Journal of Chromatography, 297 (1984) 119–127
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 386

RESOLUTION OF MULTIPLE FORMS OF CYTOCHROME P-450 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The major forms of cytochrome P-450 in the hepatic microsomes of rats pretreated with phenobarbital (PB) or 3-methylcholanthrene (3MC) were isolated by sequential chromatography on *n*-octylamino-Sepharose 4B and DEAE-cellulose columns. These preparations exhibited single protein bands corresponding to cytochrome P-450s by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). High-performance liquid chromatography (HPLC) of these preparations on an anion-exchange column yielded three peaks from the PB-induced major cytochrome P-450 and a single peak from the 3MC-induced major cytochrome P-450. That the HPLC-isolated protein peaks were various forms of cytochrome P-450 was confirmed by spectral examination and sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Examination of their absolute spectra revealed these cytochrome P-450s to be in a low-spin state. The λ_{max} of the reduced CO-complex spectra and molecular weights were found to be 450 nm and 53,000, respectively, for all the three HPLC-resolved cytochrome P-450s from PB-induced rats; and 448 nm and 56,000, respectively, for the HPLC-isolated cytochrome P-450 from 3MC-induced rats. The results demonstrate the effectiveness of HPLC in the determination of cytochrome P-450 multiplicity and charge heterogeneity.

INTRODUCTION

Cytochrome P-450, the terminal oxidase and the primary determinant of substrate specificity of the microsomal mixed function oxidase (MFO) system, plays a vital role in the metabolism of many endogeneous and exogeneous compounds[1–5]. Since cytochrome P-450 multiplicity is implicated in the heterogeneity of the MFO system, capable of catalyzing the metabolism of a wide variety of compounds of unrelated structures, the purification, characterization and determination of the number of forms of cytochrome P-450s has drawn attention for the last several years[5–9]. Cytochrome P-450s are known to be induced by several xenobiotics and this has facilitated purification of some specific forms of cytochrome P-450s[3–5]. Ion-exchange column chromatography coupled with other pre-purification methods has been commonly used for the purification of cytochrome P-450s and has yielded electrophor-

0021-9673/84/$03.00

etically apparent homogeneous forms of cytochrome P-450s from rats, rabbits and mice[5–7]. In this report, such electrophoretically apparent pure forms of cytochrome P-450s obtained from phenobarbital (PB-) and 3-methylcholanthrene (3MC)-induced rat hepatic microsomes were analyzed by HPLC on an anion-exchange columns. Results demonstrated the presence of further heterogeneity in the purified PB-induced cytochrome P-450 but not in the 3MC-induced cytochrome P-450. This report therefore, demonstrates the effectiveness of high-performance liquid chromatography (HPLC) in providing an effective tool for the separation of cytochrome P-450s based on charge differences and in supplementing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) for the determination of cytochrome P-450s multiplicity.

EXPERIMENTAL

Preparation of microsomes

Male Sprague-Dawley rats (200–250 g), obtained from Harlan Sprague-Dawley Industries, Indianapolis, IN, U.S.A., were used. Rats received commercially available rat chow and water *ad libitum* until their killing. Rats were treated with PB by inclusion of the drug (0.1%, w/v) in drinking water continuously for six days prior to being killed. Treatment with 3MC was by intraperitoneal injection of the chemical (dissolved in corn oil) at a dose of 25 mg per kg animal weight once each day for three days prior to killing. Animals were decapitated and livers were removed and perfused with ice-cold saline. Microsomes from perfused livers were prepared as reported previously[10].

Purification of cytochrome P-450

Cytochrome P-450 was isolated from the PB-induced and 3MC-induced rat liver microsomes according to the method described by Guengerich and Martin[9]. The method involved, in either case, solubilization of microsomes with sodium cholate, chromatography on *n*-octylamino-Sepharose 4B, followed by chromatography of the resulting cytochrome P-450 peak on DEAE-cellulose. Fractions under peak B_2 (Figs. 2 and 7 in ref. 9) were monitored by SDS–PAGE and the fractions exhibiting a single band on gel electrophoresis were pooled. The pooled B_2 peaks, representing the major forms of PB and 3MC-induced cytochrome P-450s, were treated with Bio-Beads SM-2 (Bio-Rad Labs., Richmond, CA, U.S.A.) to remove partially the detergent and were concentrated and dialyzed as described[9].

HPLC of cytochrome P-450s

HPLC of the cytochrome P-450s was carried out on a Waters HPLC system according to the method of Kotake and Funae[11]. The HPLC system consisted of two 6000-A pumps, System Controller, Data Module and model 440 Absorbance Detector. HPLC was performed on a 25 cm × 4.1 mm I.D. Anpak anion-exchange column (Anspec, Ann Arbor, MI, U.S.A.) and the effluent was monitored at 405 nm. HPLC solvents were: A, 20 m*M* Tris acetate buffer, pH 7.2, containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911 (Kao-Atlas, Tokyo, Japan); and B, 0.8 *M* sodium acetate in solvent A. Elution was performed at ambient temperature at a flow-rate of 1.6 ml/min with a linear gradient of solvent B (50% solvent B in 20 min)

in solvent A. At the end of 20 min the column was washed with 100% solvent B for 10 min and then with 100% solvent A for 10 min before application of the next sample.

With proper care of the column, the column efficiency was maintained for over a year during which well over a hundred samples (upto 1 mg protein) were applied to the column. After use, at the end of the day, the column was washed thoroughly with water and then with methanol and was preserved in methanol when not in use. A 5-μm pre-column filter was used to avoid frequent contamination of the column with particulate impurities that may be present in the sample. The pre-column filter was removed and cleaned quite regularly in a sonicator bath. When excess pressure (200–400 p.s.i. in excess to the back pressure of the new column) would build in the column, the column inlet frit was opened, the top 2–4 mm of column packing was scrapped off, and the column inlet was rinsed with methanol. The top of the column was then packed with Synchropak, ASC bulk support (Anspec), suspended in methanol. The inlet frit of the column was either replaced or cleaned in a sonicator bath. Such an operation on the column did not produce any noticeable change in the efficiency of the column and the column life was extended dramatically.

Other methods

SDS–PAGE was performed on 1.5-mm thick × 10-cm long gels as described by Laemmli[12], in a Hoeffer (Bio-Rad) slab gel apparatus at ambient temperature. Acrylamide concentration of the stacking and separating gel was 3 and 10%, respectively. Gels were stained with Coomassie Blue for visualization. The absolute (oxidized and reduced) and difference (sodium dithionite reduced CO-complex) spectra were recorded with an Aminco DW-2 spectrophotometer, calibrated with a holmium oxide filter. Protein was determined by a modified Lowry method[13].

RESULTS

HPLC analysis of major cytochrome P-450s purified from hepatic microsomes

The major forms of cytochrome P-450s were isolated from PB-induced or 3MC-induced rat hepatic microsomes by sequential chromatography on n-octylamino Sepharose 4B and DEAE-cellulose columns, as described by Guengerich and Martin[9]. Purified cytochrome P-450s from each of these preparations exhibited a single band of cytochrome P-450 by SDS–PAGE (Fig. 1). These cytochrome P-450s were analyzed by HPLC on an anion-exchange column and the results are shown in Fig. 2. The PB-induced cytochrome P-450 separated into three peaks with retention times of 10.6, 12.8 and 14.3 min. The 3MC-induced cytochrome P-450 yielded one major peak at the retention time of 12.3 min. HPLC of several similar preparations of PB and 3MC-induced cytochrome P-450s gave similar profiles; however, relative peak heights varied somewhat for different preparations. The retention times of all peaks shifted up to 1 min for different preparations but the relative retention times were constant.

Characterization of HPLC isolated cytochrome P-450s

Individual peaks derived from the HPLC analysis of major PB- and 3MC-induced cytochrome P-450s were collected. These were concentrated and dialyzed

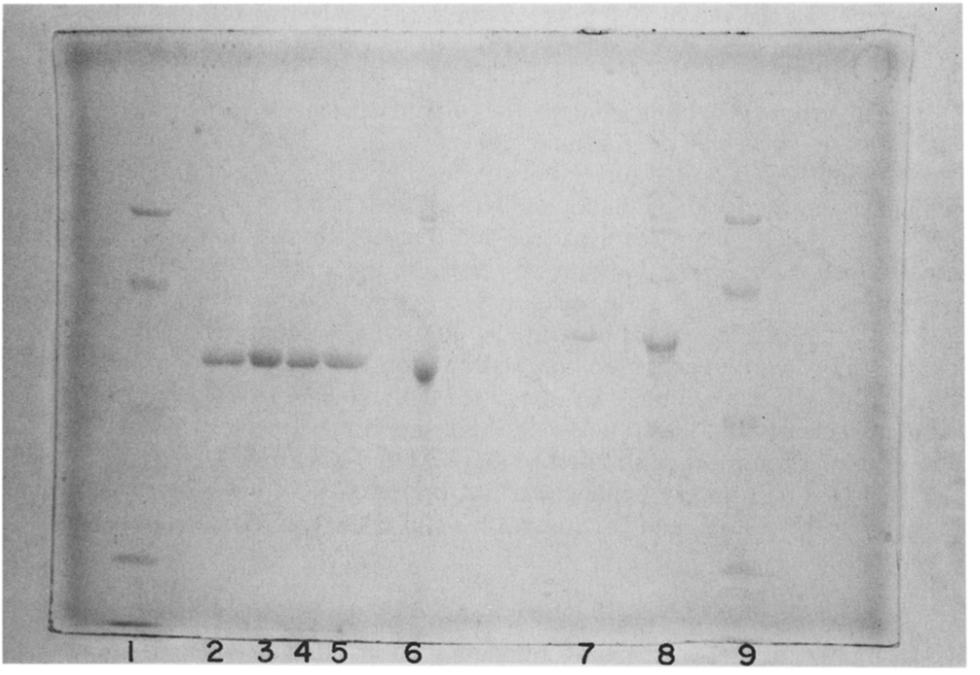


Fig. 1. SDS–PAGE of cytochrome P-450s. Electrophoresis on SDS–polyacrylamide gels was performed as described under Experimental. Wells 1 and 9 contained a mixture of standard molecular weight proteins, phosphorylase B (molecular weight 92,500, top), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000) and lysozyme (14,400, bottom). Wells 2, 3 and 4 contained HPLC-purified PB-induced cytochrome P-450s a, b and c (Fig. 2A) (4 μg protein each). Well 5 contained a mixture of equal amounts of PB-induced cytochrome P-450s, a, b and c (total protein 4 μg). Wells 6 and 8 contained 4 μg major PB-induced and 3MC-induced cytochrome P-450s before application to HPLC, respectively. Well 7 contained 2 μg of HPLC purified 3MC-induced cytochrome P-450 (Fig. 2B).

twice against 1 l each of 10 m*M* Tris–acetate buffer containing 20% (v/v) glycerol and 1 m*M* EDTA. These fractions were then subjected to SDS–PAGE and their absolute (oxidized and reduced) and difference (sodium dithionite-reduced CO-complex) spectra were recorded. Results of SDS–PAGE (Fig. 1) showed that the three fractions isolated from the PB-induced cytochrome P-450 had similar molecular weight of 53,000 and that the molecular weight of HPLC-purified 3MC-induced cytochrome P-450 was 56,000. The absolute spectra of all the three fractions isolated by HPLC from the PB-induced cytochrome P-450 and of the HPLC purified 3MC-induced cytochrome P-450 were similar (Fig. 3). The λ_{max} of the Soret band of all the oxidized cytochrome P-450s were observed between 416–418 nm. The ferric β-peak was found between 534-540 nm and the λ_{max} of the α-band was between 565–570 nm. The λ_{max} for ferrous Soret bands were observed between 420–424 nm and other ferrous spectral peaks were observed 546–556 nm. The absolute spectra, therefore, indicated that the HPLC purified PB-induced and 3MC-induced cytochrome P-450s from rat hepatic microsomes were in the low-spin state. λ_{max} of the reduced CO-complex difference spectra was at 450 nm for all the three HPLC-purified PB-induced cytochrome P-450s and was at 448 nm for the HPLC-purified 3MC-induced cytochrome P-450 (Fig. 4). The difference spectra also revealed that the denaturation of

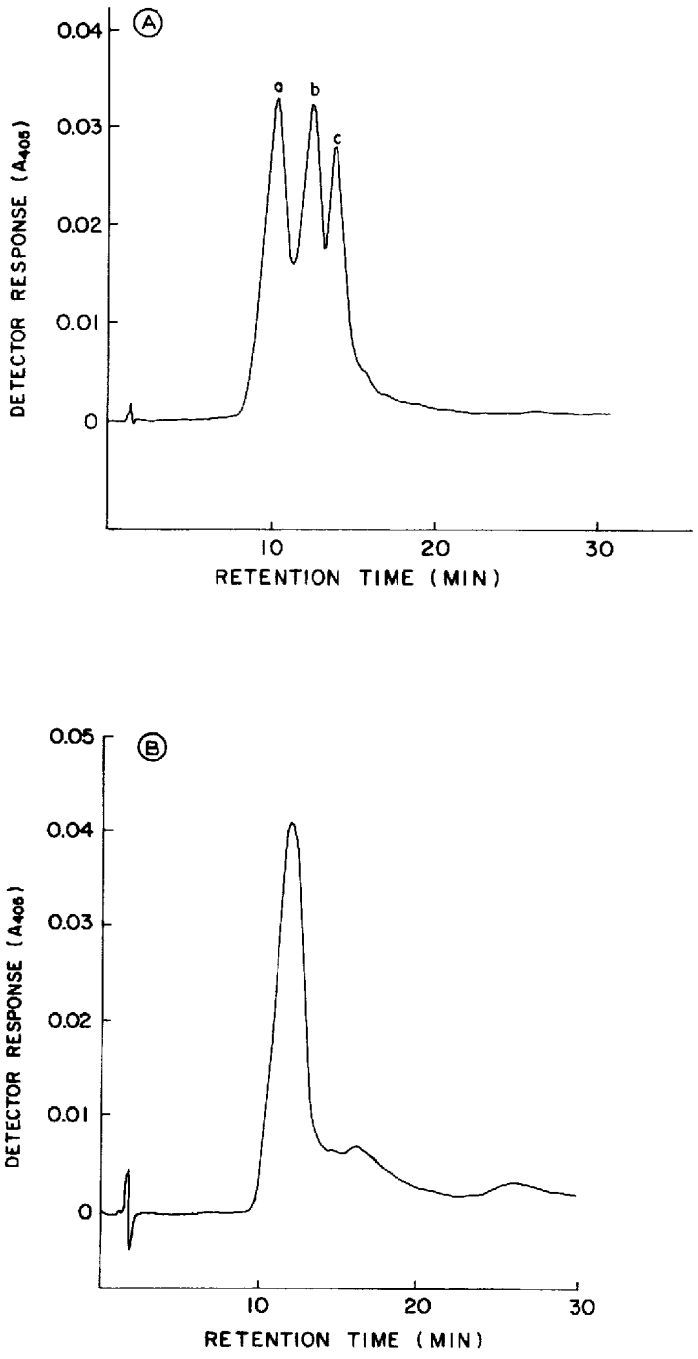


Fig. 2. HPLC separation of cytochrome P-450s. Profiles of HPLC analysis of cytochrome P-450s, isolated by sequential chromatography on *n*-octylamino Sepharose-4B and DEAE-cellulose columns, from hepatic microsomes of rats treated with (A) phenobarbital and (B) 3-methylcholanthrene. See Experimental for details.

cytochrome P-450 to cytochrome P-420 was minimal (less than 5%) for PB-induced cytochrome P-450s; greater denaturation of 3MC-induced cytochrome P-450 observed in Fig. 4 was actually due to the cytochrome P-420 already present in the preparation applied to the HPLC (data not shown). Thus, HPLC did not cause any appreciable denaturation to cytochrome P-420.

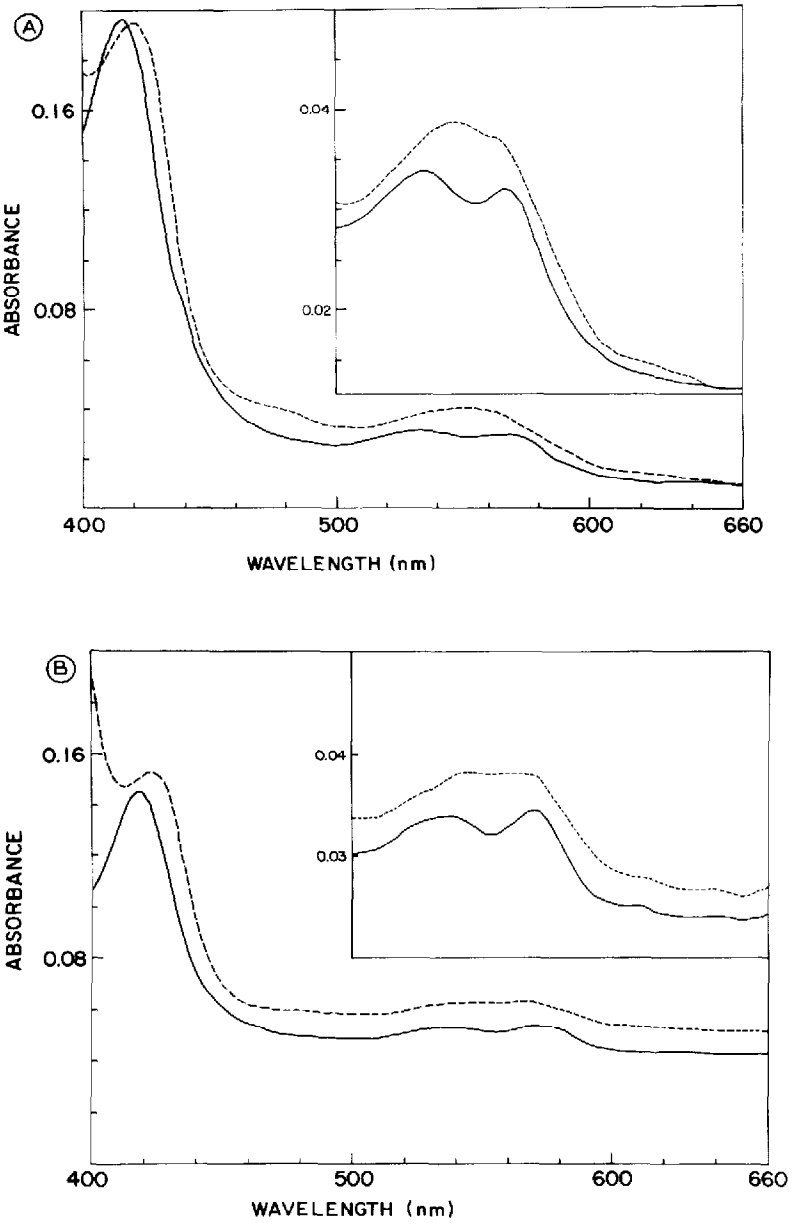
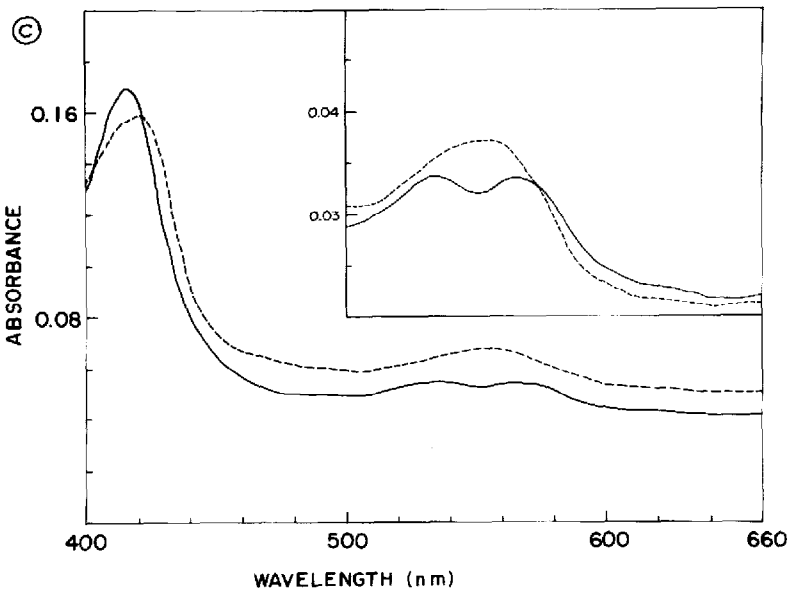


Fig. 3.



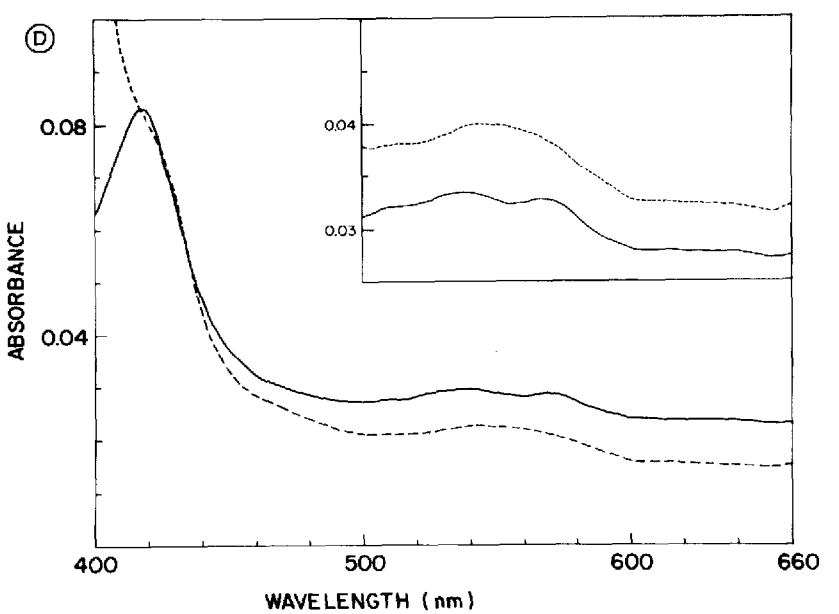


Fig. 3. Absolute spectrum of the HPLC-purified cytochrome P-450s. The oxidized (solid line) and sodium dithionite-reduced (dotted line) spectra of the HPLC-purified cytochrome P-450s were recorded as given under Experimental. (A) PBa, (B) PBb, (C) PBc, (D) 3MC. PBa, PBb and PBc refer to the HPLC-purified cytochrome P-450s from PB-induced rats (Fig. 2A) and 3MC refers to the HPLC-purified cytochrome P-450 from 3MC-induced rat (Fig. 2B). For clarity, the absorbance scale has been expanded between the wavelengths 500 660 nm as shown in the insets.

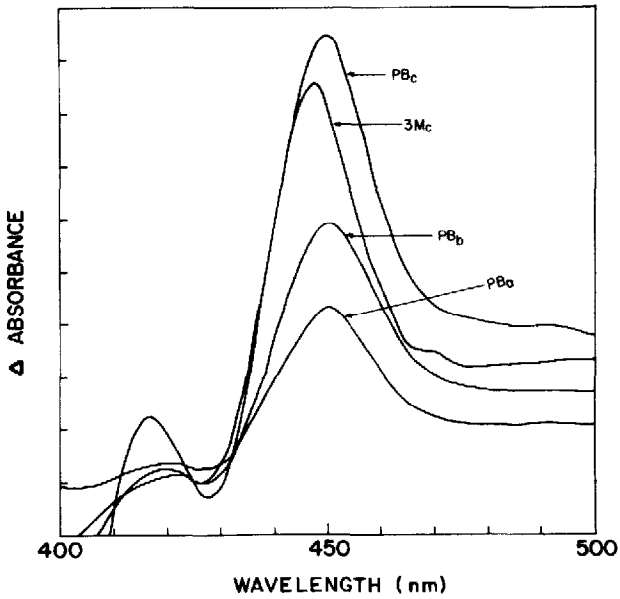


Fig. 4. Difference spectrum of the HPLC-purified cytochrome P-450s. Spectra of the sodium dithionite-reduced CO-complex of the HPLC-purified cytochrome P-450s were recorded as given under Experimentals. See legend to Fig. 3 for explanation of notations.

The HPLC-isolated cytochrome P-450s, reconstituted with NADPH–cytochrome P-450 reductase and dilauroyl phosphatidylcholine (DPC), were used to analyze the metabolism of various MFO substrates according to the procedure described elsewhere[14]. All of the HPLC-isolated cytochrome P-450s failed to support the metabolism of the tested MFO substrates.

DISCUSSION

The results reported here have demonstrated the use of HPLC in further resolution of major cytochrome P-450s isolated from rat hepatic microsomes. These results differ from those reported earlier by Kotake and Funae[11] in that we have demonstrated that the major form of cytochrome P-450 isolated from the PB-induced rats, which moved as a single band on SDS–PAGE gel, can be reproducibly resolved into three distinct HPLC peaks with retention times between 10.6 and 14.3 min. Kotake and Funae[11] reported a single HPLC peak in the major cytochrome P-450 purified from PB-induced rats used by them. Also, they observed only one major and one minor peak in the retention time interval of 8 to 14 min when HPLC analysis of the solubilized microsomes from PB-induced rats was carried out. In comparison, in the same retention time interval we have reported the existence of three HPLC peaks in the solubilized microsomes from PB-induced rats[10]; these peaks correspond to the peaks resolved here from the major form of the purified cytochrome P-450 (Fig. 2A). The reasons for the apparent differences in the resolution of the cytochrome P-450s between the two reports are not obvious at this time. In comparison to PB-induced

cytochrome P-450, the major form of cytochrome P-450 from 3MC-induced rats showed only one major peak upon HPLC (Fig. 2B).

The HPLC protein fractions isolated by us were characterized to be cytochrome P-450s based on their spectral and electrophoretic properties. Attempts to reconstitute the enzymic activities with NADPH–cytochrome P-450 reductase and DPC were unsuccessful. The cytochrome P-450s before application to HPLC were enzymatically active in a similarly reconstituted system. The cause of inactivation by HPLC could be either due to denaturative conversion of cytochrome P-450 to cytochrome P-420 or denaturation of the apoprotein. Denaturation to cytochrome P-420 was minimal, as is shown in Fig. 4. The possibility therefore exists that the apoprotein may have denatured under the high pressure of HPLC. Also, DPC was the only exogeneously added lipid in the reconstituted system. Therefore, another possible reason for inactivity of the HPLC-isolated cytochrome P-450s could be related to the absence of an essential lipid other than DPC that might have been stripped during HPLC purification. Nevertheless, the physical characteristics of the HPLC-purified proteins strongly suggested these proteins to be cytochrome P-450s. Recent publications support the presence of multiple cytochrome P-450 forms in the major PB-induced cytochrome P-450[15–17]. The current results demonstrate that multiple forms of cytochrome P-450 of different charges but similar molecular weights (inseparable by SDS–PAGE) exist in rat hepatic microsomes and that HPLC provides a fast and an effective tool for the determination of such a heterogeneity.

ACKNOWLEDGEMENTS

The authors wish to thank Miss Karen Marie Schrader for her assistance in the preparation of this manuscript. This work was supported by USPHS Grants CA-25362, 23634 and CA-24538 and an Institutional Research Grant IN-54W1 of the American Cancer Society.

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