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Immobilized metal affinity chromatography as a means of fractionating microsomal cytochrome P-450 isozymes

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ABSTRACT

Fractionation of microsomal cytochrome P-450s is usually done by chromatography on ion-exchange resins and hydroxyapatite. The resolution of the great number of similar P-450 isozymes, however, requires additional methods based on different separation parameters. For this purpose immobilized-metal affinity chromatography (IMAC) was applied to the separation of P-450 isozymes. The method in its application to rat liver microsomes is described in detail. For method optimization and for the reproducibility of analytical fractionations a completely automatic fast protein liquid chromatographic system especially designed for IMAC is presented. Optimization is done with respect to the choice of the immobilized metal ion and the elution conditions. The chromatographic resolution is markedly enhanced by using segmented *vs.* linear gradients. The efficiency of P-450 resolution is demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting, verifying the different retention behaviours of the isozymes. However, for all the isozymes analysed so far, reactivity with one particular polyclonal antibody is observed with more than two IMAC fractions of a single run. This may be explained in part by the occurrence of isozymic forms distinguishable by the pattern of chymotryptic peptides. Hence IMAC appears to be suitable for the separation of closely related isozyme forms.

INTRODUCTION

The microsomal monooxygenase system responsible for the metabolism of numerous exogenous and endogenous compounds, such as drugs, xenobiotica, steroids and eicosanoids, includes four protein components: cytochromes P-450, NADPH–cytochrome P-450 reductase, cytochrome b_5 and NADPH–cytochrome b_5 reductase. The broad spectrum of enzymatic activities catalysed by this system is due to the multiplicity of P-450 isozymes [1,2]. According to recent estimates, more than 60 different P-450 isozymes are potentially present in mammalian species [3]. Only a fraction of them is expressed constitutively [4–6], others are induced xenobiotically [7] or as a consequence of pathological alterations [8–10]. Further, the level of individual isozymes is modulated by developmental processes [5,11,12] or hormonal regulation [6,13,14] and varies with respect to sex [4]. Hence the metabolic capacity of the hepatic monooxygenase system for different substrates, such as drugs, carcinogens and endogenous steroids, is variable. A knowledge of the P-450 pattern is therefore of significance for therapeutic measures and the establishment of xenobiotic effects on an organism.

Analysis of the microsomal P-450 pattern is complex, however, because of the large number of structurally and catalytically similar isozymes [15–17]. Therefore, a comprehensive analytical procedure needs to use a combination of different methods. As discussed earlier [18], the application of high-performance liquid chromatography for analytical fractionations of P-450 isozymes has been the subject of only a few studies based on separation by ion-exchange chromatography [19–23]. We have shown that microsomal cytochrome P-450s can be separated by successive anion- and cation-exchange fast protein liquid chromatography (FPLC) into eleven P-450-containing fractions [18]. Several of these fractions still contain a mixture of different P-450 species.

Methods based on additional separation principles might be advantageous for the further chromatographic resolution of these fractions. In this paper we demonstrate the suitability of immobilized-met-

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al affinity chromatography (IMAC) for this purpose. The principle of this method is based on the property of proteins to bind to metal ions that are immobilized on a chelating gel matrix. This binding occurs essentially by histidine residues [24]. To our knowledge, IMAC has not yet been used for the fractionation of P-450 isoenzymes. In a previous paper [18] we reported preliminary experiments demonstrating its suitability for P-450 separation. This method has been further optimized using a completely automatic FPLC system to obtain highly reproducible elution profiles.

EXPERIMENTAL

Animals and animal treatment

Sprague Dawley rats (200–250 g) (Lippische Versuchstierzucht, Extertal, Germany) were treated with P-450 inducers as described by Guengerich and Martin [25] for phenobarbital and β-naphthoflavone, by Ryan *et al.* [26] for isonicotinic acid hydrazide and by Arlotto *et al.* [27] for troleandomycin.

Preparation and solubilization of rat liver microsomes

Liver microsomes of male rats were prepared as described by Guengerich [28] and solubilized in 0.8% Lubrol PX [18]. Insoluble material was removed by centrifugation at 105 000 *g* for 1 h at 4°C. The clear supernatant was passed through a 0.2-μm filter (Minisart NML, SM 16534, Sartorius) and diluted tenfold in equilibration buffer A (see below) prior to chromatographic fractionation.

Immobilized-metal affinity chromatography

For analytical IMAC runs, an automatic FPLC system (Pharmacia, Uppsala, Sweden) was designed, including two high-precision FPLC pumps, one peristaltic pump, a gradient mixer, a gradient controller, four motor valves, two solenoid valves, four sample loops and a fraction collector (Fig. 1). Protein was detected by continuous monitoring at 280 nm. Monitoring at 417 nm is impeded by a strong and variable drift in absorption which cannot be corrected by baseline subtraction. Data storage and processing were performed with a personal computer (Atari PC 3) equipped with a PC Integration Pack (Softron, Gräfeling, Germany). To obtain reproducible elution profiles and identical starting conditions, the followign obligatory washing, equilibration and elution steps were controlled by a program: (1) columns wash with water, (2) charge with appropriate metal ion, (3) column wash with water, (4) columns wash with equilibration buffer, (5) column wash with elution buffer, (6) column wash with equilibration buffer, (7) partial metal discharge by pumping first 1 ml of water and then a defined volume of 200 m*M* EDTA under reversed-flow conditions, (8) column wash with equilibration buffer from the top, (9) sample injection, (10) column wash with equilibration buffer, (11) elution from either the top or bottom (12) column regneration with 200 m*M* EDTA and (13) column wash with water.

Because of the relatively low capacity of the metal-charged chelating Sepharose for cytochrome P-450s (*ca.* 50 μg/ml gel), a column with a volume of

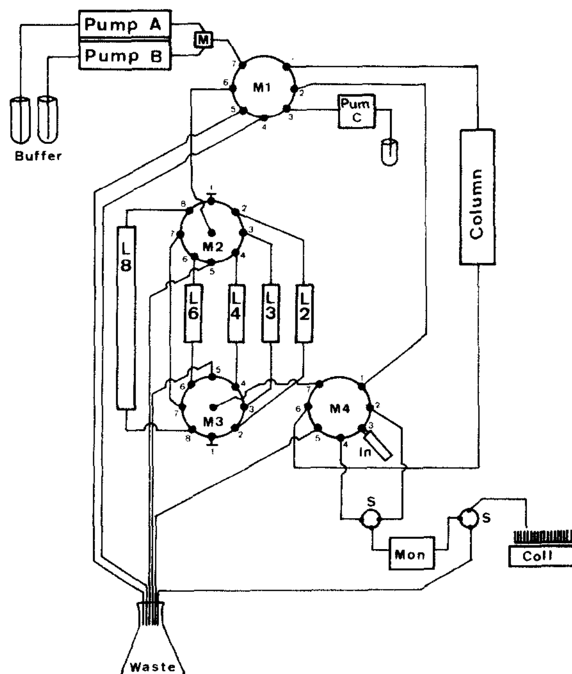

Fig. 1. FPLC system for automatically controlled protein fractionation by IMAC. Coll = fraction collector; In = input for syringe injection; L with figures for the port sets = loops for sample (2), 200 m*M* EDTA (3), metal chloride solution (4) and water (8); M = gradient mixer; Mon = monitor for UV–VIS detection; M1, M4 = motor valves (type MV7); M2, M3 = motor valves (type MV8); S = solenoid valves.

8 ml (Pharmacia, HR 10/10) was used for the analytical runs. Metal loading was done by passing 9 ml of 200 mM metal chloride solution ($NiCl_2$, $ZnCl_2$ or $CuCl_2$ in water) through the column followed by the steps given above. The composition of the equilibration buffer was 50 mM potassium phosphate (pH 7.2)–20% glycerol–0.2% Lubrol PX–0.5 M NaCl (buffer A). The elution buffer contained (B) 100 mM imidazole or (C) 2 M NH_4Cl in addition.

Analytical methods

Solubilized microsomes and column fractions were analysed by the following methods:

(i) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with an acrylamide concentration of 8% was carried out according to Laemmli [29] with subsequent silver staining of the protein bands [30] or transfer of the peptides to nitrocellulose membranes by transblotting for immunostaining [31]. To prevent non-specific antibody binding, the membranes were indubated with 10% bovine serum albumin for 2 h prior to addition of the antiserum.

(ii) Quantitative determination of total P-450 was done according to Omura and Sato [32].

(iii) The acitivity of the NADPH–cytochrome P-450 reductase was measured photometrically by cytochrome *c* reduction [33].

(iv) Protein content was determined by the method of Lowry *et al.* [34].

Enzyme purification and antibody production

P-450IA1 (= P-450c), P-450IIB1 (= P-450b), P-450IIE1 (= P-450j), P-450IIIA1 (= P-450p) and NADPH–P-450 reductase were purified as described [25,26,35–37]. Detergent was removed from the samples by chromatography on hydroxyapatite [38] prior to immunization. Rabbits were immunized by subcutaneous injections of purified enzymes emulsified in Freund's complete adjuvant for the first and incomplete adjuvant for the subsequent injections. Blood was drawn from the ear vein and unfractionated serum was used for immunoblotting.

Chemicals

Chemicals were of analytical-reagent grade and purchased from Merck (Darmstadt, Germany), except for Lubrol PX, β-naphthoflavone (Sigma, Deisenhofen, Germany), goat anti-rabbit IgG–peroxidase conjugate (Nordic, Bochum, Germany), acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine, phenobarbital (Serva, Heidelberg, Germany), hydroxyapatite (Bio-Rad Labs., Munich, Germany) and chelating Sepharose Fast Flow (Pharmacia). Troleandomycin was a kind gift from Pfizer (Karlsruhe, Germany).

RESULTS AND DISCUSSION

Automatic control of the chromatographic procedure

Analytical IMAC, as presented here, is a complex chromatographic procedure involving several consecutive equilibration, washing and fractionation steps. Automatic control of all these steps, already helpful for optimization, is essential for the reproducibility of analytical elution profiles. For this purpose we designed a completely automatic FPLC system (Fig. 1).

Depending on the composition of the samples and buffers used, the immobilized metal ion can be dissociated from the resin. For this reason, post-column rebinding of liberated ions by an uncharged gel section is necessary to avoid contamination of the protein fractions. A metal ion-free portion of the column with a defined volume is obtained by EDTA washing from the bottom. In the automatic chromatographic system reported here, all components, *i.e.*, metal ions, EDTA and sample, may be applied from either the top or the bottom of the column, thus allowing great flexibility in the design of chromatographic procedures.

Choice of the immobilized metal

A decisive parameter for P-450 fractionation by IMAC is the type of the immobilized metal ion. Of the ions Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and La^{3+}, only the first three were studied in detail and are dealt with here. Their suitability was determined by two parameters: (i) the ability to bind P-450 and (ii) the ability to release active P-450 using standard buffers (see Experimental) and a simple gradient (Fig. 2). The results are summarized in Table I. Judged by these criteria, Ni^{2+}, was found to be the most appropriate ion for our purposes because more than 80% of applied P-450 is usually retained on the column and about 50% recovery is obtained, whereas the amount of unretained P-450 is greater

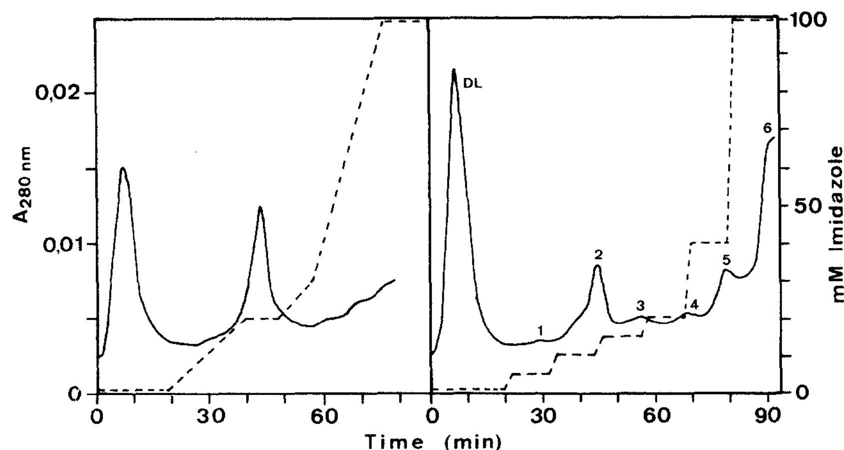

Fig. 2. Effect of the gradient form on the chromatographic resolution of microsomal proteins by IMAC. Gel: chelating Sepharose Fast Flow charged with nickel. Column dimensions: 1 cm diameter, 5.7 ml metal ion-charged gel, 1.2 ml uncharged gel section. Equilibration buffer (A): 50 m*M* potassium phosphate (pH 7.2)–20% glycerol–0.2% Lubrol PX–0.5 *M* NaCl. Elution buffer (B): buffer A + 100 m*M* imidazole. Sample: Lubrol-solubilized liver microsomes of phenobarbital-treated rats, P-450 content 0.86 nmol, protein content 0.51 mg. DL = pass-through fraction. Numbers (1–6): designation of peak fractions corresponding to those given in Fig. 5–7. The gradient forms are shown by the broken lines.

than 50% with immobilized Zn^{2+}. The superiority of Ni^{2+} over Zn^{2+} cannot be derived from the comparison of elution profiles, which appear similar (Fig. 3). In addition, spectroscopic determination of P-450 has to be included for evaluation (Table I).

The observed low capacity of the zinc-charged gel confirms previous results [18]. In contrast to our earlier study [18], however, nickel, which was deemed to be unsuitable for P-450 fractionation by IMAC, proved to be the most appropriate ion in the optimized chromatography system described here. Chelating Sepharose Fast Flow charged with Cu^{2+}

TABLE I

BINDING AND RECOVERY OF CYTOCHROME P-450s FRACTIONATED BY IMAC

Samples: Lubrol-solubilized liver microsomes of rats treated with the inducers indicated (β-NF = β-naphthoflavone; PB = phenobarbital; INH = isonicotinic acid hydrazide). Amount of P-450 applied: 1 nmol. Amounts of protein applied: 1.4 mg (β-NF), 0.6 mg (PB) and 1.8 mg (INH). Chromatographic procedure as described under Experimental. The P-450 contents of the pass-through and eluted fractions were determined spectroscopically [32].

Ion	Eluent	Recovery of applied cytochrome P-450 (%)			Sample
		Pass through fraction	Eluted fraction	Total	
Zn^{2+}	NH_4Cl	56.5	≤1	57	β-NF
Zn^{2+}	Imidazole	58.4	5.8	64.2	β-NF
Ni^{2+}	NH_4Cl	15.5	12.5	28.0	β-NF
Ni^{2+}	Imidazole	13.1	35.4	48.5	β-NF
Cu^{2+}	NH_4Cl	0	0	0	PB
Ni^{2+}	Imidazole	12.3	30.1	42.4	β-NF
Ni^{2+}	Imidazole	15.0	40.2	45.2	INH
Ni^{2+}	Imidazole	21.4	47.6	68.7	PB

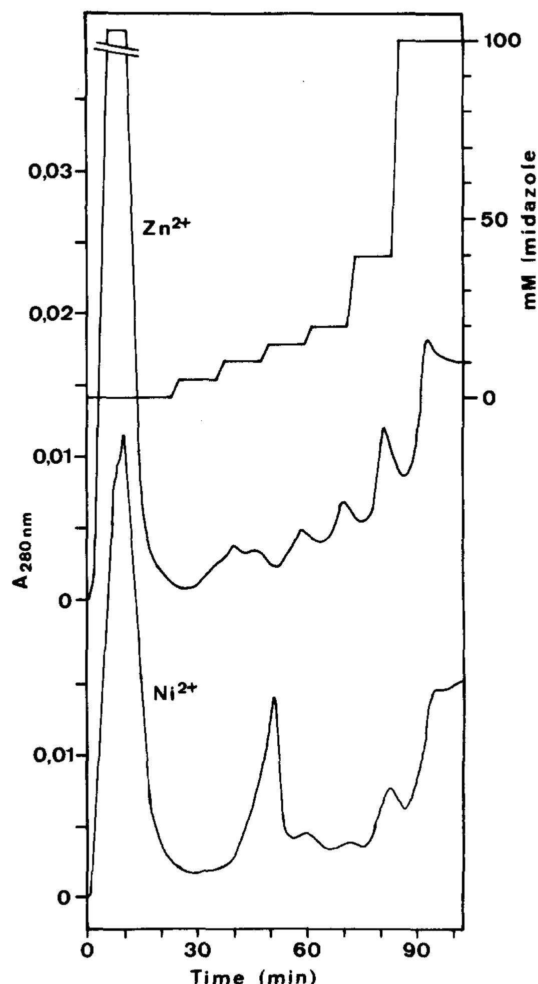

Fig. 3. Binding and elution of cytochrome P-450s of phenobarbital-treated rats to chelating Sepharose charged with nickel or zinc. Conditions as in Fig. 2. Sample: Lubrol-solubilized liver microsomes of phenobarbital-treated rats, P-450 content 0.5 nmol, protein content 0.29 mg. The shape of the gradient is given by the stepped line at the top.

leads to strong and apparently irreversible binding of P-450.

Optimization of P-450 fractionation by IMAC

Binding and recovery of cytochrome P-450s from the nickel-charged gel are dependent on several different parameters such as type of buffering component, pH, type of eluting agent and presence of detergent. For the following studies we used potassium phosphate in equilibration and elution buffers. Some organic buffer substances, such as tris(hydroxymethyl)aminomethane (Tris), are less suitable because they weaken the metal–chelate complex. In this instance dissociated metal is observed by accumulation of coloured ions in the uncharged gel section. As shown in a previous paper [18], P-450 elution by decreasing pH results in a very low recovery of spectroscopically detectable P-450, *i.e.*, about 8% of the applied cytochrome. Elution with a competitive ligand leads to higher yields (Fig. 4, Table I).

With respect to the recovery of spectroscopically detectable P-450, imidazole proved to be more advantageous than ammonium chloride as an eluting agent (Table I). This is probably due to the high concentrations of up to 2 *M* ammonium chloride necessary for P450 elution. Early studies by Imai and Sato [39] demonstrated rapid destruction of P-450 in the presence of high salt concentrations. Therefore, we used the nickel–imidazole system for further studies. Routinely, imidazole elution leads to about 50% recovery of spectroscopically detectable P-450 (Fig. 4, Table I). The exact value might be even higher, because we found that imidazole interferes with the spectroscopic determination of P-450 by inhibiting the formation of the $P\text{-}450_{reduced}$–CO complex. It has long been known that imidazole is a ligand for P-450s [39,40]. Preliminary studies showed that the extent of this inhibition is dependent on the isozyme composition of the sample, because liver microsomal P450s of rats treated with various inducers exhibit different susceptibilities for imidazole-dependent inhibition (not shown).

Successful P-450 desorption from the column requires the presence of detergent. We routinely supplement the equilibration and elution buffers with 0.2% of Lubrol PX. If detergent is ommitted from the buffers the yield of spectroscopically detectable P-450 decreases to about 35%. Simple linear gradients of the competitive ligands result in low resolution of cytochrome P-450 isozymes (Fig. 2). One or two P-450-containing peaks are obtained. Similar to ion-exchange FPLC [18,41], resolution is decisively increased by application of stepwise or segmented gradients leading to five well separated P-450-containing peaks (Fig. 2).

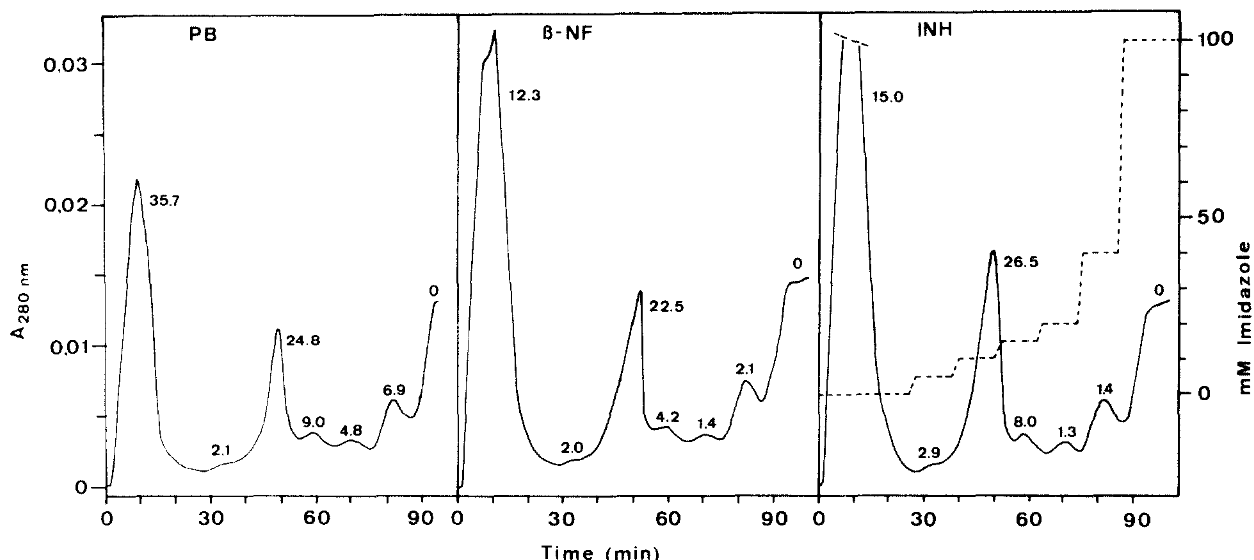

Fig. 4. IMAC fractionation of detergent-solubilized liver microsomes of rats treated with various P-450 inducers. Conditions as in Fig. 2. Flow-rate: 1 ml/min. Samples: Lubrol-solubilized liver microsomes of rats treated with various P-450 inducers. P-450 and protein content of the samples (in nmol and mg, respectively): PB-induced, 1.15 nmol, 0.67 mg; β-NF-induced, 2.3 nmol, 3.29 mg; INH-induced, 1.33 nmol, 2.42 mg. Figures give the percentage of applied cytochrome P-450 in the pooled peak fractions. Gradient form is indicated by the broken line in the chromatogram shown in the right-hand panel.

Chromatographic resolution of microsomal P-450s by IMAC

The efficiency of P-450 fractionation by IMAC was analysed by a combination of different methods, *i.e.*, UV–VIS spectrophotometry of P-450–ligand complexes, SDS-PAGE and immunoblotting. Elution profiles obtained by fractionation of liver microsomal P-450s of rats treated with various inducers are shown in Fig. 4. Although their overall shapes and their quantitative P-450 distributions in the peak fractions (Fig. 4) are very similar, the different P-450 compositions become apparent if the protein patterns of these fractions after SDS-PAGE are compared (Figs. 5–7).

Because of the large number of different isozymes with similar molecular weights, distinct protein bands cannot be assigned to definite P-450 species. Identification of individual P-450 isozymes after fractionation is achieved by immunoblotting using specific or group-specific antibodies. Some of the results of the immunochemical analyses are shown in Figs. 6 and 7 and are summarized in Table II. The differential elution of the analysed isozymes is clearly seen. Treatment of rats with phenobarbital leads to induction of cytochromes P-450 of the IIB family [36] and to a lesser extent of the IIIA family. The immunoblot analyses show that the cytochromes P450IIB1 and -IIB2 are mainly eluted with 40 m*M* imidazole, whereas P450IIIA is either found in the pass-through fraction or is mainly eluted with lower imidazole concentrations (Figs. 6 and 7).

The detection of two proteins with different electrophoretic mobilities reacting with the antibody against P450IIB isozymes (Fig. 6) can be explained by the occurrence of two very closely related isozymes (IIB1 and IIB2). Cross-reactivity of the antibody is very probably because the homology of the amino acid sequences of these two proteins is 97% [42]. Microsomes of rats pretreated with β-naphthoflavone lack isoenzymes of the IIB family [43] (Fig. 5A) but contain P450IA species [35,43] which are concentrated in peak 3 of the IMAC fractionation eluted with 15 m*M* imidazole (Table II, Fig. 5A). Similarly to P-450IIIA, P450IIE1, which can be induced by isonicotinic acid hydrazide [26], shows weak or no binding to the nickel ion-charged column (Table II). The protein band marked with 52.5 kilodalton in Fig. 5B represents the P450IIE isoenzyme as determined by immunoblotting (not shown). The 46.5-kilodalton protein shown in Figs.

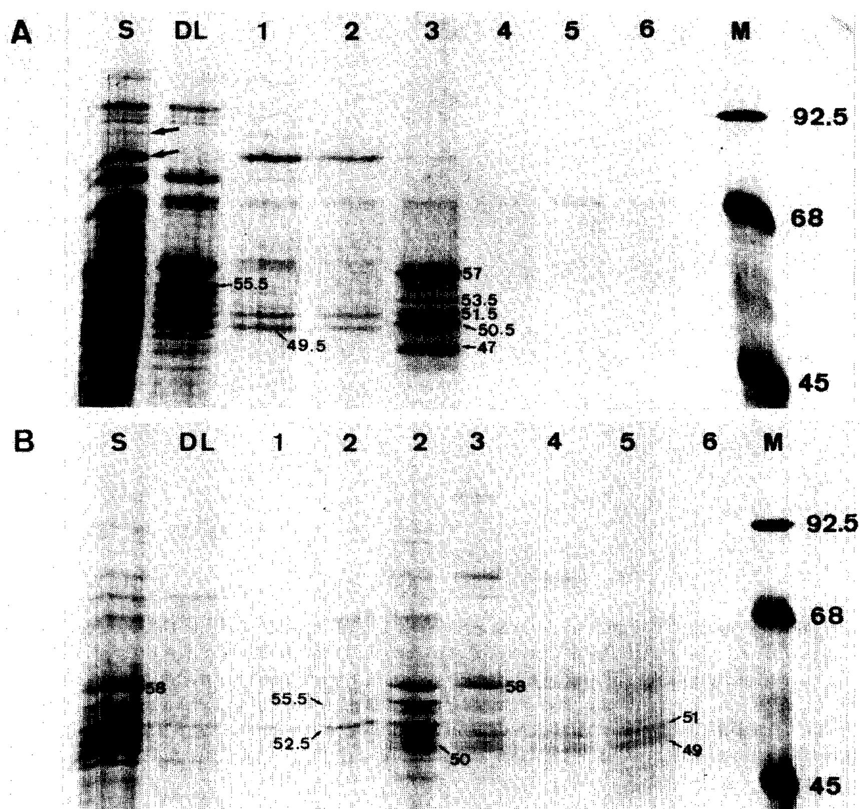

Fig. 5. SDS-PAGE analysis of P-450 fractions obtained by IMAC. Samples for the IMAC runs: Lubrol-solubilized liver microsomes of rats treated with (A) β-naphthoflavone (P-450 content 1.5 nmol, protein content 2.1 mg) and (B) isonicotinic acid hydrazide (P-450 content 1.3 nmol, protein content 2.4 mg). Fractionation: optimized IMAC as described under Experimental using the nickel–imidazole system. Column dimensions and buffers as in Fig. 2. For designation of the fractions and form of the gradient, see Fig. 2, right-hand panel. S = unfractionated sample; DL = pass-through fraction; M = molecular weight markers, with the indicated values in kilodalton. Apparent molecular weights (kilodalton) are assigned to individual protein bands. Large arrows (top left) point to protein bands which are not found in the pass-through fraction but are present in imidazole-eluted fractions showing that the column capacity was not exhausted.

6 and 7 is probably the constitutively expressed and slightly phenobarbital-inducible P450IIA1, which has the lowest apparent molecular weight of all known microsomal P-450 species. NADPH–cytochrome P-450 reductase is bound by the nickel-charghed column and can be eluted in an enzymatically active form with about 15 m*M* imidazole (Table II).

The immunoblot analyses show that isozymes detectable with a single antiserum are present in more than two peak fractions of an IMAC run. An explanation for this may be the existence and chromatographic separation of closely related isozyme forms. Such forms have been described, for example, for the P-450IIB and -IIIA protein families [44–46]. Preliminary results obtained with immunoblotting of IMAC-fractionated, chymotryptic digested P450IIIA species of rats treated with triacetyloleandomycin support this view [47]. Hence IMAC may also be suitable for the separation of closely related P-450 isozyme forms.

In addition to its suitability for analytical P-450 fractionation, IMAC may also provide a useful tool for preparative isoenzyme purification. Starting with a crude extract, *i.e.*, solubilized microsomes, fractions containing only a few proteins are obtained with a single IMAC run (Fig. 6, fractions 1 and 5).

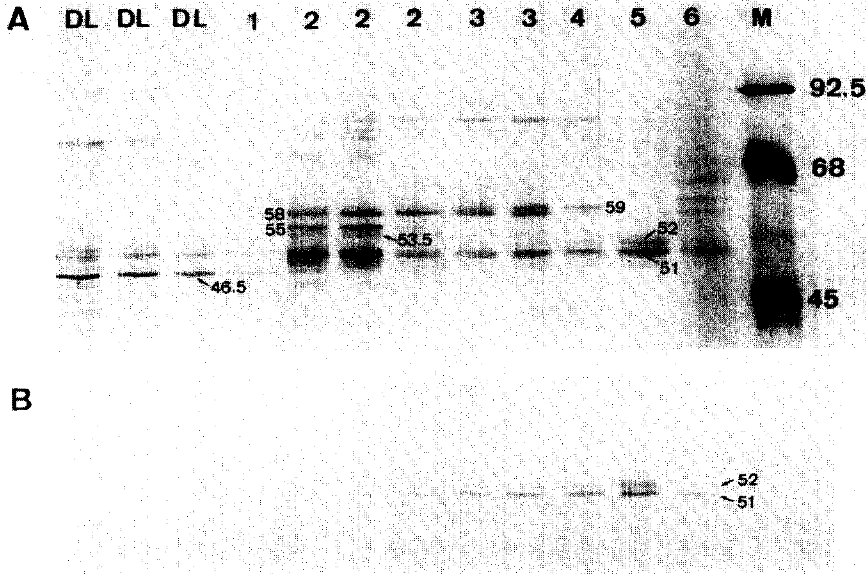

Fig. 6. Identification of P-450 isozymes in IMAC fractions by immunoblotting after SDS-PAGE. Sample for the IMAC-run: Lubrol-solubilized liver microsomes of phenobarbital-treated rats containing 0.53 nmol of cytochrome P-450 and 0.31 mg of protein. Fractionation: optimized IMAC as described under Experimental using the nickel–imidazole system. Column dimensions and buffers as in Fig. 2. For designation of the fractions and form of the gradient, see Fig. 2, right-hand panel. S = unfractionated sample; DL = pass-through fraction; M = molecular weight markers, with the indicated values in kilodalton. (A) Analysis of SDS-PAGE with (B) subsequent immunoblotting using an antibody against cytochromes P-450 IIB1 and IIB2. Apparent molecular weights (kilodalton) are assigned to individual protein bands.

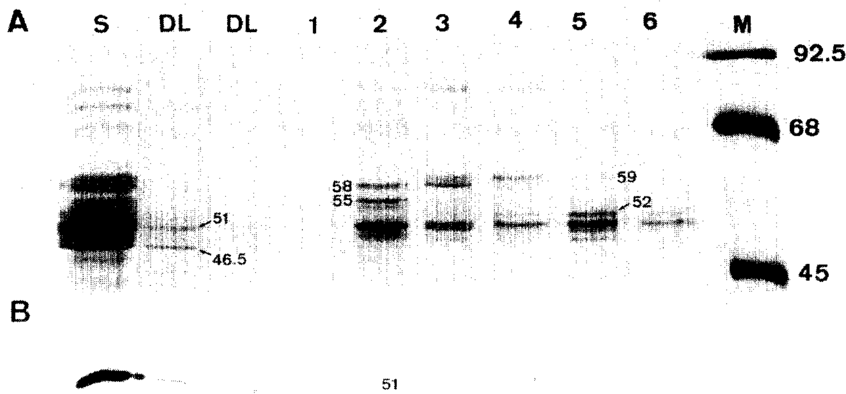

Fig. 7. Identification of P-450 isozymes in IMAC fractions by immunoblotting after SDS-PAGE. Sample for the IMAC-run: Lubrol-solubilized liver microsomes of phenobarbital-treated rats containing 1.15 nmol of cytochrome P-450 and 0.67 mg of protein. Fractionation: optimized IMAC as described under Experimental using the nickel—imidazole system. Column dimensions and buffers as in Fig. 2. For designation of the fractions and form of the gradient, see Fig. 2, right-hand panel. S = unfractionated sample; DL = pass-through fraction; M = molecular weight markers with the indicated values in kilodalton. Apparent molecular weights (kilodalton) are assigned to individual protein bands. (A) Analysis by SDS-PAGE with (B) subsequent immunoblotting using an antibody against cytochrome P-450 IIIA.

TABLE II

DISTRIBUTION OF P-450 ISOZYMES AND NADPH–P-450 REDUCTASE IN IMAC FRACTIONS AS ANALYSED BY IMMUNOBLOTTING AFTER SDS-PAGE OR ENZYMATIC ACTIVITY

Fractionation of microsomal cytochrome P-450s by IMAC was done with the optimized method as described under Experimental. Samples: Lubrol-solubilized liver microsomes of rats treated with various inducers (β-NF = β-naphthoflavone; PB = phenobarbital; INH = isonicotinic acid hydrazide; TAO = troleandomycin). Amount of P-450 applied: *ca.* 1.5 nmol. Amounts of protein applied: 0.88 mg (PB), 0.32 mg (TAO), 2.72 mg (INH) and 2.14 mg (β-NF). Peak designation: DL = pass-through fraction; numbers correspond to the peak fractions as indicated in Fig. 2. The activity of the NADPH–P-450 reductase was determined by reduction of cytochrome *c* [33]. Symbols indicate relative enzymatic activity or staining intensity after peroxidase reaction with chloronaphthol: −, (+), +, ++ and +++ = not visible, faint, slight, medium and strong staining, respectively.

Sample	Antibody to	Peak						
		DL	1	2	3	4	5	6
		Imidazole (m*M*)						
		0	5	10	15	20	40	100
PB	IIB1/IIB2	−	−	(+)	+	+	++	+
PB	IIIA1	++	+	++	+	+	+	+
TAO	IIIA1	++	(+)	+++	++	+	+	+
INH	IIE1	+	+	+	−	−	−	−
β-NF	IA1/IA2	−	−	(+)	+	−	−	−
Sample	**Activity**							
PB	Reductase	−	−	+	++	(+)	−	−

CONCLUSIONS

Our aim is to use IMAC for analytical fractionations in combination with ion-exchange FPLC to elaborate P-450 patterns in microsomal samples. The above results show the suitabiliy of IMAC for this purpose, as closely related forms can be separated and subsequently identified by immunochemical methods. The optimized chromatographic procedures presented here are part of an analytical strategy combining the application of different methods [48]. Fractionation of the cytochromes with high-resolution techniques in combination with immunochemical and spectroscopic methods should allow the detection and determination of very similar known and unknown forms in a complex mixture of P-450 isozymes. Comprehensive analyses of P-450 patterns should help in the study of P-450 induction processes, the evaluation of chemotherapeutic measures and pathologically altered P-450 patterns.

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