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SEPARATION AND PURIFICATION OF COMPONENT PROTEINS OF THE CYTOCHROME P-450-DEPENDENT MICROSOMAL MONOOXYGENASE SYSTEM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The component proteins of the hepatic microsomal monooxygenase system, including various cytochrome P-450 isozymes, were separated and isolated from liver microsomes of untreated rabbits by Aminohexyl Sepharose and high-performance liquid chromatography (TSK preparative DEAE 5-PW, Bio-Rad HPHT). In addition to the known cytochrome P-450 isozymes, two new isozymes and one variant of the major isozyme were isolated. The monooxygenase activity was reconstituted by incorporating the purified proteins into liposomal membranes.

INTRODUCTION

The hepatic microsomal cytochrome P-450 (P-450)-dependent monooxygenase system is a key enzyme system in chemical carcinogenesis and metabolism of foreign substances, such as drugs[1]. This system is unique, since all component proteins are membrane-bound, and the interaction between them takes place in the plane of the endoplasmic reticulum membrane[2–4]. Although the best way to study such a complex system is to separate and purify each component and then to reconstitute the system, this is rather difficult due to the complexity of the system, including more than 20 different P-450 isozymes, in addition to several other component proteins, and also due to the membrane-bound nature of these proteins[5,6].

The high resolution obtained by high-performance liquid chromatography (HPLC) is very useful in the separation and purification of various component proteins of the monooxygenase system, and the application of this technique to the purification of various P-450 isozymes has recently been reported[7–9]. It led not only to the rapid purification of various isozymes, but also to the purification of various isozymes which have not yet been described[9]. In the present study, we have utilized HPLC for the separation and purification of the component proteins of the monooxygenase system as well as for various P-450 isozymes.

The combination of hydrophobic interaction chromatography with ion-exchange chromatography (TSK DEAE 5-PW) and hydroxyapatite (Bio-Rad HPHT) enabled the isolation of the three proteins which supply electrons to P-450, namely

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NADPH-cytochrome P-450 reductase (fp2), NADH-cytochrome b_5 reductase (fp1) and cytochrome b_5 (b5), as well as that of various P-450 isozymes, some of which are reported for the first time, and of epoxide hydrolase. Monooxygenase activity was restored upon incorporation of the purified proteins into liposomal membrane, demonstrating the usefulness of the purification procedure in reconstitution studies.

EXPERIMENTAL

Materials

NADPH, NADH, cytochrome *c* (Type VI), 7-ethoxycoumarin, 7-hydroxycoumarin and Tergitol NP-10 were obtained from Sigma (München, F.R.G.), acrylamide, bis-acrylamide and sodium dodecyl sulphate (SDS) from Bio-Rad (München, F.R.G.), and acetonitrile (gradient grade), sodium deoxycholate and cholic acid from Merck (Darmstadt, F.R.G.). Egg yolk phospatidylcholine was obtained from Lipid Products (South Nutfield, U.K.) while trypsin (N-tosylphenylalanine chloromethyl ketone-treated) was from Interchem (München, F.R.G.). 2′,5′-ADP-Sepharose, 5′-ADP-agarose, Sephadex G-100 and Sepharose 4B were from Pharmacia (Freiburg, F.R.G.). Aminohexyl Sepharose was synthesized according to the published method[10]. TSK DEAE-HPLC columns (Toyo Soda; semipreparative, 150 mm × 21.5 mm; analytical, 75 mm × 7.5 mm) were obtained through LKB (München, F.R.G.). Mono Q columns were obtained from Pharmacia, while the Bio-Gel HPHT column (100 mm × 7.8 mm) was from Bio-Rad. A Vydac 218-TP54 reversed-phase column (250 mm × 4.6 mm) was obtained from The Separations Group (Hesperia, CA, U.S.A.). All other chemicals and biochemicals used were of the highest grade available commercially.

HPLC apparatus

An LKB HPLC system, consisting of two 2150 HPLC pumps with a high-pressure mixing chamber (LKB), was used together with a Kratos SF 575 UV–VIS detector (Kratos, Karlsruhe, F.R.G.). The conductivities of the eluates were measured continuously with a WTW LF 42 conductivity monitor with a micro-flow cell (WTW, Wilhelm, F.R.G.). All buffers used were continuously degassed using an ERC 3320 degasser (ERC, Regensburg, F.R.G.).

Preparation of microsomes

Male New Zealand white rabbits, weighing 3–4 kg, were starved overnight and killed by decapitation. Liver microsomes were prepared by differential centrifugation, as described[10]. The microsomes were washed once with 0.15 *M* KCl, containing 10 m*M* EDTA (pH 7.4), and stored at −70°C.

Solubilization of microsomes and Aminohexyl Sepharose chromatography

Microsomes were solubilized with cholate and applied directly to an Aminohexyl Sepharose column in order to lower the load for the HPLC column as well as to achieve partial separation of several enzymes[10,11]. All manipulations up to the HPLC separations were conducted at 0–4°C. The microsomes (about 3 g protein) were thawed and resuspended in 1 l of 0.1 *M* potassium phosphate buffer (pH 7.3), containing 0.6% cholic acid, 20% glycerol, 1 m*M* dithiothreitol and 1 m*M* EDTA. The

slightly turbid solution was subjected to ultra centrifugation at 150 000 *g* for 1 h. The clear supernatant was applied directly to an Aminohexyl Sepharose column (30 cm × 2.6 cm), equilibrated with the same buffer as that used for the solubilization. The column was washed with 500 ml of the solubilization buffer, containing 0.4% cholic acid, and eluted with the solubilization buffer, containing 0.4% cholic acid and 0.08% Tergitol NP-10.

The column was further eluted with the same buffer, containing 0.4% cholic acid and 0.2% Tergitol NP-10 (second elution, as indicated with an arrow in Fig. 1), and with 50 m*M* potassium phosphate buffer (pH 7.3), containing 20% glycerol, 0.15% cholic acid, 0.35% sodium deoxycholate and 1 m*M* EDTA (third elution, as indicated with an arrow in Fig. 1).

Preparative anion-exchange HPLC

The fractions obtained from the Aminohexyl Sepharose column were collected in two pools as indicated in Fig. 1, and dialyzed overnight against 10 m*M* Tris–acetate buffer (pH 7.5), containing 20% glycerol, 1 m*M* dithiothreitol and 1 m*M* EDTA. The dialysates (150–200 mg protein) were diluted three-fold with 20% glycerol, containing 1 m*M* dithiothreitol and 1 mM EDTA, and applied to a semi preparative TSK DEAE 5-PW column, which had been equilibrated with 10 m*M* Tris–acetate buffer (pH 7.5), containing 0.4% Tergitol NP-10, 20% glycerol, 1 m*M* dithiotreitol and 1 m*M* EDTA (buffer A). The column was washed with 40 ml of buffer A, and eluted with a linear gradient of 0–15% buffer B (20 mM Tris–acetate buffer pH 7.5, containing 20% glycerol, 0.4% Tergitol NP-10, 1 m*M* dithiothreitol and 1 m*M* EDTA) in buffer A in 240 min at a flow-rate of 2 ml/min.

Analytical anion-exchange HPLC

The fractions obtained from the preparative DEAE-HPLC column were re-chromatographed in an analytical TSK DEAE 5-PW column or in two Mono Q columns connected in series. The column was eluted with a linear gradient of 0–10% buffer B, incorporated into buffer A in 40 min at a flow-rate of 1 ml/min.

Hydroxyapatite HPLC

The fractions from the analytical DEAE-HPLC column were pooled and dialyzed overnight against 10 m*M* potassium phosphate buffer (pH 7.3), containing 20% glycerol and 0.1 m*M* dithiothreitol. The dialyzed fractions were applied directly to an hydroxyapatite HPLC column which had been equilibrated with buffer C (10 m*M* potassium phosphate buffer pH 7.3, containing 15% glycerol, 0.2% Tergitol NP-10, and 0.1 m*M* dithiothreitol). The column was eluted with a linear gradient of 0–100% buffer D (350m*M* potassium phosphate buffer pH 7.3, containing 15% glycerol, 0.2% Tergitol NP-10 and 0.1 m*M* dithiothreitol), incorporated into buffer C in 60 min at a flow-rate of 0.7 ml/min.

Trypsin hydrolyzate analysis

The purified proteins were diluted in 50 m*M* Tris–HCl buffer (pH 8.0) to a concentration of 0.5 mg protein/ml. To 100 μl of the sample solution, 5 μg of trypsin, dissolved in 5 μl of 1 m*M* HCl, were added, and the mixture was incubated at 37°C for 15 h. The reaction was stopped by adding 10 μl of 10% trifluoroacetic acid, and the

sample was directly injected into a Vydac 218-TP54 reversed-phase column. The column was eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow-rate of 0.7 ml/min. The elution of peptides was monitored at 210 nm.

Affinity chromatography of fp1 and fp2

The fp1-containing fractions from the preparative DEAE-HPLC column were pooled and purified to homogeneity, using a 5′-ADP-agarose column (5 cm × 1 cm), as described previously[3]. The fp2-containing fractions from the aminohexyl Sepharose column were pooled and directly applied to a 2′,5′-ADP-Sepharose column (5 cm × 1.6 cm). fp2 was purified to homogeneity as described earlier[3].

Purification of b5

Cytochrome b_5 was isolated from the third eluate from the aminohexyl Sepharose column by gel filtration chromatography on Sephadex G-100, as described previously[3].

Analytical methods

P-450 was determined from its CO-difference spectrum in microsomes and from its absorbance at 417 nm in purified, soluble form[3]. The concentration of b5 was determined from its absorbance at 413 nm (ref. 3). fp1 was determined by measuring the NADH-dependent ferricyanide reductase activity[12] or flavin absorption[13], and fp2 was determined from its flavin absorption[14]. The reconstituted monooxygenase activity was measured in 50 m*M* potassium phosphate buffer (pH 7.3) containing 300 μM ethoxycoumarin as the substrate. The reaction was started by adding 1 m*M* NADPH, and the increase in the fluorescence of the product, 7-hydroxycoumarin, was measured in a Perkin-Elmer LS-3 fluorescence spectrophotometer. The excitation wavelength was 380 nm, and the emission wavelength was set at 455 nm. Reconstitution of the monooxygenase was achieved by incorporating the purified proteins into liposomes by the cholate dialysis method as described previously[2–5]. Sodium dodecyl sulphate (SDS) gel electrophoresis was carried out according to Laemmli[15]. The protein concentration was determined by the method of Lowry *et al.*[16].

RESULTS AND DISCUSSION

Aminohexyl Sepharose chromatography

Aminohexyl Sepharose chromatography, which was first developed for the purification of the phenobarbital-inducible form of P-450 from rabbit liver[10,17] and later adapted to the purification of P-450 isozymes from various sources[18,19], was used as the first step in order to achieve the partial separation and purification of various proteins as well as to lower the load for the HPLC columns. As has been described by Imai[11], the combination of various detergents enabled the separation of fp2 and b5 from the two P-450-containing fractions (Fig. 1). Under the conditions employed, fp2 was eluted from the column as a broad peak, just after the first P-450 peak. No NADPH-cytochrome *c* reductase activity, which is catalyzed by fp2, was detected in the third eluate, as described[11]. The first P-450 peak was divided into two fractions (pools 1 and 2 in Fig. 1), dialyzed and further purified on a DEAE-HPLC

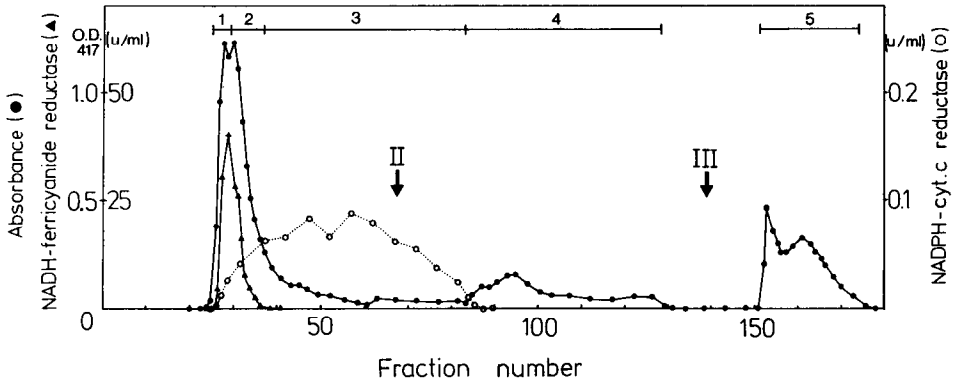

Fig. 1. Chromatography of solubilized microsomes on an aminohexyl Sepharose 4B column. The microsomes obtained from untreated rabbit liver were solubilized with cholic acid, and applied to the column. The elution buffers were changed at the times indicated by arrows. Absorption was monitored at 417 nm, and the activities of the two reductases (fp1 and fp2) were measured as described in Experimental. The fractions obtained were pooled as indicated. The column dimensions and eluent composition are described in Experimental.

column. The third pool, which contained most of the fp2, was further purified on a 2',5'-ADP-Sepharose affinity column, as described in Experimental. The peak eluted by the combination of cholate and deoxycholate contained most of the b5. Cytochrome b_5 was isolated from the pool by gel filtration chromatography as described in Experimental. The two component proteins of the monooxygenase system, fp2 and b5, were thus separated from the majority of the P-450 by Aminohexyl Sepharose chromatography, and purified to homogeneity.

Preparative DEAE-HPLC of pool 1

The first half of the main peak eluted from the Aminohexyl Sepharose column (pool 1 in Fig. 1) was further resolved on a semipreparative TSK DEAE 5-PW column, as described in Experimental. The column eluate was monitored at 417 nm, where the two haemoproteins, P-450 and b5, can be detected specifically (Fig. 2a). The collected fractions were subjected to SDS gel electrophoresis, and several proteins were immediately identified by their apparent molecular weights (Fig. 2b).

The first peak, which was only weakly bound to the column and was eluted immediately with buffer A, contained one of the two major P-450 isozymes in the uninduced rabbit liver microsomes, isozyme 3c, as well as isozyme 2, which is inducible with drugs, such as phenobarbital (Fig. 2). The last large peak which was eluted at around 6–7% buffer B (elution volume 240 ml) contained another major P-450 isozyme in the control rabbit liver microsomes, namely isozyme 3b. The identity of these isozymes was established from their chromatographic behaviour, mobility in SDS gel electrophoresis and activity. In addition to these two major forms, at least two peaks contained P-450 isozymes. One peak, eluted at the beginning of the gradient (at around 100 ml elution volume), and a small peak eluted just before the 3b peak (elution volume around 220 ml), contained proteins which migrated between isozymes 2 and 3b in SDS gel electrophoresis (marked a and b in Fig. 2b). The only

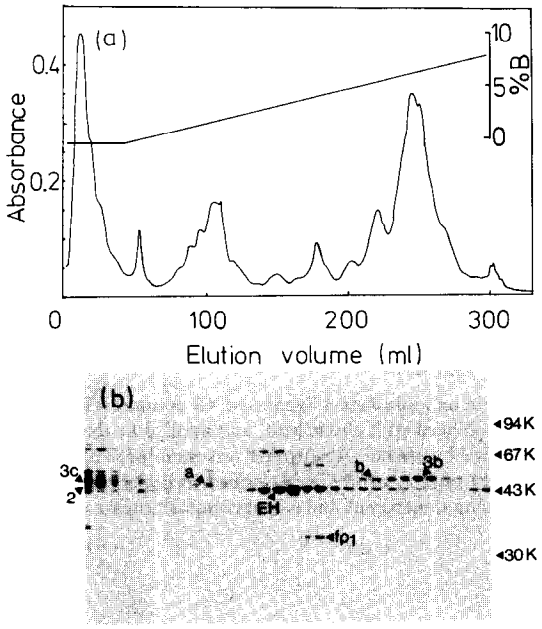

Fig. 2. (a) HPLC separation of Aminohexyl Sepharose pool 1 on a TSK preparative DEAE 5-PW column. The pooled fractions were dialyzed and applied to the column as described in Experimental. The column eluate was monitored at 417 nm. The buffer concentration in the eluate was determined from the conductivity measured continuously in a micro-flow cell. (b) SDS gel electrophoresis of the fractions obtained from the DEAE 5-PW column: 2, 3b and 3c are known P-450 isozymes, a and b are unknown isozymes. Cytochrome b_5 reductase is marked fp1, while epoxide hydrolase is marked EH.

P-450 which has been purified from rabbit liver microsomes and which migrates between isozymes 2 and 3b is isozyme 3a[20]. Since isozyme 3a shows an absorption maximum at around 390 nm, while the two isozymes separated in the present study show absorption maxima at around 416 nm, these two P-450 peaks appear to represent two as yet unidentified P-450 isozymes.

In addition to P-450 isozymes, two other proteins were identified by their activities and apparent molecular weights. Epoxide hydrolase was eluted as a rather broad peak (the peak fraction was eluted around 160 ml between a and b and is marked as EH in Fig. 2b), and showed an apparent molecular weight of 46 000, corresponding well to the published value[21]. Slightly after epoxide hydrolase, fp1 was eluted as a sharp peak at around 200 ml, and was seen in the SDS gel (Fig. 2b). The small peak around 200 ml is due to the flavin absorption of fp1. The latter was easily purified to homogeneity by affinity chromatography on 5'-ADP-agarose[3]. Epoxide hydrolase represents the major protein, and is almost pure in the peak fraction. The peak fractions were pooled and further purified by rechromatography on an analytical DEAE 5-PW column.

Preparative DEAE-HPLC of pool 2

The second half of the first main peak from the Aminohexyl Sepharose column was separately collected as pool 2, and applied on the preparative DEAE 5-PW

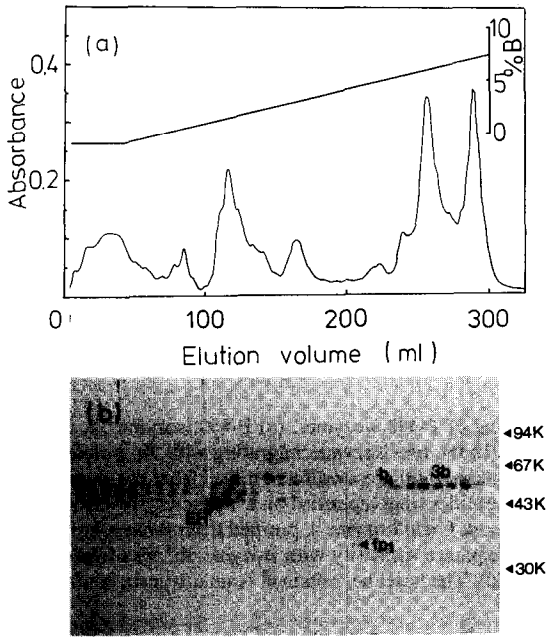

Fig. 3. (a) HPLC separation of Aminohexyl Sepharose pool 2 on a TSK preparative DEAE 5-PW column, The pooled fractions from Aminohexyl Sepharose were applied to the column and eluted as described in Experimental. (b) SDS gel electrophoresis of the fractions obtained from the DEAE 5-PW column. Several protein bands are marked as in Fig. 2. Note that the two protein bands corresponding to the last two large peaks showed similar electrophoretic mobility, while the band marked b migrated slightly more rapidly than the other two bands.

column (Fig. 3) partly because of the capacity of the HPLC column. Although there are similarities between the two chromatograms (Figs. 2 and 3), two significant differences are noted. First, the last large peak is now split into two peaks of similar size, and the two corresponding protein bands migrated together in SDS gel (Fig. 3b). These two proteins migrated at the same rate as 3b, obtained from pool 1, and were further purified separately in order to establish their identity, as will be described below. Secondly, a protein corresponding to epoxide hydrolase was eluted earlier than the P-450 isozyme marked as a, suggesting the separation of the two epoxide hydrolase isozymes in the Aminohexyl Sepharose column[22]. In contrast, other proteins, such as fp1 and P-450 isozymes 2, 3c and the two new isozymes marked as a and b, were eluted as in the resolution of pool 1, although the first peak, containing P-450 2 and 3c, was smaller, and correspondingly, less 3c was seen in the SDS gel. There is some partial resolution of various proteins in the first peak of the Aminohexyl Sepharose chromatogram.

Comparative trypsin peptide mapping of P-450s

In order to establish the identity of the two P-450 isozymes which migrated with isozyme 3b in SDS gel electrophoresis but were resolved by preparative DEAE-HPLC (Fig. 3), each peak was separately purified to homogeneity by analytical

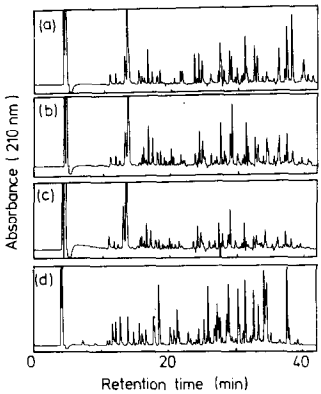

Fig. 4. Comparative HPLC trypsin peptide mapping of P-450 isozymes. (a) P-450 isozyme 3b, isolated from pool 1 of the aminohexyl Sepharose column, (b) the two isozymes migrating with 3b, isolated from pool 2 of the aminohexyl Sepharose column (first peak) and (c) preparation obtained from the second peak were treated with trypsin, and the resulting tryptic peptides were separated on a Vydac C_{18} reversed-phase column, as described in Experimental. For comparison, P-450 isozyme 4, purified from β-naphthoflavone-induced rabbit liver microsomes, which has little sequence similarity with isozyme 3b[6] was treated with trypsin, and the peptides obtained were separated (d). The baseline, obtained from a trypsin control, was subtracted by using a Shimadzu C-R3A integrator.

DEAE-HPLC and HPLC on hydroxyapatite columns. The purified P-450 isozymes were treated with trypsin, and the resulting tryptic peptides were separated on a Vydac C_{18} reversed-phase column, as described in Experimental. As shown in Fig. 4, the two isozymes isolated from pool 2 showed almost the same peptide pattern as isozyme 3b isolated from pool 1, although some differences were noted. These results suggest that the three preparations are at least very similar in primary structure. Several lines of evidence, which suggest the presence of variants of isozyme 3b in rabbit liver microsomes, have recently been reported[23], although the isolation of these variants has not yet been achieved. The preparations obtained in the present study, therefore, may represent these isozyme 3b variants. Studies are now in progress to clear up the relationship between these preparations.

Reconstitution of monooxygenase activity

Three proteins isolated from rabbit liver microsomes as described above were then incorporated into liposomal membranes by the cholate dialysis method[2–5] in order to examine the intactness of the purified proteins. Proteins fp2 and P-450 isozyme 2, from the first peak of the preparative DEAE-HPLC column, which has been further purified by HPLC on hydroxyapatite, and various amounts of cytochrome b_5 were reconstituted into liposomes of egg yolk phosphatidylcholine. As shown in Fig. 5, the inclusion of b5 in the system stimulates the monooxygenase activity. This confirms the previous observations that the second of the two electrons needed for the monooxygenase reaction is preferentially supplied by b5[3] and shows clearly that the enzyme preparations obtained in the present study by HPLC keep their structure intact, and are able to interact properly with each other in the liposomal membranes as in intact microsomes.

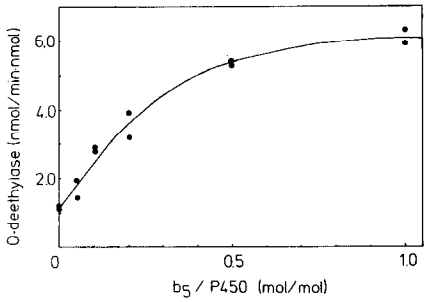

Fig. 5. Effect of b5 on the reconstituted monooxygenase activity. Purified cytochrome P-450 isozyme 2, fp2, and various amounts of b5 in a molar ratio of fp2–P-450–lipid = 1:1:400 were incorporated into liposomes of egg yolk phosphatidylcholine, and the NADPH-dependent monooxygenase was measured with ethoxycoumarin as a substrate as described in Experimental.

CONCLUSION

The combination of conventional hydrophobic interaction chromatography (Aminohexyl Sepharose column) with HPLC on resin-based ion-exchange columns and hydroxapatite columns, developed for protein purification, enabled a rapid simultaneous resolution and isolation of various component proteins of the hepatic microsomal monooxygenase system. In addition to the three proteins which supply electrons to P-450 (fp1, fp2, b5), various P-450 isozymes, including two unknown isozymes, were easily purified. At least two variants of P-450 isozyme 3b were separated by anion-exchange HPLC. The purified proteins conserved their ability to bind to liposomal membranes, and monooxygenase activity was restored when these proteins were incorporated into membranes. The HPLC techniques are, therefore, very useful in the study of complex membrane-bound enzyme systems.

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