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Journal of Chromatography B, 684 (1996) 107–131

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Chromatographic separation and behavior of microsomal cytochrome P450 and cytochrome b_5

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Abstract

The methods used for separation of the multiple mammalian cytochrome P450 enzymes by liquid chromatography are reviewed. In addition to the chromatographic techniques, preparation and handling of samples and prefractionation procedures are considered. Conditions that affect stability and chromatographic resolution of cytochromes P450 are also discussed. Special emphasis is put on useful methods which are not routinely used for P450 separation, such as immobilized metal affinity or hydrophobic-interaction chromatography. Applications of low- and high-pressure methods with regard to preparative and analytical separations are compared. It is shown that high- and medium-pressure ion-exchange chromatography are suitable tools for separation of closely related P450 enzymes, especially when specific detection methods are available. In addition to fractionation of cytochromes P450, the isolation and chromatographic behavior of cytochrome b_5 is discussed.

Keywords: Reviews; Cytochromes; Enzymes

Contents

0378-4347/96/$15.00
PII S0378-4347(96)00018-7

1. Introduction

Cytochrome P450 has been described originally as a CO-binding pigment with an absorption maximum of the ligand complex at 450 nm [1,2]. In the subsequent years after its discovery, the heterogeneity of this pigment became apparent by spectroscopic and chromatographic investigations [3–8]. Today more than 300 different P450 species of bacteria, plants and animals have been characterized either by nucleotide or amino-acid sequences [9]. In a single animal species such as the rat, more than 40 different P450 enzymes have been found so far and there is no reason to believe that all existing P450 enzymes have been detected. Recently some new P450 species were added to the list of known enzymes [10,11] and there is evidence obtained by chromatographic [12,13] and electrophoretic analyses [14,15] of liver microsomal samples that the diversity of P450 is much greater than currently believed [16].

In mammalian species the greatest quantity and diversity of cytochromes P450 is found in the endoplasmic reticulum of liver parenchymal cells. P450 enzymes are also expressed in other organs, tissues and cells such as lung, kidney, intestine, skin, liver macrophages and several types of circulating leukocytes [17–20]. Their total microsomal P450 content is generally lower than in the liver, i.e., 1/10 or less based on microsomal protein [21,22]. Furthermore, the enzyme profile of extrahepatic tissues is less complex because a smaller number of P450 species is expressed. Some P450 forms, however, occur only in some specialized tissues, e.g., CYP2G1 in the nasal mucosa [23].

The microsomal monooxygenase system includes cytochromes P450, NADPH–cytochrome P450 reductase, cytochrome b_5 and NADH–cytochrome b_5 reductase. It functions in the phase I metabolism of many drugs and environmental chemicals (xenobiotics) in the course of detoxification and elimination processes. In addition, endogenous compounds such as steroids, eicosanoids, fatty acids and vitamin D_3 are substrates of this system. Its ability to metabolize a large number of compounds is not only caused by the existence of numerous P450 enzymes but also by the fact that one distinct enzyme can recognize several substrates. Vice versa, most substrates can be metabolized by more than one P450 enzyme. This means that, among the numerous known substrates, only a few can be considered more or less specific for an individual P450 enzyme and can thus be used as diagnostic tools in chromatography. Overlapping properties of P450 enzymes are not restricted to substrate specificity but also concern molecular size, ligand binding and spectroscopic characteristics of P450 ligand complexes, inhibitor specificity, immunoreactivity and chromatographic behavior. Therefore, together with the multiplicity of P450 enzymes, biochemical and chromatographic dissection of the P450 system is difficult. The chromatographist and/or the analyst is confronted with two problems: the separation of similar enzymes and the identification of the separated species.

Several factors influence the expression of individual P450 enzymes. Thus, the microsomal P450 enzyme profile varies with respect to age and sex and may be modified in response to diseases [24–28]. A number of P450 enzymes are induced by xenobiotics, thereby increasing the metabolic capacity of the microsomal monooxygenase system for certain

substrates which often includes the inducer itself. This process of metabolic adaptation can be exploited for the isolation of inducible enzymes by increasing their absolute and specific content in the source material, the liver microsomes.

In the present paper, chromatographic procedures for the purification and separation of microsomal cytochromes are reviewed with a special focus on mammalian enzymes of the cytochrome P450 gene superfamily. If not stated otherwise, only cytochromes P450 from rat liver microsomes are considered. Cytochromes which are involved in electron transfer chains of chloroplasts, mitochondria or prokaryotes are not dealt with here (applications for their isolation have been compiled by Froud [29]). However, chromatographic properties of cytochrome b_5, a component of the microsomal monooxygenase system, will be summarized. Methods for P450 monitoring during chromatographic separation, for general identification of cytochrome P450 and recognition of individual enzymes will be discussed.[1]

2. Cytochromes P450 – a challenge for the chromatographer

As mentioned above, the chromatographic purification of mammalian cytochromes P450 is fraught with problems arising from the multiplicity and structural similarity of P450 enzymes present in the endoplasmic reticulum. In liver microsomes, more than 20 different but often very similar P450 enzymes can be present concomitantly. Low levels of individual enzymes amidst a bulk of similar enzymes are another problem for purification. As amphipathic membrane-bound enzymes, cytochromes P450 require solubilization prior to their chromatographic separation. However, detergents used for membrane solubilization exhibit several undesirable effects. Because destruction of the membrane environment by detergents can abolish the structural integrity of P450 enzymes, measures to prevent denaturation are necessary (Section 4). Another problem is that detergents can interfere with enzymatic assays used for monitoring the purification (Section 9.2). Furthermore, interactions of detergents with column resins can decrease chromatographic resolution and binding capacity of a column (Section 8.2). Thus, the effects exerted by a detergent have to be considered in the design of a chromatographic strategy. Therefore, not only the chromatographic procedures for P450 separation but also measures to increase the yields of total or individual P450 enzymes will be discussed.

3. Preparative and analytical P450 separations

For preparative chromatography of cytochromes P450, several strategies have been developed which vary due to the purposes, i.e., isolation of single or several P450 enzymes or concomitant purification of further components of the microsomal monooxygenase system along with P450. For the different purposes, suitable conditions and procedures were elaborated. These include selection of source material (microsomes of untreated or inducer-treated animals) and detergent for membrane solubilization, inclusion of a prefractionation step and the combination of consecutively applied chromatographic methods. All these steps will be discussed in the following sections.

Requirements for analytical fractionations are often very different from those of preparative enzyme purifications. Whereas purity and yield of an enzyme are decisive criteria for preparative separations, high resolution and reproducibility of a chromatographic step are required for analytical procedures. The latter may be met with by application of medium- or high-pressure chromatographic methods. High-resolution chromatographic techniques, such as HPLC and FPLC, have been applied only infrequently for the separation of cytochromes P450. As an analytical tool in P450 research, the use of HPLC is reported in only a few studies [30–33]. FPLC has been applied for this purpose by Sakaki et al. [12] to show the distinctness of purified P450 enzymes, and by Kastner and Schulz [34] to differentiate between P450 patterns in liver microsomes of marmoset monkeys treated with various inducers. Optimization of ion-exchange FPLC and IMAC for analytical purposes has been carried out in our laboratory. We have shown that chromatographic

[1] For additional information on this subject see also Jansen et al. in this volume [J. Chromatogr. B, 684 (1996) 133-145].

Table 1
Recovery of cytochromes P450 by precipitation of detergent solubilized membranes with poly(ethylene glycol) (PEG)

Species	Organelle	Rat treatment	PEG concentration	P450 recovery (%)	Ref.
Rat	Microsomes	Untreated	7–15%	70.3	[39]
Rat	Microsomes	Phenobarbital	7–15%	71.4	[39]
Rat	Microsomes	3-Methylcholanthrene	7–15%	75.8	[39]
Rabbit	Microsomes	β-Naphthoflavone	8–12%	65.2	[40]
Rat	Microsomes	Untreated	10–16%	53.0	[41]
Rat	Microsomes	Untreated	7–15%	59.5	[42]
Rat	Microsomes	β-Naphthoflavone	5–15%	41.3	[43]
Rat	Mitochondria	β-Naphthoflavone	5–15%	64.6	[43]
Rat	Mitochondria	Untreated	5–15%	44.5	[43]

resolution of both methods can be remarkably increased by introduction of detailed segmented gradients [13,35,36].

Besides the chromatographic strategy, sample preparation and handling are crucial for analytical separations. For preparative P450 separations, prefractionation procedures are usually applied subsequent to membrane solubilization with detergents. Neither prefractionation by PEG (Table 1) nor by chromatography on 8-aminooctyl-Sepharose [37] is useful for analytical separations because both steps result in a severe loss of cytochrome P450, altering the original P450 composition of the sample. The pass-through fraction of an 8-aminooctyl-Sepharose column contains considerable amounts of both cytochrome P450 and P420 (the enzymatically inactive form of cytochrome P450). Because of its low specific P450 content, this fraction is not used for preparative purposes. Nevertheless, it might include P450 forms not present in the P450-enriched eluate. Furthermore, overall P450 recovery amounts to about 50% only. Therefore, direct application of detergent-solubilized microsomes to chromatography is necessary for analytical separations [13,35,36].

Membrane solubilization with detergents is an acceptable preparatory step for analytical P450 separations because P450 recovery is very high (>95%). Lubrol PX has been found especially suitable for analytical chromatography of cytochromes P450. Its solubilization efficiency for microsomal cytochrome P450 is about 96%. In contrast to sodium cholate Lubrol does not interfere with strong anion-exchangers such as Mono Q which are useful for analytical P450 separations. Furthermore, acceptable chromatographic resolution of cytochromes P450 on Mono Q is achieved in the presence of Lubrol alone. An advantage of Lubrol for detection is that it does not absorb at 280 nm, so that protein monitoring at this wavelength is possible during chromatography (Table 2) [35,38].

4. Purification of enzymatically active cytochromes P450

For most purposes, the maintenance of the structural and functional integrity of chromatographically separated cytochromes P450 is desirable. Generally, cytochromes P450 are destabilized when their membrane environment is destroyed. It has been found, however, that polyols such as glycerol can counteract this effect [44]. Therefore, glycerol in a concen-

Table 2
Properties of widely used detergents in P450 chromatography

Property	Sodium cholate	Emulgen 911	Lubrol PX
Interference with strong anion exchangers	Yes	No	No
Absorption at 280 nm	No	Yes	No
Dialyzable	Yes	No	No
Strong inhibitory effect on P450 activities	No	Yes	Yes

tration of 20% is generally used during all chromatographic steps starting with membrane solubilization by detergents. While non-ionic, zwitterionic and some anionic detergents such as sodium cholate do not show deleterious effects on cytochromes P450, others such as the cationic detergents benzalkonium chloride and cetyl-triethylammonium bromide lead to irreversible P450 damage or conversion to the inactive P420 form [35,38]. In some cases a mixture of a non-ionic detergent and sodium cholate is required for elution of intact P450 as shown for P450d(=CYP1A2) bound to hydroxyapatite. In this case, elution in the presence of a non-ionic detergent alone results in loss of heme [45]. Sundin et al. prevented heme loss during chromatographic purification of a cytochrome P450 from rat prostate by using preconditioned stationary phases which have been pretreated with hemoglobin [46]. Conversion of P450 to P420 occurs at pH values below 6.0 and above 8.0 which is not critical for most applications [47]. However, acidic conditions can be necessary for elution in immunoaffinity chromatography [48] or immobilized metal affinity chromatography [36]. Apparently, formation of P420 can also be promoted by Lubrol PX when present in the assay for quantitation of P450 as CO complex. Dilution of the sample with 0.05–0.2% Renex 690 can prevent this effect [49].

5. Solubilization of membrane-bound cytochromes P450

For solubilization of cytochromes P450, detergents are most often used to disrupt the membranes, although for analytical purposes cleavage by proteases appears also suitable [50,51]. Several aspects are important for the selection of a suitable detergent, including efficiency of solubilization, compatibility with the chromatographic procedure and effects on the stability and enzymatic activity of the cytochromes P450. For preparative but especially for analytical purposes, the solubilization efficiency of a detergent should come close to 100%, either to obtain high yields or to exclude loss of any P450 species. Some typical results for solubilization of liver microsomes obtained with a variety of detergents are presented in Table 3.

With the exception of CHAPS, a zwitterionic detergent, P450 is solubilized to a high degree (about 95%) by the detergents listed in Table 3. In fact, sodium cholate, Emulgen and Lubrol represent the reagents most commonly used for P450 solubilization. In many cases, solubilization by detergents leads to a slight enrichment of cytochrome P450 on a protein basis (Table 3). Especially CHAPS, although less efficient than the other compounds, appears to be suitable for selective P450 solubilization (2–3-fold enrichment).

Cationic detergents, such as hexadecyl-trimethylammonium bromide or benzalkonium chloride, are not suitable for P450 solubilization because they either convert most of the cytochrome to the enzymatically inactive P420 form or lead to complete and irreversible damage. For the same reasons, octylglucoside, Tween-20 and N-dodecyl-N,N-dimethylpropane-sulfonate are not recommended [38]. In Table 2 those properties of the most suitable detergents are listed which have an impact on the chromatographic strategy.

6. Prefractionation procedures

Prefractionation procedures are applied prior to the subsequent enzyme separations for elimination of phospholipids and general enrichment of cytochromes P450. Different methods have been used for prefractionation of P450, including precipitation by PEG or ammonium sulfate, as well as chromatographic steps such as application of 8-aminooctyl-Sepharose.

Ammonium sulfate is used in concentrations of 40–50% for precipitation of cytochromes P450. Recovery of spectroscopically detectable P450 amounting to about 20% is low compared to other prefractionation methods [55]. The advantage of ammonium sulfate is that it is readily dialyzable. However, P450 recoveries with non-dialyzable PEG are usually in the range of 50 to 70% (Table 1). A similar degree of P450 recovery (45–60%) is obtained by chromatography on 8-aminooctyl-Sepharose [7,39,49,56]. The advantage of this method is that other components of the microsomal monooxygenase system such as cytochrome b_5 and NADPH–cytochrome P450 reductase can be isolated concomitantly [57]

Table 3
Efficiency of detergents for solubilization of microsomal membranes

Detergent	Yield (%)		Ratio P450/protein	Rat treatment	Ref.
	P450	Protein			
Sodium cholate					
0.4%	92	82	1.12	Phenobarbital	[37]
3.0%	95	92	1.03	Untreated	[41]
0.5%	97	62	1.56	Untreated	[35]
0.3%	71	72	0.99	Untreated	[35]
0.1%	35	51	0.69	Untreated	[35]
Renex 690					
1.5%	92	82	1.12	β-Naphthoflavone	[52]
Emulgen 911					
0.2%	98	82	1.20	Untreated	[53]
0.2%	85	51	1.67	Untreated	[54]
Lubrox PX					
0.8%	96	69	1.39	Untreated	[35]
0.4%	93	59	1.58	Untreated	[35]
0.2%	79	47	1.68	Untreated	[35]
CHAPS					
0.5%	83	43	1.93	Untreated	[35]
0.3%	63	20	3.10	Untreated	[35]
0.1%	40	21	1.90	Untreated	[35]

7. Properties of P450 enzymes used for separation

The possibilities to separate different proteins depend on their structural features and physicochemical properties such as size, surface characteristics and binding domains for specialized structures. For separation of cytochrome P450 species, size-exclusion chromatography will not be useful because of similar molecular masses of the various P450 enzymes which are in the range of 48 to $56 \cdot 10^3$ [47]. Most purification protocols for P450 enzymes include one or more ion-exchange chromatographic steps. In contrast to surface charges, other surface properties of P450 have been much less exploited for chromatographic strategies. Interaction with hydrophobic domains has been used to purify cytochromes P450 by chromatography on phenyl- or octyl-Sepharose [58–61]. The occurrence of accessible histidine residues at the protein surface allows fractionation of P450 enzymes by immobilized metal affinity chromatography [13,35,36,62,63]. Probably other surface properties or modifications such as protein phosphorylation can be exploited by this technique when different immobilized metal ions are applied [64]. In some cases special ligands for P450 enzymes have been immobilized on gel matrices for successful use in affinity chromatography. Relevant applications will be discussed in the respective section (Section 8.6). Separation of cytochromes P450 based on differences in p*I* has been done by isoelectrofocusing [14,65,66]. Chromatofocusing, the corresponding liquid chromatographic method, appears to have been applied for P450 purification in a few cases only [51,67–69].

8. Types of chromatography used for separation of P450 enzymes

8.1. LPLC, FPLC and HPLC

For separation of cytochromes P450, conventional low-pressure techniques (LPLC) are frequently used which in most cases apply only a few chromato-

graphic principles such as anion-exchange and retention on calcium phosphate–hydroxyapatite matrices [7,47,49,70,71]. The application of high-resolution HPLC methods for P450 fractionation, introduced by Kotake et al. [30], is limited and has been carried out in most cases in combination with low-pressure methods [39,56,61]. On a preparative scale, HPLC is sometimes used late in purification protocols for P450 [30,39,42,60,63,72–74]. Because of the higher resolution compared to LPLC, several HPLC/FPLC methods have also been applied as single separation step [59,75]. Generally, P450 recoveries are higher with the HPLC/FPLC techniques than with LPLC, although specific P450 contents are comparable. Some examples for successful purification of cytochromes P450 by HPLC are given in Table 4.

HPLC is also suitable as an analytical tool and has been applied for differentiation of P450 fractions obtained by conventional LPLC. The results are impressive with regard to P450 resolution. For example, Bornheim and Correia [33] analyzed DE-52 fractions of a broad, confluent peak by HPLC on a TSK–DEAE–3SW column and thereby resolved the cytochromes P450 of individual fractions into three well separated peaks. Similarly, Bansal et al. [32] could further resolve fractions from a DEAE–Sepharose column by anion-exchange HPLC (Anpac). A cytochrome P450 of phenobarbital treated rats purified by aminooctyl-Sepharose and DEAE–Sepharose and judged to be homogeneous by SDS-PAGE was further resolved into three distinct P450 species by anion-exchange HPLC [31].

Like HPLC, the medium-pressure FPLC has been applied for purification of P450 enzymes [56,58,63], to demonstrate differences between purified P450 enzymes [12] and to differentiate between P450 patterns in liver microsomes of animals treated with various inducers [13,34–36]. Optimized high-resolution chromatographic methods are suitable tools to investigate microheterogeneity of P450 species. Their application has often yielded unexpected results and shown the existence of multiple P450 forms with otherwise similar properties [12,13,30,60].

8.2. Ion-exchange chromatography (IEC)

There are few purification protocols for cytochromes P450 which do not include ion-exchange chromatography (see, e.g., the schemes for the isolation of P450a to P450k given by Ryan and Levin [47]). Weak and strong anion and cation-exchange resins have been used in low, medium and high-pressure liquid chromatography. From the wealth of available data some general rules can be deduced which are discussed in the following sections. Several parameters may influence the chromatographic resolution of cytochromes P450 on ion-exchange resins such as the density of charged residues on the gel matrix [76], gradient shape [35] and type of the eluting agent [34]. The most critical

Table 4
P450 recovery and specific P450 content of fractions obtained with several methods on HPLC/FPLC basis

Species	Inducer	Prefractionation	HPLC method	Recovery (%)	Specific P450 content (nmol P450/mg microsomal protein)	Ref.
Human	–	Protease	Chromatofocusing (Mono P)	47	11.5	[51]
Marmoset	TCDD	AO-Sepharose	Ion-exchange (Mono Q, Mono S)	0.6–9.3	9–16	[75]
Rat	PB	AO-Sepharose	IMAC (Chelating Superose)	33–66	14.3–15.1	[62]
Rat	PB	PEG and AH-Sepharose	Anion-exchange (DEAE-5PW)	8.3	8.56	[39]
Rat	3MC	PEG and AH-Sepharose	Anion-exchange (DEAE-5PW)	7.7	12.7	[39]

TCCD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxine; PB = phenobarbital; 3MC = 3-methylcholanthrene; AH-Sepharose = 6-aminohexyl-Sepharose; AO-Sepharose = 8-aminooctyl-Sepharose.

parameter, however, appears to be the detergent composition of the eluent.

Resolution of cytochromes P450 by ion-exchange chromatography is strongly dependent on the detergents present in elution as well as in equilibration buffers. Usually a combination of an anionic (sodium cholate in most cases) and a non-ionic detergent is used in LPLC with weak anion-exchangers such as DEAE–Sephadex, DEAE–Sepharose or DEAE–cellulose, whereas in cation-exchange chromatography non-ionic detergents are applied exclusively [47]. Dutton et al. [77] have shown that good resolution of cytochromes P450 in anion-exchange LPLC is also achieved in the presence of the zwitterionic detergent CHAPS. In contrast to weak anion-exchangers, strong binding resins with quaternary ammonium groups such as Mono Q do not allow the use of sodium cholate because of strong interactions between matrix and detergent. As a consequence, irreproducible elution patterns are obtained [34,35,78]. Cholate probably also affects retention of cytochromes P450 on weak anion-exchangers. For example, Ryan and Levin [47] report that resolution of P450 enzymes on DEAE–Sepharose is dependent on the volume of detergent-containing buffers used for column equilibration. The effect of different detergents on recovery and resolution of cytochromes P450 has been investigated for the strong anion-exchanger Mono Q [34,35] (Fig. 1). According to these studies, Emulgen 911 and Lubrol PX appear to be the most suitable detergents for this purpose. Emulgen 911 as sole detergent has also been chosen for P450 separation by weak anion-exchangers in HPLC [30–33,60,72].

High resolution of cytochromes P450 in ion-exchange chromatography can be achieved by application of medium and high-pressure chromatographic methods used either for analytical [13,32–35] or preparative P450 separations [30,39,56,58,60,61]. It has been shown that chromatographic resolution of cytochromes P450 on Mono Q can be remarkably increased by the introduction of elaborate segmented instead of simple linear salt gradients [35] (Fig. 2). Up to eight separated peaks including the pass-through fraction can be obtained in this way. Furthermore, cytochromes P450 present in the pass-through fraction bind quantitatively to the strong cation-exchanger Mono S, allowing further resolution into four P450-containing fractions by elution with step-wise NaCl gradients [35].

The conditions described by several authors for the separation of cytochromes P450 by conventional ion-exchange LPLC are very similar. Usually sample application and elution are performed at ambient temperature [47,49]. The stability of cytochromes P450 allows their elution as enzymatically active proteins under these conditions. Most commonly, Tris–HCl and potassium phosphate serve as buffer substances in low concentrations (10–20 m*M*) at slightly alkaline pH values (7.2–7.8). The presence of 20% glycerol is obligatory to maintain the activity of P450 enzymes. Further additives include detergents (usually 0.5% sodium cholate–0.2% Emulgen 911), EDTA and dithiothreitol. For elution, linear NaCl gradients are usually applied.

The efficiency of ion-exchange steps for P450 purification varies considerably. Whereas anion-exchange chromatography used in an isolation procedure for CYP2E1 increases the specific P450 content by a factor of 1.06 only [79], an increase from 1.67 to 11.0 nmol P450/mg protein is achieved in a protocol for purification of CYP2D1 [80]. Descriptions of the several available ion-exchange resins together with respective applications for P450 separation have been reviewed recently by Ryan and Levin [47] and are therefore not discussed here.

8.3. Hydroxyapatite

Hydroxyapatite is widely used in chromatography of cytochromes P450 for either separation of P450 enzymes or removal of detergents from the final P450 preparation. The mechanisms of protein retention and elution concerning hydroxyapatite are not as clear as for other chromatographic matrices. However, model studies have been performed with a variety of well-characterized proteins which allow limited predictions on the chromatographic behavior of an individual enzyme on hydroxyapatite [81,82]. According to these studies, cytochromes P450 which have p*I* values between 7.0 and 8.0 [14,65] might be eluted from hydroxyapatite by phosphate, fluoride and chloride, but not thiocyanate or calcium. However, there is no systematic study on the chromatographic behavior of cytochromes P450 on hydroxyapatite as a function of different ions. Instead, P450

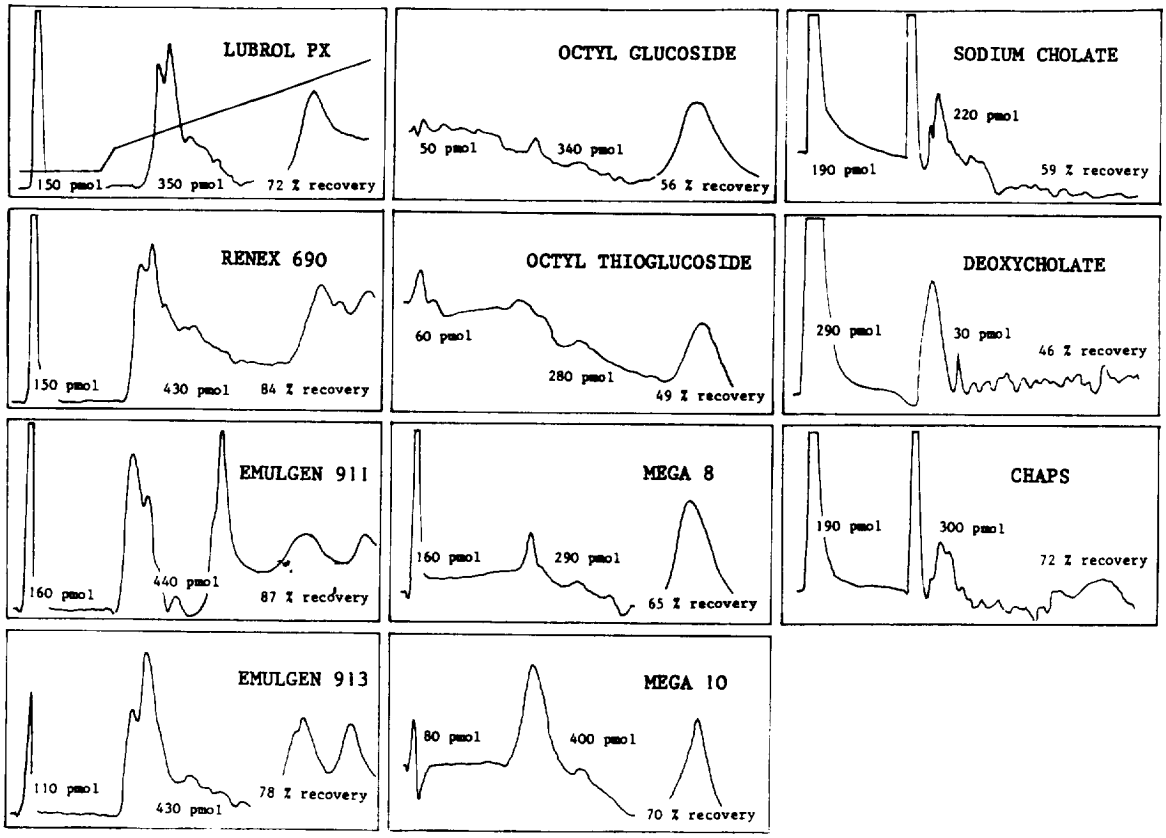

Fig. 1. Effect of various detergents in the eluent buffer on resolution. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, filtered through Sephadex G-75; sample size, 690 pmol P450=1.1 mg protein; column, Mono Q, 50×5 mm I.D.; flow-rate, 1 ml/min. Buffer A, 10 m*M* Tris–HCl (pH 7.7)–0.1 m*M* EDTA–20% glycerol–0.5% detergent; buffer B, 1 *M* NaCl in buffer A; gradient, 0–40–200 m*M* over 28 ml (see upper left profile); detection, 405 nm at 0.01 AUFS. P450 elutes throughout the chromatograms. Recovery data are a summation of the recoveries in several parts of the chromatogram (reprinted from Ref. [34]).

is generally eluted by step gradients of phosphate [39,47,75,83].

For P450 purification, hydroxyapatite has been applied in different stages of chromatography protocols. It served as an initial step for the isolation of P450j (=CYP2E1) and as the final step for purification of P450a (=CYP2A1) [47] (see Section 9.3, Table 6). In the latter case, this step concomitantly removes detergent from the preparation. For successful application of hydroxyapatite in P450 purification, detergent composition of the eluting buffer can be critical in some cases with the possible consequence of heme loss under improper conditions [45]. Furthermore, the source and quality of hydroxyapatite has remarkable effects on P450 recovery. This has been well documented in a study by Kastner and Neubert who determined capacity for and recovery of cytochromes P450 using hydroxyapatite from several manufacturers [75]. The variation in recovery ranges from 48 to 100% of applied P450. Some examples for successful application of hydroxyapatite are purifications of the rat P450 enzymes CYP1A1, CYP2A1, CYP2B1, CYP2B2, CYP2C6 and CYP2E1 by Ryan and Levin [47]. Remarkable increases in the specific P450 content by chromatography on hydroxyapatite (from 8.1 to 14.0 nmol P450/mg protein or 4.8 to 15.5 mol P450/mg protein) were achieved in the purification of CYP2E1 [79,84].

Detergent removal from P450 preparations by

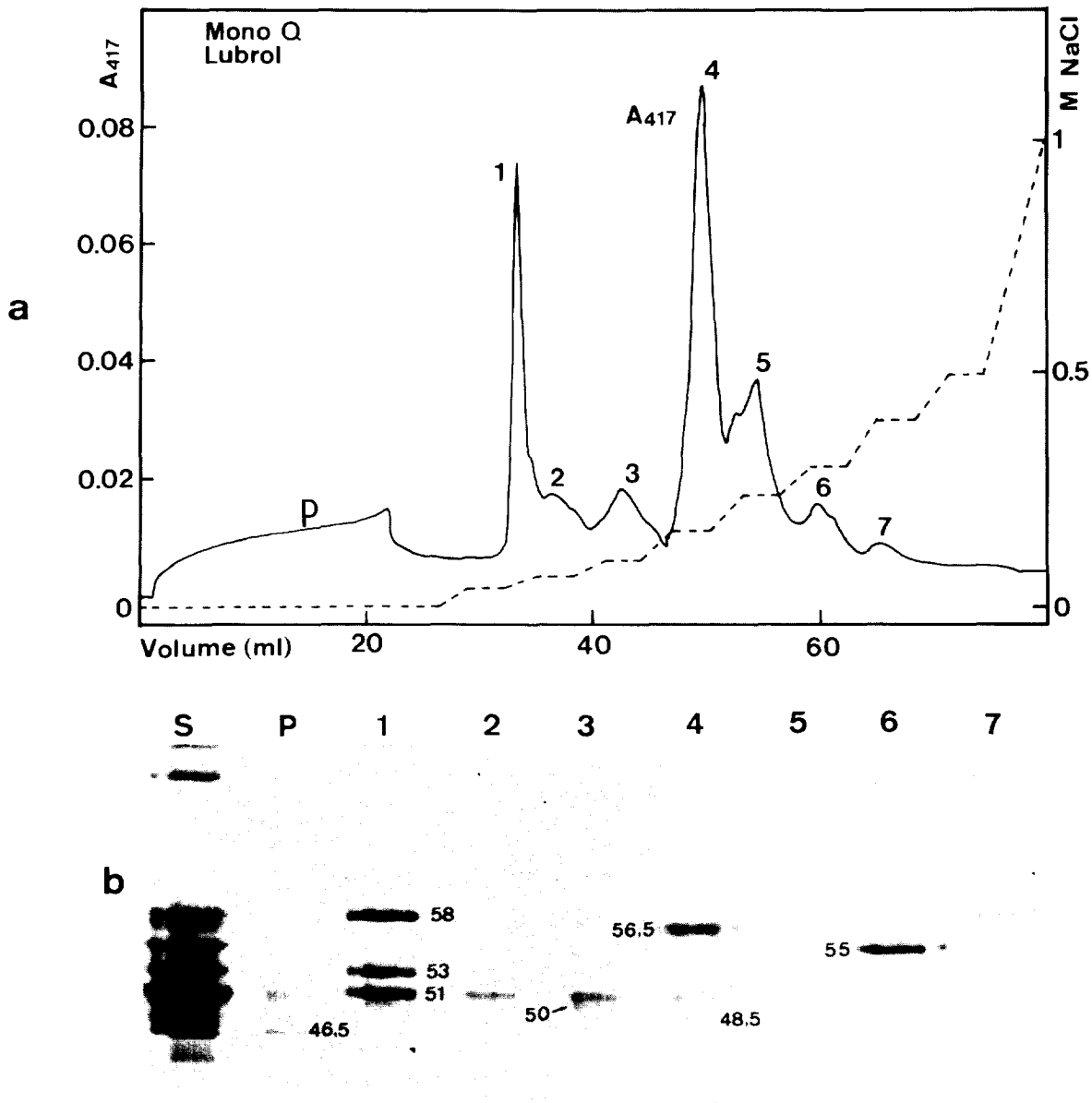

Fig. 2. Chromatographic resolution of P450 species by optimized gradient elution on Mono Q. Column, Mono Q HR 5/5; sample, Lubrol-solubilized liver microsomes of rats pretreated with phenobarbital; P450 content, 3.8 nmol. (a) Chromatogram showing absorption at 417 nm (solid line) and gradient shape (broken line). (b) SDS-PAGE of the Mono Q fractions, apparent molecular masses are assigned to individual bands. S, unfractionated sample; P, unbound material in the pass-through fraction (reprinted from Ref. [35]).

chromatography on hydroxyapatite has been investigated in detail by Kastner and Neubert [75]. They have shown that efficiency and P450 recovery are dependent on the flow-rate and the quality of hydroxyapatite. Funae and Imaoka [39] included sodium cholate at a low concentration (0.05%) to elute cytochrome P450 from hydroxyapatite after removal of Emulgen 911.

8.4. Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) has been used for separation and isolation of cytochrome P450 enzymes in a few cases only. This is probably due to the notion that P450 release from the immobilized metal results in loss of its enzymatic activity. Compared to other types of liquid chromatography, a greater number of variables has to be considered in an IMAC run. Their appropriate setting is decisive for successful binding and elution of cytochromes P450. After elaboration of suitable conditions [36,62], IMAC has been applied for analytical P450 separations [13,36], and for purification of enzymatically active cytochromes P450 which appeared homogeneous by SDS-PAGE and spectroscopic data [62,63]. Because this technique has never been reviewed in the context of cytochrome P450 and promises to be a useful tool for P450 separation, it will be discussed in more detail.

IMAC has been introduced for separation of proteins by Porath and co-workers in 1975 [85]. Since then there have been extensions for its application and IMAC has been widely used for purification of proteins and peptides [86–89]. IMAC exploits the ability of certain surface residues of proteins to interact with metal ions which are immobilized by chelation on a modified stationary phase. Proteins bind to immobilized transition metal ions, such as Cu^{2+}, Ni^{2+} and Zn^{2+}, preferentially by their histidine and to a lesser extent by their cysteine and tryptophan residues [85,90], whereas immobilized Al^{3+} interacts with phosphoryl groups [64]. Only the first type of interaction has been used in P450 research and will be considered here.

To adapt IMAC for separation of cytochromes P450 several operating parameters influencing binding and desorption of the sample have been investigated. These are binding capacity as a function of the immobilized metal ion, effect and strength of various eluents on desorption of spectroscopically intact P450, effect of pH on elution, effect of loss of metal ions by bleeding and prebleeding on column capacity and P450 yield, effect of buffer components on stripping-off of metal ions, effect of detergents on the elution profile and effect of the gradient shape (Fig. 3) on the resolution of P450 enzymes [35,36,62]. The results of these investigations can be

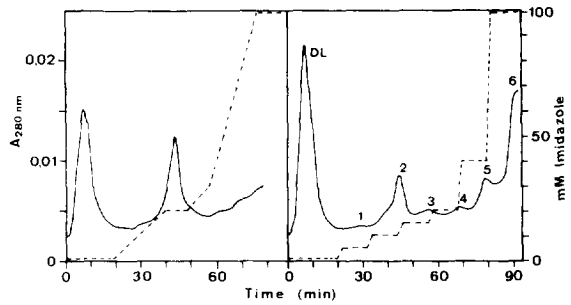

Fig. 3. Effect of the gradient form on the chromatographic resolution of microsomal proteins by IMAC. Gel, Chelating Sepharose Fast Flow charged with Ni^{2+}. Column dimensions, 1 cm diameter, 5.7 ml metal-ion-charged gel, 1.2 ml uncharged gel section. Equilibration buffer (A), 50 m*M* potassium phosphate (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; P450 content 0.86 nmol; protein content 0.51 mg. DL, pass-through fraction; 1–6, peak fractions. Solid line, absorption at 280 nm; broken line, gradient shape (reprinted from Ref. [36]).

summarized as follows: From the various examined metal ions, Ni^{2+} has been found most suitable for P450 chromatography because a high amount of cytochrome P450 (usually >80%) is bound to the stationary phase. Furthermore, appropriate elution results in a recovery of 30–80% of spectroscopically intact P450, depending on the type of eluent and the enzyme composition of the applied sample [13,62]. Suitable eluents for a nickel-charged column are imidazole and glycine. Apparently, the high affinity of immobilized copper for cytochromes P450 does not allow their elution in a spectroscopically or enzymatically active form. On the other hand, immobilized Zn^{2+} shows only a very low affinity for cytochromes P450 and is therefore not suitable for their chromatographic separation [13,62]. Thus, the affinity of cytochromes P450 for several immobilized metal ions follows the same order as observed for other proteins, i.e., $Cu^{2+}>Ni^{2+}>Zn^{2+}$ [91]. Some components and buffer substances such as EDTA or citrate must be absent in sample, washing or elution buffers because they strip off the immobilized metals efficiently. There are other compounds that should be avoided. Among them are some common organic buffer substances such as tris-(hydroxymethyl)-aminomethane (Tris), morpholinoethanesulfonic acid (MES) and N,N-bis-(2-hydroxyethyl)-2-amino-

ethanesulfonic acid (BES). They cause desorption of metal ions and compete with protein binding [36,91,92]. To circumvent these problems, phosphate buffered solutions are recommended which in addition result in narrower peaks as compared to Tris-buffered solutions [62]. Resolution of cytochromes P450 with an optimized IMAC system are shown in Fig. 3 and Fig. 4 (elution profile and SDS-PAGE of individual fractions).

Kastner and Neubert [62] used optimized IMAC for purification of a phenobarbital-inducible liver microsomal cytochrome P450 of Wistar rats. With an 8-aminooctyl-Sepharose pool as starting material they obtained a homogeneous P450 protein in one step by chromatography on immobilized Ni^{2+} and elution with glycine. It was identified as CYP2B1 by its molecular mass of $50–51 \cdot 10^3$, by immunoblotting with specific antibodies and by its reconstituted pentoxyresorufin *O*-depentylase activity.

IMAC was also successfully applied for the purification of a pig liver cytochrome P450 involved in a step of morphine biosynthesis, i.e., the ring closure reaction leading from (*R*)-reticuline to salutaridine [63]. In the purification procedure, IMAC is used in the final step after cation-exchange chromatography on S-Sepharose Fast Flow and results in an increase of the specific P450 content from 5.1 to 20.0 nmol P450/mg protein. The purified P450 is homogeneous by SDS-PAGE, shows an absorption maximum of the CO complex of 450 nm and specifically catalyzes the formation of salutaridine from reticuline in a reconstituted system.

Another application of IMAC using a Ni^{2+}/imidazole system has been the separation of closely related P450 enzymes of the CYP3A subfamily [13]. Starting with Lubrol-solubilized liver microsomes of dexamethasone-treated male rats, cytochromes P450 were separated by IMAC with an optimized stepwise imidazole gradient [36]. Subsequently, the separated P450 species were digested with V8 protease and the peptides analyzed by immunoblotting with specific antibodies for CYP3A after separation by SDS-PAGE. Different peptide patterns indicated the separation of closely related CYP3A species. Taken together, the limited studies concerning separation of cytochromes P450 by IMAC demonstrate the suitability of this technique to purify enzymatically active P450 enzymes and to resolve structurally related P450 enzymes.

8.5. Hydrophobic interaction chromatography

Two types of chromatography can be distinguished here with regard to separation of cytochromes P450, namely pure hydrophobic interaction chromatography (HIC) using phenyl or alkyl-substituted stationary phases and a mixed-type chromatography using 8-aminooctyl-, 6-aminohexyl- or N-lauryl-aminohexyl-derivatized Sepharose. These mixed-type procedures probably exhibit ion-exchange or ligand-binding mechanisms, respectively, in addition to the hydrophobic interactions. Before discussing any applications, an inaccuracy in the nomenclature of diaminooctane substituted Sepharose should be mentioned. Originally Imai and Sato [37] introduced 8-aminooctyl-Sepharose (AO-Sepharose) as an affinity matrix for purification of cytochromes P450. It is synthesized by coupling of 1,8-diaminooctane to CNBr-activated Sepharose. Unfortunately, this resin was subsequently referred to as

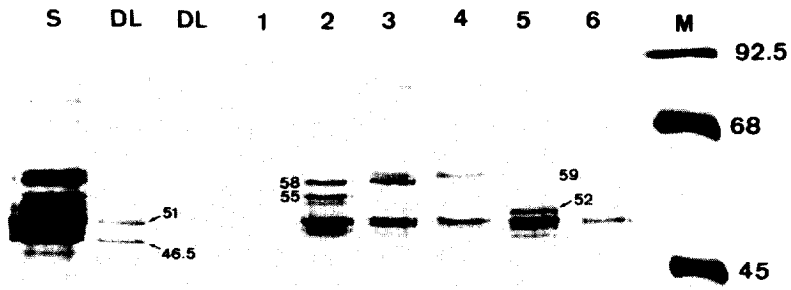

Fig. 4. SDS-PAGE analysis of IMAC fractions. For conditions and designation of fractions, see Fig. 3. S, unfractionated sample; M, molecular mass marker (kDa). Reprinted from Ref. [36]).

octyl-Sepharose by several authors although the latter material was also in use. *n*-Octyl-Sepharose 4B has been studied in parallel with 8-aminooctyl-Sepharose for its suitability in P450 separation. It was found that cytochromes P450, as well as cytochrome b_5 bind very strong to the former and that for achievement of good separation both the alkyl side chain and the terminal amino group of AO-Sepharose are essential [37]. The effect of the alkyl chain length on retention of cytochromes P450 has also been examined. 6-Aminohexyl-Sepharose is characterized by a lower capacity for P450 binding as compared to AO-Sepharose [37,78], whereas strong binding of cytochromes P450 to 10-aminodecyl-Sepharose impairs their elution [37].

Chromatography on AO-Sepharose is widely used as prefractionation step in P450 purification (see Section 6). In contrast, pure hydrophobic interaction resins have been applied in a few studies only. Phenyl-Sepharose has been successfully used for purification of different P450 enzymes. Kiffel et al. [59] have obtained a homogeneous P450 preparation by this method in one chromatographic step. The starting material was an ammonium sulfate precipitate of detergent-solubilized liver microsomes isolated from clofibrate-treated rats. The dissolved material was chromatographed on phenyl-Sepharose CL-4B by elution with a linear gradient of Lubrol running from 0.05 to 1%. The P450 fraction which eluted with 1% Lubrol was enzymatically active as lauric acid hydroxylase and showed a specific P450 content of 3.0 nmol/mg protein. This value is low for a homogeneous P450 and may be explained by overestimation of the protein content or by loss of the heme moiety, leading to an underestimation of cytochrome by spectroscopic quantitation [93]. In contrast, Kastner et al. [56] obtained a cytochrome P450 fraction from phenyl-Sepharose with acceptable recovery of the spectroscopically intact protein. As the last step in the chromatographic strategy of these authors, HIC removed a major contaminant of the Mono S fraction and increased the specific P450 content from 11.6 to 14.8 nmol/mg protein. In a subsequent paper, Kastner and Neubert [58] applied HIC on a FPLC basis for P450 purification with phenyl-Superose as stationary phase. This resin allows P450 separation in shorter times compared to conventional phenyl-Sepharose and yields more sharply defined peaks. Chromatographic resolution is improved by introduction of step gradient elution with Emulgen 911 [58].

8.6. Affinity chromatography

Because of its specificity, affinity chromatography can be used for the isolation of P450 enzymes with similar binding characteristics for a ligand such as orthologous proteins of different animal species or closely related isozymes. Furthermore, it can serve for group-specific prefractionation or as a final purification step of an individual enzyme. Two types of affinity ligands coupled to a stationary phase can be distinguished: (a) small molecules that interact with the substrate binding site and (b) proteins such as cytochrome b_5 or antibodies that bind to surface domains of the cytochrome P450 molecule.

8.6.1. Ligand-affinity chromatography

Several immobilized ligands have been exploited for purification of cytochromes P450 by affinity chromatography. Although some remarkable results have been obtained, this type of chromatography is usually not applied for P450 isolation. An exception is the widely used prefractionation for P450 enzymes via 8-aminooctyl-Sepharose which can also be considered as a type of ligand-affinity chromatography.

Remarkable results with respect to a minimum number of chromatographic steps and resultant highly purified P450 enzymes have been obtained with immobilized *p*-chloroamphetamine and adamantane-1-carboxylic acid [46,94]. *p*-Chloroamphetamine coupled to an N-hydroxysuccinimide activated resin (Affi-Gel 10) proved useful for the isolation of cytochromes P450 from tissues with low P450 contents such as rat prostate and brain with about 20 and 100 pmol P450/mg microsomal protein, respectively [46,95]. By the affinity step and subsequent anion-exchange chromatography on DE52 cellulose, Sundin et al. [46] obtained a homogeneous P450 preparation from rat prostate. Warner et al. enriched cytochromes P450 from rat brain by chloroamphetamine affinity chromatography [95]. In both cases, elution from the affinity matrix was achieved by a mixture of 0.5% sodium cholate and 0.2% Emulgen, indicating that binding of cytochrome P450 to the immobilized ligand is due to hydro-

phobic interactions. This is in accordance with the notion that the binding energy of cytochrome P450 ligand complexes is mainly based on the hydrophobic effect [96].

Detergent-solubilized cytochromes P450 from liver microsomes of phenobarbital-treated rats bind to a high degree to aminohexyl-Sepharose to which adamantane-1-carboxylic acid was coupled by carbodiimide activation. Recovery of cytochromes P450 by elution from this column amounts to 58% [94]. By two additional chromatographic steps (DEAE–Sephacel and DE32) a homogeneous P450 fraction with a specific content of 14.1 nmol/mg protein and a yield of 16% was obtained.

A more widely used affinity matrix is lauric acid immobilized on 6-aminohexyl-Sepharose as introduced by Gibson and Schenkman [54]. Apparently, this resin can be compared with 8-aminooctyl-Sepharose with regard to its efficiency and selectivity. However, differential elution of P450 enzymes with buffers of varying detergent and salt concentrations can be achieved by lauric acid affinity chromatography. For example, Gonzalez et al. [53] could resolve bound P450 enzymes into three fractions containing the following enzymes: (1) LAω (=CYP4A1) and PCN1 (=CYP3A1), (2) db1 and db2 (=CYP2D1 and CYP2D2) and (3) PB1 (=CYP2C6). As indicated by the investigations of Schenkman, N-lauryl-(6-aminohexyl)Sepharose is a group-specific adsorbent. It has been shown that cytochromes P450 bound to this matrix could be resolved subsequently into 14 different species by cation-exchange chromatography [97]. Some further applications of lauric acid affinity chromatography can be found in publications cited by Ryan and Levin [47].

8.6.2. Immunoaffinity chromatography

Detection of orthologous P450 enzymes in different animal species is possible with P450 antibodies because cross-reactivity is frequently observed. By this method several human P450 enzymes have been isolated using antibodies against the orthologous rat enzyme. For example, Wrighton et al. [98] purified the constitutively expressed and isoniazid- or ethanol-inducible human P450 by immunoaffinity chromatography with an antibody against rat P450j (=CYP2E1). In the same way the human ortholog of rat P450d (=CYP1A2) has been isolated [99]. However, polyclonal and even monoclonal antibodies often do not only recognize the P450 enzyme against which they were raised but also structurally related P450 species. This has been well documented in several studies [100,101]. Therefore, not only orthologous but also other evolutionary closely related enzymes may be isolated by immunoaffinity chromatography. Subsequent characterization of immunopurified P450, however, is complicated by the fact that the conditions necessary to dissociate the P450 antibody complex result in complete or partial loss of enzymatic activities [102–104]. This is also indicated by preferential elution of the inactive P420 species. As shown by Friedman et al. [48] some activity is retained by desorption of cytochrome P450 with 0.1 *M* glycine at pH 3.0.

In order to study the expression of different P450 species reacting with a defined antibody, initial adsorption of detergent solubilized microsomal P450 on an immunoaffinity column can be used. This method has been used by Sakai et al. [105] who studied the expression of three immuno-identical phenobarbital inducible P450 species (H, M and L). After elution from the affinity column with 3 *M* KSCN, the cytochromes P450 were subsequently analyzed by SDS-PAGE.

8.6.3. Protein-affinity chromatography

In the membrane layer microsomal cytochrome P450 interacts specifically with NADPH–cytochrome-P450 reductase and cytochrome b_5. The interaction domains on the proteins can be exploited in affinity chromatography. Immobilized cytochrome b_5 which has been isolated either after trypsin cleavage or detergent solubilization of membranes has been used for the purification of several proteins (see Section 10). However, the native b_5-protein is more suitable for P450 purification than the trypsin cleaved cytoplasmic domain which exhibits decreased binding efficiency for cytochromes P450 [106,107]. Immobilized NADPH–cytochrome P450 reductase has also been reported as affinity ligand for cytochromes P450 [108]. In analogy, mitochondrial cytochromes P450 have been purified by affinity chromatography on adreno-ferredoxin (adrenodoxin) Sepharose 4B [109].

8.7. Chromatofocusing

Although chromatofocusing might be a useful tool for separation of cytochromes P450 only a few applications are reported. Its special advantage for separation of membrane proteins is due to the ionic milieu during the chromatography, i.e., the low salt concentrations as compared to ion-exchange chromatography which counteract the aggregation of membrane proteins [78]. It has been shown that some purified P450 enzymes such as CYP2B1 and CYP2B2 can be further resolved into several enzyme species by isoelectrofocusing [14]. Their p*I* values range from 7.4 to 7.9, thus differing sufficiently for separation by chromatofocusing [68].

P450 separation by chromatofocusing has been used for different purposes, namely as an effective first [67] or last purification step [69], as an analytical tool to characterize P450 profiles [51] and as a means to distinguish closely related enzyme species [68]. The three forms of mitochondrial P450scc (CYP11A) separated by chromatofocusing did neither differ by their enzymatic activities or amino-terminal sequences, nor could any heterogeneity due to glycosylation be detected. The authors assume that post-translational modifications other than glycosylation are responsible for the differential chromatographic behavior [68]. Marriage and Harvey [110] resolved liver microsomal cytochromes P450 of untreated and phenobarbital-treated mice by chromatofocusing into 8 and 7 cytochromes P450, respectively.

9. Isolation of individual P450 enzymes

9.1. Induction of P450 enzymes

The P450 enzyme profile of the endoplasmic reticulum is variable and is influenced by a great number of xenobiotics. Selective induction of P450 enzymes can thus be exploited to obtain higher yields. One can distinguish three groups of P450 enzymes on the basis of their expression pattern in rat liver: (1) constitutively expressed enzymes which are not or only slightly inducible (2C7, 2C11, 2C12, 2C13, 2D1, 2D2); (2) constitutively expressed enzymes which are considerably increased by xenobiotics (1A2, 2A1, 2B2, 2C6, 2E1, 3A2, 4A1) and (3) enzymes which are not expressed constitutively or in negligible amounts only, but can be induced by xenobiotics (1A1, 2B1, 3A1). As starting material for the purification of enzymes of the first group, microsomes of untreated animals are used, whereas for isolation of inducible enzymes microsomes of pretreated animals are suitable. However, inducing agents are often not isozyme-specific, i.e., they lead to induction of several P450 enzymes and may possibly suppress others. For example, by treating rats with phenobarbital the microsomal content of CYP2B1 and CYP3A isozymes is markedly increased. Dexamethasone is also an efficient inducer of CYP3A and concomitantly causes suppression of CYP2C11. For an orientation, Table 5 gives the constitutively expressed levels of major P450 enzymes and their inducibility by several xenobiotics. The microsomal P450 enzyme profile also varies with respect to the sex. Often, however, the picture is not as clear as in the expression of the strictly male- or female-specific enzymes CYP2C11 and CYP2C12. Closely related, still poorly characterized P450 species can be differentially expressed in males and females [11,15]. Furthermore, cytochromes P450 are expressed tissue- or organ-specific. For example, CYP2C6, CYP2C7 and CYP1A2 have been found exclusively in the liver, whereas the expression of other cytochromes P450 appears to be restricted to special extrahepatic tissues, such as CYP2G1 of the nasal mucosa [23,67].

9.2. Detection of cytochrome P450 and identification of individual P450 enzymes

Routinely, elution of cytochromes P450 is followed by monitoring the absorption at 405 nm or 417 nm. Additionally, the absorption at 280 nm can be monitored if suitable non-absorbing detergents such as Lubrol PX (Table 2) are used in the buffers. The 417/280 ratio can give first hints on the purity of a P450 preparation. However, this method has a number of limitations. Firstly, cytochrome b_5, another hemoprotein of the microsomal fraction, is also detected at 417 nm. Secondly, P450 enzymes have different extinction coefficients at 417 (405) nm. Thirdly, P450 apoproteins can be present which

Table 5
Effect of various xenobiotics on the level of cytochrome P450 enzymes in liver microsomes of male rats (pmol P450/mg protein)

P450	Untreated		PB	DEX	PCN	TAO	3MC	INH	CLO	KEP
	Male	Female								
Total	900[l]	820[l]	1300[l]	1900[l]	1390[m]	4390[l]	2080[l]	1000[l]	1000[g]	1935[g]
1A1	26[c]		22[c]				1427[c]			
1A2	43[c]		44[c]				439[c]			
2A1	52[c]		131[c]				256[c]			
2B1/2	<10[l]	<10[l]	750[l]	380[l]						
2C6	196[k]		499[k]				131[k]			
	350[a]									
2C7	93[g]	130[g]	83[g]		50[g]	110[g]	48[g]	38[g]	31[g]	270[g]
2C11	280[d]	n.d.	190[d]	100[d]			130[d]			
2C12	<10[b,i]	350[i]	<10[b]							
2C13	86[g]	<1[g]	13[g]		100[g]	110[g]	26[g]	46[g]	34[g]	66[g]
2D1	110[h]	110[h]								
2E1	66[e]		37[e]		47[e]	42[e]	26[e]	310[e]	50[e]	
3A	110[l]	7[l]	315[l]	1250[l]		3430[l]	19[l]			
4A1	15[f]		10[f]				15[f]			

PB, phenobarbital; DEX, dexamethasone; PCN, pregenenolone 16α-carbonitrile; TAO, triacetyloleandomycin; 3MC, 3-methylcholanthrene; INH, isoniazide; CLO, clofibrate; KEP, kepone. References: [a] [111]; [b] [112]; [c] [113]; [d] [114]; [e] [154]; [f] [115]; [g] [116]; [h] [80]; [i] [83]; [k] [21]; [l] [117] and Roos (unpublished data); [m] [118]. n.d.=not detected.

do not absorb at this wavelength. Apparently, some P450 enzymes are susceptible to heme loss [47].

For qualitative and quantitative analysis of P450-containing chromatographic fractions, several methods have been used. Their advantages and limitations will be discussed briefly. Collective quantitation of cytochrome P450 enzymes can be done spectroscopically after formation of a CO-complex of the P450 sample reduced with sodium dithionite [93]. The advantage of this method is that all P450 enzymes are involved. Complex formation, however, is not achieved with apo-cytochromes P450 and P450 enzymes whose ligand binding sites are blocked by a metabolic intermediate. The latter is the case, for example, after treating rats with triacetyloleandomycin or isosafrole. These substances effectively induce CYP3A isoenzymes and CYP1A2 and concomitantly convert them to stable enzymatically inactive P450 metabolite complexes in vivo [119,120]. Dissociation of the metabolite complexes can be achieved by oxidation with potassium ferricyanide. Due to its stability, complete dissociation of the CYP1A2 complex with isosafrole requires conditions which lead to partial denaturation of the enzyme [120].

Identification of individual P450 species or differentiation between several P450 enzymes in a chromatographic fraction requires more specific methods. However, the required specificity of a method or a reagent with respect to an individual P450 species is frequently not achieved. For example, antibodies, even monoclonal ones, often cross-react with several P450 enzymes [101,121]. Substrate and product specificity of enzymatic reactions may also overlap [122,123]. For monitoring the purification of P450 enzymes, immunochemical [41,124] and enzymatic analyses [63,80] are rarely applied. In this context, a comment is given concerning the term 'recovery' for purification of P450 enzymes. In most cases, recovery is determined spectroscopically by the P450 CO complex [93], i.e., it is related to the total of P450 enzymes and not to the enzyme under consideration. Thus, the recovery values obtained in this way are always underestimates with respect to an individual P450 enzyme. Real recovery can only be determined by quantitation of the respective P450 enzyme in chromatographic fractions by specific immunochemical, enzymatic or spectroscopic methods.

For analytical purposes, such as detection of

closely related P450 isozymes after fractionation on highly resolving gel matrices in HPLC or FPLC, immunoblotting proved to be very suitable [13,36]. It can be conducted either with the native proteins or with peptide fragments after proteolysis and subsequent separation by SDS-PAGE [13]. By the latter method structurally related P450 enzymes can be distinguished as has been shown for liver microsomal CYP3A enzymes of the rat [15]. Two distinct CYP11B enzymes from rat adrenal mitochondria were also recognized after separation on octyl-Sepharose by their peptide patterns [125].

Analysis of chromatographic cytochrome P450 fractions can also be performed by further resolution of their components using ion-exchange FPLC on Mono Q. By this approach, Sakaki et al. [12] recognized several 3-methylcholanthrene inducible P450 species which were otherwise indistinguishable by enzymatic, gel electrophoretic and spectrosopic methods. To summarize, a number of general (CO complex), group (immunoblot with native enzymes) or isozyme-specific detection methods (immunoblot of proteolytic fragments, anion-exchange HPLC) for P450 enzymes are available. Which type of detection system is applied depends on the experimental goals, e.g., the purification of a single enzyme or recognition of closely related isozymes

9.3. Separation protocols for selected enzymes

9.3.1. P450a to k, slight variations in separation strategies

Purification protocols of the cytochrome P450 species a to k isolated in the laboratory of Ryan and Levin have been summarized recently [47]. Table 6 gives an overview of the consecutive chromatographic steps for the various P450 enzymes. It can be seen that only a few chromatographic principles are applied, i.e., ion-exchange chromatography, absorption on calcium phosphate matrices and in a few cases immuno-affinity chromatography. Most of the numerous purification protocols for cytochromes P450 published so far are based on these methods which are applied in varying orders. However, the sequence of chromatographic steps may have a significant influence on the resolution of P450 enzymes. It has been shown that the capability of a column to separate an individual P450 enzyme is influenced by the presence of other cytochromes P450 [75,78].

Table 6
Consecutive chromatographic steps (from top to bottom) for the isolation of cytochrome P450 enzymes according to Ryan and Levin [47]

P450*	a	b	c	d	e	f	g	h	i	j	k
CYP**	2A1	2B1	1A1	1A2	2B2	2C7	2C13	2C11	2C12	2E1	2C6
Treatment	Aroclor	Aroclor	Aroclor	Isosafrole	Aroclor	–	–	–	–	Isoniazid	PB
Sex	Male	Male	Male	Male	Male	Male	Male	Male	Female	Male	Male
Prefraction.	PEG	PEG	PEG	AS	PEG	–	–	–	–	OA-Seph	OA-Seph
$Ca\text{-}PO_4$				+							
DEAE				+							
DE52	+	+	+	+	+	+	+	+	+		
DE53	+					+	+	+	+		
DEAE			+		+						
HA	+	+	+		+					+	+
DEAE										+	
DE51,52,53											+
CM-Seph						+	+	+	+	+	
Phosphocell								+		+	+
Immuno				+	+	+					+
$Ca\text{-}PO_4$		+		+	+	+	+	+	+	+	+

Samples are cholate-solubilized rat liver microsomes. *P450 nomenclature of Ryan and Levin [47]; **modern P450 nomenclature [9]. PB, phenobarbital; PEG, poly(ethylene glycol); AS, ammonium sulfate; OA-Sepharose, 8-octylamino-Sepharose; $Ca\text{-}PO_4$, calcium phosphate matrix; HA, hydroxyapatite; DE51, 52, 53, application of DEAE–cellulose DE51, DE52 and DE53 in tandem; CM-Seph, CM-Sepharose; Immuno, immunoaffinity step; Phosphocell, phosphocellulose.

9.3.2. CYP2A2 and the importance of the monitoring system

By means of an affinity purified antibody against P450a(=CYP2A1) Arlotto et al. [124] detected two P450 enzymes in microsomes of untreated male rats which were structurally related to P450a. Using immunoblotting to monitor the purification process, these enzymes, named P450m and P450n, were isolated by LPLC. Otherwise, the purification strategy for P450m is similar to that of P450a as described by Ryan et al. [126] (Table 6). Because neither anion-exchange chromatography on DE 53 nor subsequent fractionation on hydroxyapatite resulted in complete separation of P450a and P450m, an additional CM-Sepharose step was introduced. On this cation-exchanger the two P450 enzymes behave very different. Whereas P450m is found in the pass-through fraction or is eluted with low NaCl concentrations, P450a is tightly bound to the resin. Further characterization of purified P450m, now termed CYP2A2, revealed great similarity to CYP2A1 with regard to the amino-terminal sequence and enzymatic activities. The successful purification of P450m is a good example to show that the monitoring method can be as important for P450 separation as the chromatographic procedure itself

9.3.3. CYP3A and isozyme multiplicity

CYP3A enzymes were discovered as the major P450 components in rats treated with pregnenolone 16α-carbonitrile [127] and were later isolated from liver microsomes of rats treated with various inducers such as triacetyloleandomycin and dexamethasone [128–130]. Such microsomes contain high amounts of CYP3A enzymes and are thus a suitable source for their purification. For example, treating rats with triacetyloleandomycin can increase the liver microsomal P450 content to about 4.5 nmol/mg protein, with CYP3A making up about 78% of total P450 [117] (Table 5). CYP3A enzymes have been isolated using different purification protocols [128,131,132]. Thereby, homogeneous P450 fractions as estimated by SDS-PAGE are obtained. However, it has been shown later that the apparently homogeneous enzymes can be further resolved into enzyme species with slightly different properties as regards amino-acid sequence, electrophoretic mobility and peptide cleavage pattern [118,129,130]. For example, from a fraction previously reported to be homogeneous [132] three different but very similar P450 enzymes were purified by anion-exchange chromatography on DEAE–Sephacel [118,129]. Later, four members of the CYP3A subfamily (6β_1–6β_4) were recognized and purified by Nagata et al. [130]. They used liver microsomes of phenobarbital- and dexamethasone-treated rats and, after prefractionation on 8-aminooctyl-Sepharose, applied either a combination of anion- and cation-exchange chromatography (for 6β_1 and 6β_3) or fractionation on hydroxyapatite in combination with ion-exchange methods (for 6β_2 and 6β_4). The diversity of CYP3A enzymes on the protein level is also found on the genomic level as shown by recognition of several distinct genes [11,133,134]. Enzyme multiplicity in the CYP3A subfamily, however, may even be greater as indicated by chromatographic resolution obtained in our laboratory with immobilized metal affinity chromatography [13,36]. As shown in Fig. 5, several CYP3A-containing fractions can be obtained by fractionation of solubilized microsomes from phenobarbital-treated rats. Subsequent detection by immunoblotting allows specific recognition of CYP3A

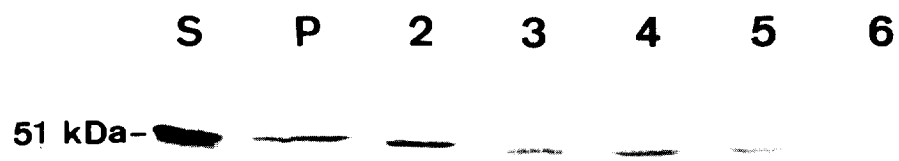

Fig. 5. Immunochemical identifcation of CYP3A enzymes separated by metal chelate affinity chromatography. Column, Chelating Sepharose Fast Flow, 80×10 mm; immobilized metal ion, Ni^{2+}. Sample for chromatography, Lubrol-solubilized liver microsomes of phenobarbital-treated male rats. Buffers and shape of imidazole gradient as described in Ref. [36]. The unfractionated sample (S), the pass-through (P) and several peak fractions (2–6) were analyzed by SDS-PAGE and subsequent immunoblotting with a CYP3A-specific polyclonal antibody. Protein content of the samples used for SDS-PAGE, 1 μg; S, unfractionated Lubrol-solubilized microsomes.

Table 7
Chromatographic methods for the purification of enzymes from the cytochrome P450 subfamilies 2D, 2G, 2H, 4A and 4B

P450 enzyme (species, tissue)	Notes on the separation strategy (sequence of steps)	Ref.
2D (rat, liver)	N-Lauryl-aminohexyl-Sepharose, CM-Sepharose, DEAE–cellulose	[53]
2G (rat, nasal mucosa)	PEG, Polybuffer exchanger, CM-Sepharose, S-Sepharose, HA	[67]
2H (chicken, liver)	Octyl-Sepharose, TSK–DEAE–cellulose, HA	[60]
4A (rat, liver)	AO-Sepharose, HA, DEAE–Sephacel, CM-Sephadex	[135,136]
4B (rat, lung)	OA-Sepharose, DEAE–5PW, HA	[61]

AO-Sepharose, 8-aminooctyl-Sepharose; OA-Sepharose, *n*-octylamino-Sepharose; HA, hydroxyapatite.

enzymes and reveals enzyme species with slightly differing electrophoretic mobilities. Further differentiation of the separated enzymes can also be done by immunoblotting after proteolytic digestion [13]. As in the isolation of P450m (see above), the monitoring system plays a decisive role in the recognition of chromatographic resolution.

9.3.4. Further P450 enzymes and further chromatographic methods

Members of some P450 families and subfamilies have not yet been dealt with. Published strategies for their separation will not be discussed here, except when uncommon methods were applied for their purification. However, brief notes are given in Table 7 on the separation of some further P450 enzymes.

For the isolation of CYP2G from rat nasal mucosa, primary fractionation by chromatofocusing has been applied successfully [67]. This technique was also used for separation of cytochromes P450 from human liver biopsy material, which also requires handling of small P450 quantities [51]. Separation strategies for cytochromes P450 which include uncommon chromatographic methods such as metal chelate affinity, protein affinity and hydrophobic interaction chromatography have been discussed already in Section 8.4, Section 8.5 and Section 8.6.

10. Purification and chromatographic properties of cytochrome b_5

Besides cytochromes P450, microsomal membranes contain another hemoprotein, cytochrome b_5. This redox component is involved in several reactions, such as desaturation and elongation of fatty acids and in prostaglandine synthesis. Furthermore, it is connected to the monooxygenase system because it may transfer an electron to cytochromes P450 acting in a synergistic way together with NADPH–cytochrome P450 reductase. It has been shown in vitro that electron transfer via cytochrome b_5 increases enzymatic activities of some P450 enzymes [130]. Cytochrome b_5 itself is reduced by a specific membrane-bound NADH-dependent reductase (for purification of this component, see Ref. [137]).

Because several structural and chromatographic aspects of cytochrome b_5 have been studied in detail, it appears especially suitable for chromatographic considerations. Different chromatographic principles such as anion-exchange chromatography, HIC and IMAC were applied for isolation of cytochrome b_5 (see Table 8). HPLC separation of proteolytic cytochrome b_5 peptides has also been performed [138]. Detailed knowledge of its three-dimensional structure [139,140] including position and accessibility of histidine residues [141] is important for the understanding of its behavior in immobilized metal affinity chromatography. Moreover, the wild type and some mutant cytochrome b_5 forms have been used in a model study for proteins in anion-exchange chromatography [142,143]. With these informations, the relationship between structure and chromatographic behavior of cytochrome b_5 can be discussed. Furthermore, cytochrome b_5 itself has been used as immobilized ligand in affinity chromatography.

The liver microsomal content of cytochrome b_5 is in the range of 0.3 to 0.9 nmol/mg protein and is not influenced considerably by treatment with a variety of P450 inducers [83,144–147]. However, intraperitoneal application of $CoCl_2$ results in a dramatic decrease of the microsomal cytochrome b_5 content to

Table 8
Chromatographic conditions for desorption of cytochrome b_5 from various stationary phases

Stationary phase	Eluting conditions	Ref.
Rat		
Aminooctyl-Sepharose	50 m*M* K-phosphate, pH 7.25–20% glycerol, *0.15% sodium cholate, 0.35% sodium desoxycholate*	[57]
DEAE–cellulose	*150 mM Na-phosphate*, pH 7.2	[148]
DEAE–cellulose	10 m*M* Tris-acetate, pH 7.4–20% glycerol, 1 m*M* EDTA, *300 mM KCl*	[149]
DEAE–cellulose	10 m*M* Tris-acetate, pH 8.1, *90 mM NaSCN*	[137]
Anpac anion-exchange	20 m*M* Tris-acetate, pH 7.2–0.2% Emulgen 911–20% glycerol, *400–800 mM Na-acetate*	[39]
Mono Q	20 m*M* Tris–HCl, pH 7.7–20% glycerol, 0.2% Lubrol PX, *240 mM NaCl*	[35]
Chelating Sepharose, Ni^{2+}	In pass-through	[13,36]
Phenyl-Sepharose	25 m*M* Tris-acetate, pH 8.1, *0.001 to 0.01% Triton X-100*	[137]
Hydroxyapatite	10 m*M* Tris–HCl, pH 7.4, *500 mM K-phosphate*	Roos (unpublished)
Chicken		
TSK–DEAE–5PW	10 m*M* Tris-acetate, pH 7.5%–20% glycerol, 0.4% Emulgen 911, *240–800 mM Na-acetate*	[60]
Mortierella hygrophila (Mycophyta)		
Mono Q	100 m*M* Tris-acetate, pH 8.1–15% glycerol, 0.3% Triton X-100–1 m*M* EDTA, *140–160 mM NaSCN*	[150]

The eluting components are italicized.

about 20% compared to that of untreated rats [146]. Thus, liver microsomes of rats which are either untreated or treated with P450 inducers are an equally suitable source for cytochrome b_5.

The procedure for the isolation of cytochrome b_5 may depend on the further experimental purposes. Thus, methods have been developed for purification of cytochrome b_5 concomitant with either cytochromes P450, NADPH–cytochrome-P450 reductase or NADH–cytochrome-b_5 reductase [57,137,144]. In earlier studies, cytochrome b_5 has been dissociated from the microsomal membrane by cleavage with proteases, leaving behind the membrane binding domain of the protein [144]. However, the native protein can be isolated after solubilization of microsomes with detergents. Inclusion of phenylmethylsulfonylfluoride (PMSF) during the purification is necessary. Otherwise, cleavage of the carboxy-terminal domain may occur at amino acids 95 (serine) or 97 (threonine) [138].

Purification of cytochrome b_5 usually starts with a prefractionation procedure. If concomitant isolation of cytochromes P450 and NADPH–cytochrome-P450 reductase is desired, a prefractionation on ω-aminooctyl-Sepharose is recommended. After elution of cytochromes P450, epoxyde hydrolase and P450 reductase, the still bound cytochrome b_5 can be eluted with 0.15% sodium cholate–0.35% sodium desoxycholate [57]. This fraction is more than 90% pure, as estimated by SDS-PAGE. Most of the remaining contaminants can be separated by an additional chromatography on a strong cation-exchanger (Q-Sepharose, Mono Q) with a stepwise NaCl gradient as described [35]. After a NaCl step of 160 m*M*, cytochrome b_5 is eluted with 20 m*M* Tris–HCl, pH 7.4–240 m*M* NaCl–0.2% Emulgen 911.

Recently, a method for rapid isolation of cytochrome b_5 has been developed [137] including prefractionation with PEG and subsequent chromatography on DEAE-cellulose and phenyl-Sepharose. By this method, the cytochrome is purified about 125-fold with a specific content of 50 nmol/mg protein and a yield of 22% (rat) or 32% (rabbit). To obtain highly purified cytochrome b_5 application of purification steps in addition to the routine pro-

cedures can be necessary. For these purposes, eluting conditions for various stationary phases are listed in Table 8. A combination of these methods which are based on different chromatographic principles might prove useful in eliminating contaminants.

The behavior of cytochrome b_5 in IMAC is especially interesting in view of its known surface structure. As mentioned above, Ni^{2+} ions immobilized on Sepharose derivatized with iminodiacetic acid preferentially bind histidine residues within proteins. According to Sulkowski [86], the retention of proteins on iminodiacetic acid–Me^{2+} columns reflects the number of accessible surface histidine residues. Hemdan et al. [151] found that at least two histidines are necessary for protein binding to a nickel-charged column, while for binding to a Cu^{2+}-charged gel a single histidine residue is sufficient. Interestingly, rat cytochrome b_5 with six histidine residues does not bind to the Ni^{2+}-charged gel. Its presence in the pass-through fraction can be shown spectroscopically by the method of Omura and Takesue [144] and by immunoblotting (see Ref. [13]; and Roos, unpublished results]. Judged from the three-dimensional structure of calf-liver cytochrome b_5 [139] and from sequence comparison with the orthologous rat enzyme [138], at least four histidine residues appear to be exposed at the protein surface. In the rat protein His-39 and His-63 are coordinated to the heme iron. From the four remaining histidines, His-26 appears to be the most exposed at the protein surface, whereas His-15 and His-80 are involved in intramolecular hydrogen bonding and His-27 interacts with the carboxy-terminal segment [141]. Thus, it appears that only His-26 is able to participate in complex formation with the immobilized Ni^{2+} which is not sufficient for a stable binding to the stationary phase. Binding, however, may be achieved on a Cu^{2+}-charged column. Among the cytochromes P450 the ethanol-inducible P450 2E1 with twelve histidines is also preferentially found in the pass-through fraction [13,36]. Judged from these results, the chromatographic behavior of membrane proteins in IMAC does not seem to conform to the rules established for soluble proteins. One explanation for this may be that the interaction of the metal-chelate with surface histidine residues of membrane proteins is partly abolished, possibly by bound lipids or detergents.

Using a heterologously expressed truncated form of cytochrome b_5, a model study for the retention behavior of a protein in ion-exchange chromatography has been performed by Roush et al. [142]. This form of cytochrome b_5 missing the membrane anchoring region has been chosen to minimize hydrophobic interactions with the stationary phase in order to approximate an idealized model in which coloumbic forces mainly contribute to the observed retention. Data analysis allowed the calculation of the apparent number of binding sites in the contact region as a function of protein concentration and temperature. Further studies with cytochrome b_5 mutants showed that a cluster of negatively charged glutamate residues (E41, E42, E47, E48) mainly determines the binding affinity of cytochrome b_5 to anion-exchange resins. Transitions from glutamate to glutamine obtained by site directed mutagenesis result in a disproportionally large decrease in binding affinity when located within the glutamate cluster [143].

As has been shown in several studies cytochrome b_5 itself can be used as an affinity ligand covalently attached to a stationary phase. For this purpose, either the cytoplasmic domain or the native protein including the membrane binding region can be used. Coupling is performed with cyanogen bromide-activated Sepharose 4B [152]. Proteins purified via cytochrome b_5–Sepharose include linoleoyl-CoA desaturase [153], Δ^7-sterol 5-desaturase [106], 4-methyl sterol oxidase, NADH–cytochrome b_5 reductase and a number of cytochromes P450 among them CYP2C7 [107].

11. Concluding remarks

Separation and purification of cytochromes P450 by liquid chromatography is an important tool to identify and study the expression of the numerous P450 enzymes. Neither quantitative determinations of mRNA levels nor enzymatic analyses can substitute these investigations. The presence of a distinct P450 mRNA species is no proof for the expression of the respective P450 protein, whether or not it is enzymatically active. On the other hand, enzymatic activities often do not allow discrimination of closely related P450 species. Application of chromatograph-

ic techniques with high resolution have brought up the existence of P450 species with slightly differing properties. However, in most cases, it remains to be elucidated whether these P450 forms constitute distinct gene products or proteins that are post-translationally modified by phosphorylation or glycosylation [12,13,36]. Progress in chromatographic separations and detailed analysis of the separated P450 species will shed light on the observed microheterogeneity of cytochromes P450 which might be connected to fine tuning of the P450 enzymatic activities or regulation of P450 degradation.

12. List of abbreviations

AO	8-Aminooctyl
DEAE	Diethylaminoethyl
FPLC	Fast protein liquid chromatography
HPLC	High-performance liquid chromatography
IMAC	Immobilized metal affinity chromatography
LPLC	Low-pressure liquid chromatography
PEG	Poly(ethylene glycol)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Acknowledgments

The author thanks Prof. Dr. W.G. Hanstein (Bochum) for critical comments on the manuscript and Mrs. D. Strotkamp for the kind permission to reprint immunoblotting results from her master thesis (Fig. 5).

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