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Isolation of cytochrome P-450 components from marmoset liver microsomes by high-performance liquid chromatography☆

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ABSTRACT

A fast protein liquid chromatographic (FPLC) system with pre-packed and laboratory-packed columns was used for the analytical and preparative isolation of marmoset monkey cytochrome P-450 (P450) and NADPH–P450-reductase. Chromatographic separations also allowed the recovery of cytochrome b_5, NADH–b_5-reductase and epoxide hydratase. Cholate-solubilized liver microsomes from phenobarbital-induced marmosets were crudely purified on 8-aminooctyl-Sepharose or 6-aminohexyl-Sepharose and then fractionated into several isoenzyme groups using hydroxyapatite. Further purification on Mono S or CM-Sepharose and finally on phenyl-Superose, phenyl-Sepharose or octyl-Sepharose yielded a P450 fraction which was apparently homogeneous as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis in the automated Phast system using silver staining. Removal of excess of non-ionic detergent was effected by hydroxyapatite columns, and this was compared with other methods. For the isolation of P450 isoenzymes from untreated marmosets, Mono Q columns were employed and yielded at least two highly purified forms. NADPH–P450-reductase was recovered from the 8-aminooctyl-Sepharose column or crudely fractionated on DEAE-Sepharose Fast Flow. Subsequent purification via 2′,5′-ADP-Sepharose and Superose 12 chromatography resulted in a homogeneous preparation.

INTRODUCTION

Cytochrome P-450 (P450) is a collective term for a wide variety of chromatographic closely related monooxygenases which, depending on animal species and induction type, cover an increasing number of different proteins [1]. NADPH–P450-reductase, on the other hand, seems to exist in only one form per species. Purification of monooxygenases is, in general, difficult as most of these membrane proteins have molecular weights between 47 000 and 55 000 dalton and isoelectric points of about 5.5–6.5, a range shared by many other proteins [2].

P450 has been examined mainly and exhaustively using rat liver microsomal tissue [2,3]. Additional difficulties arising with P450 from primate as against rodent sources almost invariably include lower specific contents, lower metabolic activities and genetic polymorphisms. Studies involving primate P450 forms usually involve human tissue, but the use of non-human primates as a model has the advantage that these can be induced in a controlled way.

The purpose of this work was twofold. First, marmoset P450 and reductase were to be isolated and characterized for subsequent comparison with corresponding rodent forms [4,5], and second, sufficient amounts were to be prepared that would permit the establishment of a metabolizing system in organ culture [6,7]. A wide range of commercially available, prepacked fast protein liquid chromatographic (FPLC) columns and laboratory-packed columns were employed. This paper describes their usefulness and applicability for such purification.

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EXPERIMENTAL

Chemicals and chromatographic gels

Sodium cholate was purchased from Serva (Heidelberg, Germany), sodium deoxycholate from Merck (Darmstadt, Germany), 2′-AMP, NADPH, polyethylene glycol and Amberlite XAD-2 from Sigma (St. Louis, MO, USA), Emulgen 911 from Kao-Atlas (Tokyo, Japan) and Renex 690 from Atlas-Chemie (Essen, Germany). 8-Aminooctyl-Sepharose 4B was synthesized as described [8]. HA-Ultrogel hydroxyapatite was obtained from Serva (Munich, Germany) and Bio-Beads SM-2 from Bio-Rad Labs. (Munich, Germany). Hydroxyapatite prepared according to Mazin *et al.* [9] was also employed. AH-Sepharose (6-aminohexyl-Sepharose), 2′,5′-ADP-Sepharose, phenyl-Superose, phenyl-Sepharose, octyl-Sepharose, Superose 12, Mono Q, Mono S, DEAE-Sepharose Fast Flow and CM-Sepharose Fast Flow were obtained from Pharmacia (Uppsala, Sweden). The prepacked FPLC-grade gels each had a consistent particle size of 10 μm.

General conditions

Liver microsomes from phenobarbital-induced and from untreated male marmoset monkeys (*Callithrix jacchus*) were obtained and solubilized as described [5]. Buffer solutions usually contained 20% glycerol, 0.1 m*M* EDTA and 0.2 m*M* phenylmethylsulphonyl fluoride (PMSF). The temperature for all chromatographic steps was 18°C except for affinity, hydroxyapatite and hydrophobic interaction chromatography, which were carried out at 4°C. In this instance, the columns were maintained in a refrigerator or held in an ice-bath. Absorbances were recorded at 280 and 405 nm, and fractions were collected in a rack filled with ice.

Except where noted otherwise, all chromatographic equipment was purchased from Pharmacia. Laboratory packing of gels in high-resolution (HR) glass columns was carried out as follows: the initial packing was done in the customary manner [10] using *ca.* 1 m hydrostatic pressure. The column was then attached to the FPLC system and washed over "load/inject" with 5–10 gel bed volumes of 50% glycerol and increasing flow-rates from 0.1 to 1 or 2 ml/min until the back-pressure began to exceed 4.0 MPa. Laboratory packed columns were equilibrated and developed using flow-rates corresponding to 0.1–0.2 MPa.

Purification of P450

Laboratory-packed 8-aminooctyl-Sepharose 4B or 6-aminohexyl-Sepharose in an HR 16/10 column (10 cm × 16 mm I.D.) were equilibrated with 100 m*M* potassium phosphate (pH 7.4) and 0.6% sodium cholate, loaded with a centrifuged solution of solubilized microsomes, and then washed with 0.4% cholate in the same buffer [8]. Elution with 0.33% cholate and 0.06% Emulgen 911 yielded a single pool consisting of all P450 forms originally present. Further washing with 0.33% cholate and 0.35% sodium deoxycholate resulted in a broad peak of epoxide hydratase followed by a narrow peak of NADPH–P450-reductase.

The P450 fraction derived from phenobarbital-treated marmosets was then diluted with 20 volumes of 20% glycerol (pH 7.2) and loaded onto an HR 10/10 column (10 cm × 10 mm I.D.) packed with HA-Ultrogel hydroxyapatite which was equilibrated with 5 m*M* phosphate and 0.5% Emulgen 911 (pH 7.2). Successive washings with 20, 50, 100, 200 and 500 m*M* phosphate and 0.5% Emulgen 911 were collected. The 200 m*M* phosphate fraction was concentrated on an Amicon PM 30 ultrafiltration chamber, desalted on pre-packed or laboratory-packed HR 10/10 columns of Sephadex G-25 Superfine using 10 m*M* phosphate and 0.5% Emulgen 911 (pH 6.8) as running buffer and then loaded onto HR 5/5 (5 cm × 5 mm I.D.) pre-packed Mono S or laboratory-packed CM-Sepharose Fast Flow columns. The flow-rates were 1.0 and 0.2 ml/min, respectively. After application of a 20-ml continuous gradient of 0–250 m*M* NaCl in the above buffer, fractions were collected between 100 and 150 m*M* NaCl and freed from residual detergent on hydroxyapatite as described below. The Mono S or CM-Sepharose Fast Flow fraction (now in 200 m*M* phosphate buffer) was then loaded onto HR 5/5 phenyl-Superose, phenyl-Sepharose or octyl-Sepharose columns. For phenyl-Superose the flow-rate was 1.0 ml/min and for the Sepharose columns 0.3 ml/min. Subsequent to washing with 100 m*M* phosphate and 0.4% cholate, proteins were eluted with a linear or step gradient of 0–1.0% Emulgen 911 in this buffer. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) indicated that a 0.05–0.1% Emulgen 911 pool was homogeneous.

In contrast to an 8-aminooctyl-Sepharose frac-

tion of phenobarbital-induced microsomes, an 8-aminooctyl-Sepharose fraction of untreated marmoset microsomes was purified by a further chromatographic step on an HR 5/5 Mono Q column. A flow-rate of 1.0 ml/min and a 20-ml 0–200 m*M* NaCl gradient in 10 m*M* Tris buffer containing 0.5% Renex 690 resulted in highly purified forms.

Purification of NADPH–P450-reductase

8-Aminooctyl-Sepharose chromatography as described above yielded a crude fraction of reductase. In place of this, a large-scale fractionation of solubilized microsomes from phenobarbital-treated rats or rabbits on a 50 cm × 5 cm I.D. column of DEAE-Sepharose Fast Flow equilibrated with 0.6% sodium cholate and 0.8% Renex 690 was also used for elution with a step gradient between 120 and 360 m*M* KCl [11]. In either case, the reductase-containing fractions were then loaded directly onto a laboratory packed HR 10/10 column of 2′,5′-ADP-Sepharose which had been equilibrated with 300 m*M* phosphate and 0.1% Renex 690 or Emulgen 911. Using a flow-rate of 1.0 ml/min, the column was washed with this buffer and with 30 m*M* phosphate containing 0.15% deoxycholate, and then the flow direction was reversed. After application of 5 m*M* 2′-AMP in the same buffer, the reductase eluted in a sharp band. The eluent was concentrated over Amicon PM-30, divided into small aliquots suitable for gel filtration, frozen in liquid nitrogen and stored at −80°C. Finally, contaminating proteins, AMP, detergent and PMSF were removed via Superose 12 where the running buffer was devoid of detergent, EDTA and PMSF. An HR 10/30 pre-packed column (30 cm × 10 mm I.D.) accommodated a 200-μl loop load; a laboratory-packed HR 16/50 column (50 cm × 16 mm I.D.) of Superose Prep grade allowed 2-ml loads. In order to eliminate as much as possible the dead volume between the load valve and the column, the column prefilter was removed.

Removal of detergents using hydroxyapatite

An HR 10/2 column (20 mm × 10 mm I.D. was packed with hydroxyapatite and equilibrated with 20 m*M* phosphate (pH 7.2). The P450 fraction to be freed from excess of non-ionic detergent was diluted with 20% glycerol (pH 7.2) to the same phosphate concentration. After loading at a reduced flow-rate of 0.1–0.2 ml/min and if necessary recycling the pass-through fraction, the column was washed with 20 m*M* phosphate until excess of detergent was removed. This could be evidenced for detergents such as Emulgen or Renex by following the decrease in absorbance at 280 nm. A wash with 15–20 bed volumes was sufficient and, after reversal of the flow direction and elution with 300 m*M* phosphate, the P450 was recovered in a sharp peak.

Assays

P450 contents, NADPH–P450-reductase activities, reconstitution and substrate activities and protein assays correcting for non-ionic detergents were carried out as reported previously [4–5]. SDS-PAGE analyses were performed with an automated Phast system (Pharmacia) using 10–15% gradient gels and silver staining.

RESULTS AND DISCUSSION

Choice of detergent

For the general reader, a comment on the detergents used may be of value. Anionic cholate and deoxycholate are powerful membrane solubilizers and tend to dissociate interactions between proteins more effectivley than non-ionic detergents. Deoxycholate is more capable of complete dissociation. For most of the conventional column chromatographic separations of P450 forms, a combination of cholate and a non-ionic detergent such as the polyoxyethylene alkyl aryl ethers Renex 690 or Emulgen 911 has been preferred [2,3]. Protocols for the purification of many different P450 forms using several conventional anion-exchange resins may also involve various concentrations of both ionic and non-ionic detergents together [2,3]. For FPLC- and HPLC-quality anion-exchange gels, however, only non-ionic detergents were used in this work (see below). Renex 690 and Emulgen 911 were found to give similar results as regards stability and resolution of these enzymes. The fact that these two detergents absorb at 280 nm (in contrast to *e.g.*, Lubrol PX) was considered advantageous in that this property allowed for monitoring during their removal.

Other types of chromatographic gels, notably aminoalkyl-Sepharose, which exploit the amphipathic character of such proteins, have also utilized

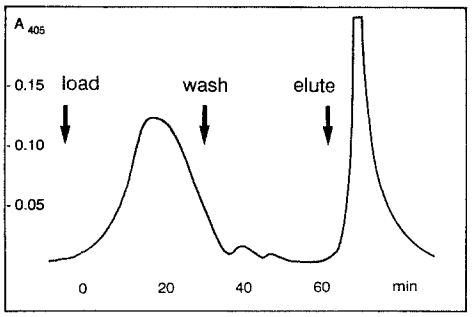

Fig. 1. Affinity chromatography using 2',5'-ADP-Sepharose 4B. Sample: 143 μmol (cytochrome *c* reduced) per minute of phenobarbital-treated marmoset liver microsomal NADPH–P450-reductase prefractionated on 8-aminooctyl-Sepharose. Load: in 300 m*M* phosphate (pH 7.7)–0.1% Renex 690–20% glycerol–0.4% PMSF–0.1 m*M* EDTA. Wash: 30 m*M* phosphate–0.1% deoxycholate–20% glycerol. Elution: 5 m*M* 2'-AMP. Column: HR 10/10. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.2 a.u.f.s. Yield: 85–90%.

several concentrations and combinations of detergents [2,3,8]. In almost all instances, this was determined empirically.

8-Aminooctyl- and 6-aminohexyl-Sepharose chromatography

This was found to be a good choice as a first step in reducing to a minimum especially low-molecular-weight, non-monooxygenase proteins as has been reported for rat P450 [8]. This type of chromatography is apparently based more on a combination of hydrophobic interaction and ion exchange than on haeme–ligand affinity [3]. Successive elutions as described under Experimental then yielded a pool of collected P450 forms in 65–70% yield and the reductase in 80–90% yield. Gels used and washed even three or four times were routinely found to bind more than 20 nmol/ml of marmoset P450 and to increase the specific content about threefold. Commercially available 6-aminohexyl-Sepharose has also been used [12,13] and gave basically similar results here, except that the yield and capacity were both about 20% lower.

Polyethylene glycol (6000 or 8000 dalton) was also employed as used for rat material in an initial step prior to hydroxyapatite or ion-exchange chromatography [14]. This was found to increase the

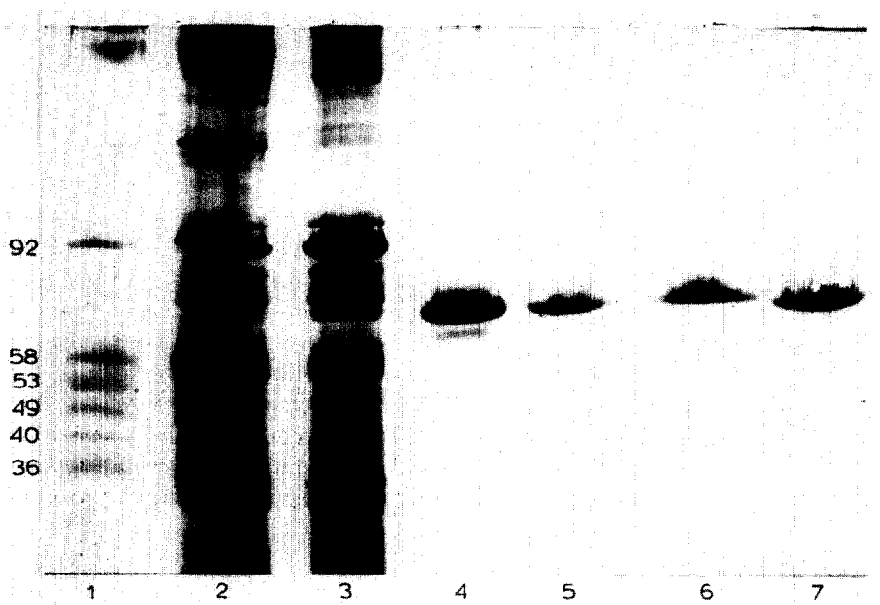

Fig. 2. SDS-PAGE gel of phenobarbital-treated marmoset NADPH–P450-reductase in various steps of purification. The anode is at the bottom, and the 10–15% gradient gel was stained with silver. Lanes: 1 = marker proteins phosphorylase *a* (molecular weight 92 kilodalton), catalase (58), glutamate dehydrogenase (53), fumarase (49), aldolase (40) and lactate dehydrogenase (36); 2 = solubilized microsomes; 3 = 8-aminooctyl-Sepharose fraction; 4 = ADP-Sepharose fraction; 5 = Superose 12 fraction as described in the text; 6 = as in lane 5 but from rat; 7 = as in lane 5 but from rabbit.

P450 specific content slightly more than as was described above, but in consideration of the poorer yields was not used further.

2',5'-ADP-Sepharose chromatography

In contrast to 8-aminooctyl-Sepharose, 2',5'-ADP-Sepharose chromatography required as sample load a certain degree of prefractionation of solubilized microsomes. This was achieved by using the 8-aminooctyl-Sepharose pool described above or a DEAE-Sepharose pool as described under Experimental [11]. It is likely that these two types of chromatography may thereby eliminate amounts of phosphatases which are capable of destroying the ADP groups bound to Sepharose [15]. A 1-ml volume of this resin was capable of binding at least 150 μmol (cytochrome *c* reduced) per minute. Fig. 1 shows a chromatographic profile of this affinity chromatography. The great increase in purification during this step is also evident in the SDS-PAGE gel (Fig. 2, lane 4). Instead of NADPH as recommended for elution by the manufacturer of this gel, 2'-AMP was used as subsequent substrate reactions with reconstituted enzyme systems required the addition of NADPH for initiation.

Superose 12 chromatography

In the SDS-PAGE gel (Fig. 2), it can be seen that a very slight degree of contaminating protein is still present in the ADP-Sepharose fraction (lane 4). Originally, for rat or pig reductases, this lower molecular weight protein was removed by Ultrogel AcA 34 gel filtration [11], which has a separation range of 20 000–350 000 dalton. FPLC-quality Superose 12 (separation range 1000–300 000 dalton) in a pre-packed HR 10/30 column was utilized in this work and yielded an almost quantitative recovery of this enzyme from the ADP load in much less time. This constituted the first, main peak shown in Fig. 3. At higher elution values, lower molecular weight protein, AMP and PMSF then appeared. As for our embryotoxicological studies [6,7] not only P450 but also excess stoichiometric amounts of reductase are required, this chromatographic step is considered to be especially useful. In contrast to an earlier report [11], the present description of separating minimal protein contaminants resulted in a great increase in the specific activity of the reductase.

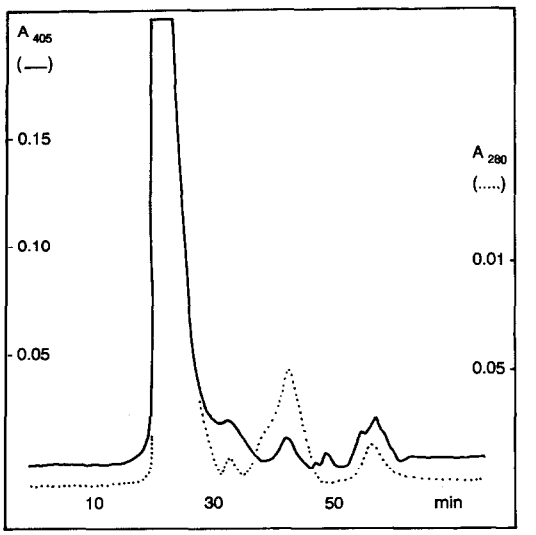

Fig. 3. Gel filtration chromatography using Superose 12. Sample: 48 μmol (cytochrome *c* reduced) per minute of phenobarbital-treated marmoset NADPH–P450-reductase prefractionated on ADP-Sepharose. Column: HR 10/30. Buffer: 50 m*M* phosphate (pH 7.7)–20% glycerol. Flow-rate: 0.4 ml/min. Detection: solid line, 405 nm with 0.02 a.u.f.s.; and dotted line, 280 nm with 0.2 nm. Yield: 80–90%.

In addition to purification of marmoset reductase as described above, we have also prepared with the same scheme the corresponding reductases from rat and rabbit. Judging from the preformed Phast 10–15% gradient gels (Fig. 2, lanes 5–7), the relative molecular weights of these are all about 77 000 dalton.

As indicated above for our work with organ culture, the conditions are very much dependent on highly purified, highly active reductase. To this end we have found it convenient to maintain aliquot portions of 200 μl of ADP-Sepharose reductase at −80°C, which can then be fractionated on Superose 12 at a flow rate of 0.4–0.5 ml/min in about 30 min just prior to use in culture. Tenfold larger sample volumes were loaded onto an HR 16/50 column filled with the bulk gel Superose 12 Prep-Grade, in which case the elution time was fivefold.

Sephadex G-25 chromatography

Desalting of sample loads for ion-exchange chromatography was accomplished by gel filtration on laboratory-packed or prepacked HR 10/10 columns of Sephadex G-25 Superfine. Load volumes of 0.5–

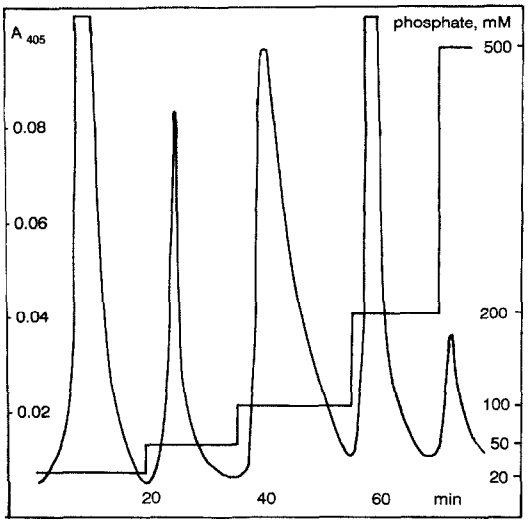

Fig. 4. Hydroxyapatite chromatography. Sample: 51 nmol of phenobarbital-treated marmoset P450 prefractionated on 8-aminooctyl-Sepharose. Buffer: 5 m*M* phosphate (pH 7.2)–20% glycerol–0.5% Emulgen 911. Gradient: 20–500 m*M* phosphate. Column: HR 10/10. Flow-rate: 0.5 ml/min. Detection: 405 nm with 0.1 a.u.f.s. Yield of P450 in the 200 m*M* fraction, 35%; in all fractions, 65–75%.

1.0 ml could be desalted with 90% protein recovery in 5 min. As ascertained by conductivity measurements, at least 95% of the salt was removed. Up to 2.5-ml loads were conveniently desalted on disposable PD-10 or NAP-25 cartridges (Pharmacia) at atmospheric pressure, and gave the same recovery but required 40 min each.

Hydroxyapatite chromatography

This type of purification was found to be useful for an initial separation into different P450 groups as was shown earlier for rat P450 [16]. At the same time, NADH–cytochrome b_5-reductase could be removed during washes with up to 50 m*M* phosphate (Fig. 4). Application of various gradient steps between 50 and 500 m*M* resulted in several fractions characterized by varying yields, electrophoretic purities, specific contents and metabolic activities [5]. Of all hydroxyapatite fractions, the 100–200 m*M* step apparently had the highest purity and yield and, as at this time the main aim was to isolate only one or the "main" P450 form present in phenobarbital-induced marmosets, this fraction alone was purified further. In comparison with the 8-aminooctyl-Sepharose fraction itself, this hydroxyapatite fraction displayed a considerable degree of electrophoretic purity (Fig. 5, lanes 2 and 3).

HA Ultragel gel could be used for more runs and at higher flow-rates than could granulated hydroxyapatite [9], but (at the same flow-rate) the latter was seen to bind P450 samples more strongly. Both types of gel were each capable of binding more than 25 nmol/ml of P450, and were not seen to cause any loss of haeme.

Hydroxyapatite gels of the Bio-Gel HT or HTP type were not used routinely as they were repeatedly found to compress on chromatography, resulting in greatly elevated back-pressures, even at reduced flow-rates.

Detergent removal

Excess amounts of non-ionic detergents not only interfere with protein determinations and substrate assays, but are also toxic towards tissue and organ culture [6,7]. Detergents present in fractions of marmoset P450 were removed with Bio-Beads or Amberlite [14,16] in 2–3 h but for the above-mentioned biological purposes, detergent removal by these resins was found to be too incomplete. Polyethylene glycol precipitation [17] offered the advantages of simultaneous desalting and concentration of the enzymes but was too cumbersome and gave too little recovery of enzyme. The same applied with calcium phosphate extraction [14], which had the advantage, however, of batchwise application. As already implemented for rat P450 [13,18], we found for marmoset enzymes that hydroxyapatite column chromatography was the most suitable method for removal of excess detergent, especially for *in vitro* assays.

Mono Q chromatography

When a sample of 8-aminooctyl-Sepharose pool was desalted as described above and loaded onto a Mono Q column (strong anion exchanger) and then washed and eluted as described under Experimental, P450 was recovered in both the pass-through fraction and the gradient fraction. The usefulness of this column at this stage of purification was found to be dependent on the type of marmoset induction: when phenobarbital was used as inducing agent and an 8-aminooctyl-Sepharose pool was loaded, only a modest resolution was found (Fig. 6a). This is in

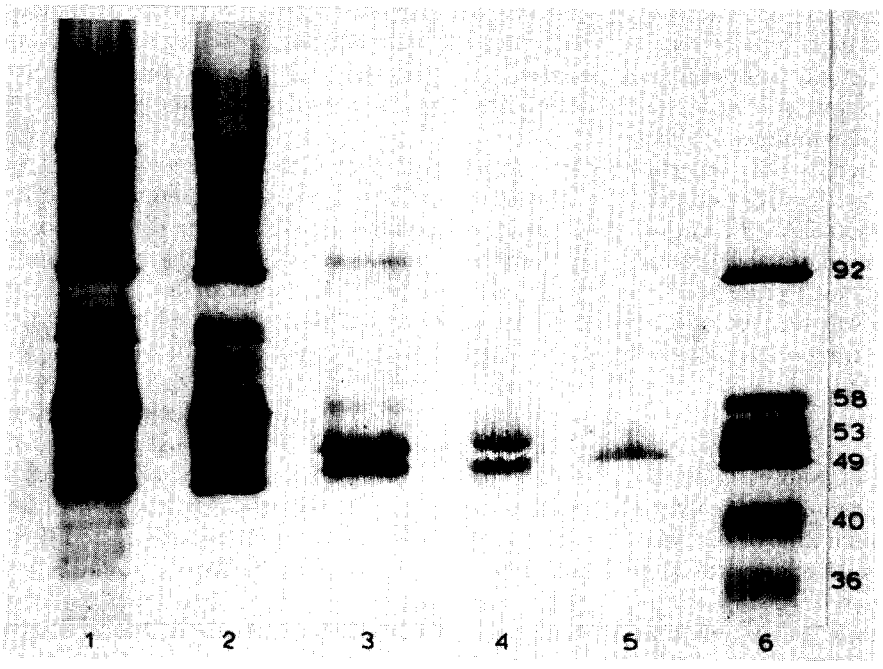

Fig. 5. SDS-PAGE gel of phenobarbital-treated marmoset P450 in various steps of purification. Lanes: 1 = solubilized microsomes; 2 = 8-aminooctyl-Sepharose fraction; 3 = hydroxyapatite fraction; 4 = Mono S fraction; 5 = phenyl-Superose fraction; 6 = marker proteins in kilodalton as described in Fig. 2.

accordance with that found for Mono Q fractionations of solubilized marmoset microsomes [4] and, recently, solubilized rat microsomes [19]. In SDS-PAGE analyses of the fractions obtained by such separations, the protein in the pass-through fraction was more homogeneous than that recovered in the gradient fraction (results not shown). We therefore conclude that this type of gel is more suitable for the simultaneous isolation of several P450 forms when the starting material is already more highly purified [5]. On the other hand, Roos [19] has recently shown that detailed segmented gradients on Mono Q columns are capable of yielding more discrete fractionations of variously induced P450 samples.

When non-induced marmoset 8-aminooctyl-Se-

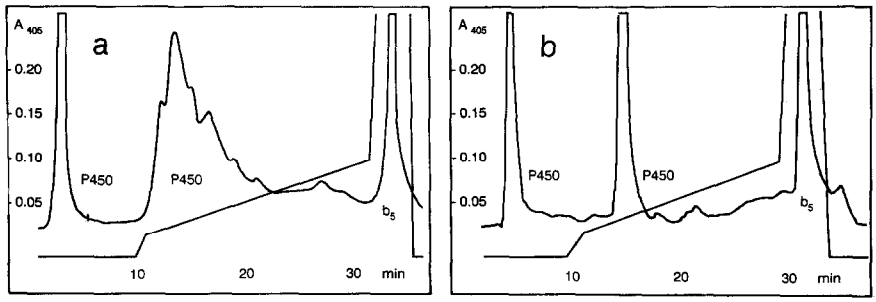

Fig. 6. Anion-exchange chromatography on Mono Q of (a) 6.3 nmol of phenobarbital-treated and (b) 6.1 nmol of untreated marmoset P450, each prefractionated on 8-aminooctyl-Sepharose. Column: HR 5/5. Buffer A: 10 m*M* Tris (pH 7.8)–20% glycerol–0.5% Renex 690. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–40–200–1000–0 m*M* NaCl. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.25 a.u.f.s. Yield: 70–90%.

pharose samples where fractionated on Mono Q columns, a good separation of P450 into two well defined, more homogeneous fractions was obtained (Fig. 6b). In this instance, in comparison with phenobarbital-induced P450, a smaller number of effective proteins were to be separated, so that this column may be considered adequate for this sample.

Mono Q fractionations of P450 derived from induced or non-induced marmoset material led to the isolation of cytochrome b_5 by NaCl gradients between 230 and 250 m*M*. P420 was recovered, if at all, mainly in the pass-through fraction as reported by us earlier [4] and by Roos [19].

DEAE chromatography

For phenobarbital-induced marmoset material, DEAE-Sepharose Fast Flow columns revealed a greater resolution of P450-containing peaks than did Mono Q columns. As this difference was also apparent between DEAE-Sepharose Fast Flow and Q Sepharose Fast Flow columns, we assume that this is indeed due to the different anion-exchange moieties, and not to any differences in gel matrix composition. It has often been reported [2,3] that for separations involving conventional anion-exchange of P450 on several types of DEAE matrices, a combination of anionic and non-ionic detergents results in better resolution than when using non-ionic detergents alone. In contrast to DEAE-Sepharose, DEAE-Sephacel or Whatman DE-52 [2,3], however, use of the anionic detergent sodium cholate with Mono Q is not possible, as this combination can cause substantial detergent-to-matrix binding which leads to a serious increase in back-pressure [4]. We have recently found that use of cholate with HPLC-quality DEAE gels also results in increased back-pressure and lower resolution, the latter of which has been reported earlier [13].

Mono S and CM-Sepharose chromatography

Mono S or CM-Sepharose chromatography of the 8-aminooctyl-Sepharose fraction (of phenobarbital-induced marmoset P450) using an NaCl gradient also resulted in a poorly defined profile. In contrast to Mono Q, Mono S yielded more homogeneity in the gradient fractions. For Mono S also, however, better separation of P450 forms alongside each other is to be expected when the load material is already more purified. The classical example, of course, in recovering a wide variety of P450 forms present is to load the pass-through fraction of an anion exchanger onto a cation exchanger and then utilize the gradient portion [13,16,19]. Again, Roos [19] has found that the use of segmented gradient can greatly enhance the resolution of Mono S separations.

Fig. 7a and b illustrate profiles of linear and step gradients in Mono S separations incorporating as the load a 100–200 m*M* phosphate pool derived from hydroxyapatite chromatography. Division of the gradient into the steps incorporated was determined by SDS-PAGE. The 100–150 m*M* fraction was the most homogeneous and, as seen in Fig. 5, lane 4, consists of a clear double band.

Phenyl-Superose, phenyl-Sepharose and octyl-Sepharose

After none of the above-discussed types of chro-

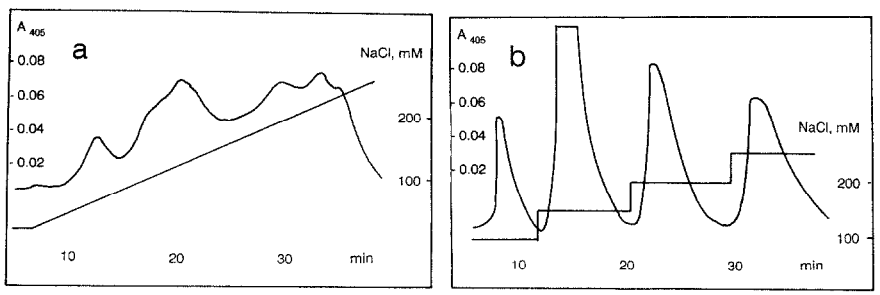

Fig. 7. Cation-exchange chromatography on Mono S using (a) linear and (b) step gradients. Sample: 5.6 nmol of phenobarbital-treated marmoset P450 prefractionated on hydroxyapatite (200 m*M* pool). Column: HR 5/5. Buffer A: 10 m*M* phosphate (pH 6.8)–20% glycerol–0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 10–300 m*M* NaCl. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.1 a.u.f.s. Yield of P450 in the 100–150 m*M* fraction, 65–75%; in all fractions, 85–95%.

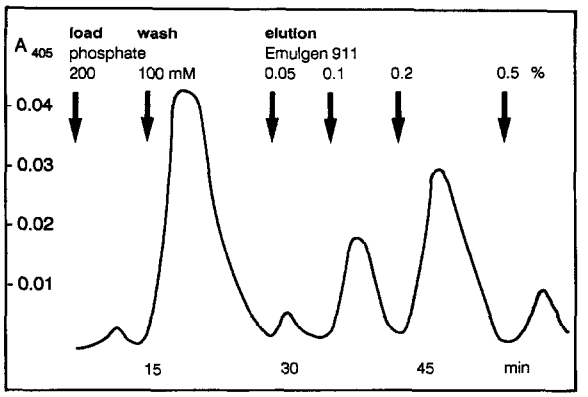

Fig. 8. Hydrophobic interaction chromatography on phenyl-Superose. Sample: 7 nmol of phenobarbital-treated marmoset P450 prefractionated on hydroxyapatite (200 m*M* pool). Column: HR 5/5. Load and wash: phosphate (pH 7.2)–20% glycerol. Elution: increasing concentrations of Emulgen 911. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.05 a.u.f.s. Yield in the 0.1% Emulgen fraction, 20%; in all fractions, 80%.

matographic gel types either alone or in combination had led to homogeneous fractions of marmoset P450, it seemed of interest to exploit differences in hydrophobicity. Starting from the high phosphate and low detergent concentration of a 100–200 m*M* phosphate gradient on hydroxyapatite (after dilution) or from a Mono S gradient (after detergent removal on hydroxyapatite), phenyl-Superose proved to be capable of further significant separation of this sample. After loading the sample, decreasing the salt concentration to 100 m*M* phosphate and then applying a gradient of Emulgen 911, several different P450 fractions were obtained (Fig. 8). The 0.1% Emulgen fraction was homogeneous as judged by SDS-PAGE (Fig. 5, lane 5), but did not represent the largest recovery among the different fractions of P450 from this column. The prepacked phenyl-Superose column, which had a bed volume of 1.0 ml, bound at least 30 nmol of P450.

Laboratory-packed phenyl-Sepharose and octyl-Superose columns gave essentially similar chromatographic profiles and SDS-PAGE patterns; separations with these gels, however, took three to four times longer and yielded less sharply defined peaks. The finding that these latter two gels themselves behaved so similarly with regard to the applied sample may indicate that the individual P450 forms fractionated here are not especially hydrophobic. If this were indeed the case, then separations on octyl-Sepharose (in comparison with phenyl-Sepharose) should have resulted in the elution peaks being more retarded. In support of this is the finding that among various membrane proteins, only averagely high LIVM values (percentage of leucine, isoleucine, valine and methionine as an expression of hydrophobicity) were registered for P450 (forms) [20].

Relationships between various results and strategies for column use

In FPLC, as in conventional chromatography, ion exchange is an integral step, applicable at almost any stage of purification, particularly at the onset and where large volumes are to be applied. The Mono Q and Mono S columns used here probably represent the most useful steps, and showed good reproducibility. Hydrophobic interaction was found to complement ion-exchange chromatography, and can also be used as a first step. On the other hand, P450 forms differing mainly in hydrophobicity can be easily separated, for example with phenyl-Superose, in a last step subsequent to hydroxyapatite as after this latter method the salt (phosphate) concentration is already elevated. Gel filtration was found to be much faster for desalting than, say, dialysis but is limited to small sample volumes. Hydroxyapatite was found to be useful for general purification, especially as a last step where non-ionic detergents can be removed and the sample is concentrated.

CONCLUSIONS

The FPLC system utilized here was found to be well suited to the rapid isolation of marmoset monooxygenase components for which little or no chromatographic information was available. Also, the sizes of the columns employed seemed to represent a good choice in that they were small enough to enable a good number of analytical and optimization runs in one day, yet were large enough to yield amounts sufficient for use in organ culture or immunological studies.

In the marmoset, as in many other animal species after induction with different agents, a large number of P450 forms probably also exist, and FPLC may prove useful in accelerating their isolation. To this end, we consider that over and above the tradi-

tional anion- and cation-exchange fractionations, a wider variety of gel types in prepacked and laboratory-packed columns should be exploited.

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