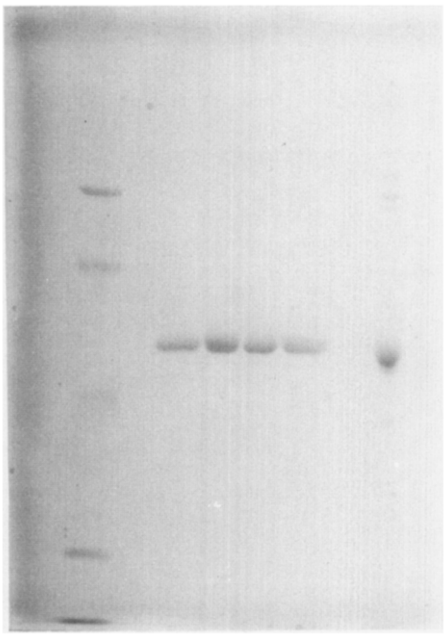

Figure 2: SDS-PAGE of purified cytochrome P450s. The wells (from left to right) contained a mixture of standards with molecular weights of 92,500 (top), 66,200, 45,000, 31,000 and 14,400 (bottom), equal amounts (4 μg protein each) of HPLC purified cytochrome P-450s a, b and c (Figure 1B), a mixture of equal amounts of these three forms, and major PB-inducible cytochrome P-450.

against two changes of 1 liter 10 mM tris-acetate buffer, pH 7.4 containing 20% (v/v) glycerol and 1 mM EDTA. These cytochrome P-450s were subjected to SDS-PAGE and their spectral properties were determined. The results are shown in Table I. All three cytochrome P-450s moved with the same mobility on SDS-PAGE and exhibited a molecular weight of 55,000 calculated by co-electrophoresis with molecular weight standards. The molecular weight of

Table I
Physical Properties of HPLC Isolated Cytochrome P-450s from the Major PB-Inducible Cytochrome P-450

Cytochrome P-450	λ_{max} (nm) Oxidized	Reduced	Fe^{2+}-CO Complex	Mol. Wt.
a	416, 535, 567	421, 548	450	55,000
b	416, 535, 568	423, 540	450	55,000
c	417, 534, 568	420, 553	450	55,000

55,000 was similar to the molecular weight of the major PB-inducible cytochrome P-450. λ_{max} of the CO-reduced difference spectrum of all three forms of cytochrome P-450 was at 450 nm and their absolute spectra were consistent with their being in the low spin state (9,17). However, some differences in the λ_{max} in the absolute spectrum of the oxidized and reduced forms were evident (Table I).

Discussion

Presence of a single band by SDS-PAGE is one of the important criteria for testing the homogeneity of enzymes. Using such a criterion the major PB-inducible cytochrome P-450 obtained in several laboratories (6-8) would be considered homogeneous. However, doubts have been expressed concerning the homogeneity of such a preparation (2,6,9,11). Thomas et al. (11) have provided immunological evidence for the presence of three species in the major PB-inducible cytochrome P-450. Recently Waxman and Walsh (9) isolated two cytochrome P-450s from the major PB-inducible cytochrome P-450. In this report we have shown the presence of three cytochrome P-450s in the major PB-inducible cytochrome P-450. The reason why these forms were not discernible by SDS-PAGE is clear from their similar molecular weights and behavior on SDS-PAGE (Table I and Figure 2). The three forms had similar mobility on SDS-PAGE and did not separate when electrophoresed as a mixture (Figure 2). Kotake and Funae (14) reported a single major HPLC peak from the purified cytochrome P-450 isolated from phenobarbital-pretreated Sprague-Dawley rats. They also reported only one major peak in the HPLC profile of the PB-induced microsomes in the retention time interval of 8 to 14 min. However, here we demonstrate the presence of 3 major peaks in the same retention time interval.

In conclusion, the isolation of three forms of cytochrome P-450s from the major PB-inducible cytochrome P-450 provides an evidence for the presence of charge heterogeneity among the cytochrome P-450s of similar molecular weights and furthermore HPLC provides a fast and an easy tool for the determination of this heterogeneity.

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