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High-performance metal chelate affinity chromatography of cytochromes P-450 using Chelating Superose

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ABSTRACT

High-performance metal chelate affinity chromatography [immobilized metal ion affinity chromatography (IMAC)] using Chelating Superose (iminodiacetic acid adsorbent) was investigated for its suitability in purifying phenobarbital-induced rat liver microsomal cytochrome P-450 isozymes (P450) and optimized for preparative purposes. Starting with an 8-aminooctyl-Sepharose fraction of partially purified P450, it was found that only Ni^{2+}- and Cu^{2+}-charged columns could bind P450. No binding was ever observed when Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Fe^{3+}, Fe^{2+} or Tl^{3+} ions were employed. Of eight commonly used elution buffers, imidazole and tryptamine were found to cause some denaturation of P450. For desorption of proteins bound to Ni^{2+}-charged columns, the following order of decreasing elution buffer strength was determined: cysteine $\approx$ histidine > glycine > histamine > tryptophan > ammonium chloride. During protein desorption with some of these buffers, metal ions were found to bleed from the gel, resulting in P450 denaturation. This could be eliminated by prebleeding the charged columns prior to sample application and had an effect on product recovery and homogeneity. Ni^{2+} and glycine were chosen as a standard for further optimization involving sample adsorption conditions as influenced by equilibration buffer, detergent, load capacity and flow, gradient and temperature conditions. In this way, potassium phosphate (pH 7.75) and 0.4% Emulgen 911 were used to equilibrate a 1.6-ml column and purify 20–50 nmol of P450 (5–15 mg of protein) within 15 min. One gradient fraction consisted of a single sodium dodecyl sulphate–polyacrylamide gel electrophoresis band as judged by silver staining and represented about 25% of the total P450 applied to the column; total recoveries were usually more than 80%. Comparison with the molecular weights and spectral, catalytic and immunological properties of P450 forms isolated according to established procedures indicated that the form isolated here using Chelating Superose comprises mainly P450 2B1 (PB-B). A method is described for fully automated, programmable column regeneration and sample runs.

INTRODUCTION

The history and principles of high-performance metal chelate affinity chromatography [immobilized metal ion affinity chromatography (IMAC)] have been dealt with in recent reviews [1–3] and will not be discussed here. To our knowledge, the use of this type of chromatography in separating forms of cytochrome P-450 isozymes (P450) for preparative purposes has not been reported. IMAC has, however, recently been examined by Roos [4,5], who employed Chelating Sepharose Fast Flow on an analytical basis for fractionating these enzymes. Today, endeavours to isolate preparative amounts of P450 may still be encountered, however, for example in *in vitro* testing of drugs [6] and in characterizing P450 forms derived from hitherto unstudied animal species [7]. More often than not, classical purifications involving, for example, ion-exchange and hydroxyapatite gels, even in fast protein liquid chromatographic (FPLC) or high-performance liquid chromatographic (HPLC) systems, result in poor yields, inhomogeneous products or both. For this reason, we considered IMAC as a possible approach to this task, and attempted to optimize systematically a number of parameters affecting this method. For a new protein, we expect that, as in any affinity chromatography, a certain amount of empiricism will be unavoidable.

In view of the fact that each run necessitated a column regeneration involving sequential treatment with up to seven different solutions, an increased number of FPLC pumps and valves were utilized, and the automation of this is described.

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EXPERIMENTAL

Sample preparation

An 8-aminooctyl-Sepharose pool of combined P450 forms derived from phenobarbital-induced male Wistar rats was prepared as described [7] and kept at −80°C until further use. Prior to IMAC, salt removal and detergent exchange were effected by gel filtration on HR 10/10 columns of Sephadex G-25 Superfine or disposable NAP-25 columns (Pharmacia, Uppsala, Sweden). The buffer used for gel filtration elution was always the same as the start buffer described below. Samples for IMAC usually consisted of 2–5 nmol of P450. The specific content of this particular sample lot was 3.4 nmol of P450 per milligram of total protein.

Chemicals

All substances were of the highest purity commercially available. Imidazole was recrystallized once from toluene and then once from ethanol. Tryptamine was recrystallized twice from ethanol.

FPLC

All parts of the equipment necessary for all runs were purchased from Pharmacia. Chelating Superose, consisting of the chelating group iminodiacetic acid (IDA) covalently linked to a Superose 12 base matrix of 13-μm particle size, was used as supplied in a prepacked HR 10/2 (20 mm × 10 mm I.D., 1.6 ml) glass column. Chromatography was carried out as described for this column by the manufacturer and necessitated the following steps for each single run.

(a) Regeneration by successive washings with 6 ml of 40% acetic acid, 6 ml of 500 m*M* NaCl in water (pH 5–6), 4 ml of 200 m*M* metal chloride (usually nickel chloride, see below) in water (pH 5–6) and then 8 ml of water (pH 5–6). When a different metal ion was to be charged, the regeneration procedure was preceded by a wash with 16 ml of 50 m*M* EDTA and 500 m*M* NaCl in water (pH 5–6).

(b) Equilibration with 6 ml of buffer A: 50 m*M* start buffer [usually potassium phosphate (pH 7.5)], 20% glycerol and 500 m*M* NaCl. Detergents were always present and consisted in most instances of 0.4% (w/v) Lubrol PX (Sigma, St. Louis, MO, USA). Prebleeding (see below) consisted of a wash with usually 2 ml of buffer B (elution buffer as given in Table I) at a concentration of 50–500 m*M* in buffer A, and then 6 ml of buffer A alone.

(c) Sample load and injection via motor valve and 2-ml sample loop.

(d) Sample run. After the pass-through fraction had eluted, a gradient of buffer B in buffer A was applied over a volume of 20 ml. Detection was at 280 and 405 nm simultaneously and the flow-rate was (in most instances) 2.0 ml/min. The temperature was 25°C and fractions of 1 ml were collected in a rack filled with ice.

Automation

Full automation of all steps outlined above from the beginning of column regeneration to the end of fraction collection was facilitated by integrating the following accessories: a third high-pressure pump (P-500), a 15-pole 24-V relay switch-box, a peristaltic pump (P-1) and three motor valves (MV-8). Illustrations of similar assemblies have been presented in detail by the manufacturer in an FPLC Technical Note on automation under the headings Multidimensional Chromatography and Column Cleaning and will be reiterated here only briefly. The third high-pressure pump, filled with water, allows continuous column regeneration without having to empty and refill the buffer solution originally present in pump A or B between each run. A novelty in this work is the relay switch-box which allows programmable automation of the third pump, including solvent exchange via a port (number 3 or 4) from the controller. The peristaltic pump and one motor valve allow successive washings with each of the column regeneration solutions described above. Finally, addition of one more motor valve (MV-8) between the peristaltic pump and the original motor valve (MV-7) makes it possible to alternate between load via peristaltic pump (for regeneration solutions) and load via syringe (for sample solution). For chromatographic runs, variation of options such as prebleeding or charging with a new metal ion, and also evaluation, was carried out by combining in one program any number of sub-programs controlled by the software package FPLC-manager.

Optimization strategy

A list of parameters noticeably affecting any IMAC separation will include several items not en-

countered in more conventional types of chromatography, such as ion exchange, hydrophobic interaction, hydroxyapatite or gel filtration [1]. As the optimum results for most parameters will also depend on the order of selection of these, the following sequence was followed: choice of metal ion, elution buffer, bleeding and prebleeding, suitable metal ion–elution buffer pairs, variation in further chemical parameters such as pH, and lastly physical parameters such as flow-rate.

Assays

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 7.5% homogeneous and 4–15% gradient gels with silver staining in an automated PHAST system (Pharmacia) as described previously [7]. Use of the word homogeneity in this work refers in SDS electrophoreses to the area of the main band relative to the total area of all bands in the same lane, as computed by a laser densitometer (Elscript 400, Hirschmann, Unterhaching, Germany). Assays concerning spectral, magnetic and catalytic properties [7] and immunological characterizations [8] were also performed as described.

RESULTS AND DISCUSSION

Chelating ligands

The chelating ligand investigated towards its affinity chromatographic properties was iminodiacetic acid (IDA). Coupling of tris(carboxymethyl)ethylenediamine (TED) yields a metal-chelating gel which, in relation to IDA, has been shown elsewhere to result in stronger retention of metal ion and thus weaker retention of protein [1,9]. Use of this chelating ligand for the purification of P450 was not investigated here.

Metal ions

When Chelating Superose was charged with Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Fe^{3+}, Fe^{2+} or Tl^{3+} ions and equilibrated using the standard conditions described above [with start buffer 50 m*M* potassium phosphate (pH 7.5), 500 m*M* NaCl, 20% glycerol and 0.4% Lubrol) and then loaded with 5 nmol of P450, no binding of this protein was ever observed. Using Chelating Sepharose Fast Flow, Roos [4] found that only Zn^{2+}-charged columns were capable of binding a similar preparation of P450, but this may possibly reflect a difference between properties of the gel matrices. The recoveries of P450 (in the pass-through fractions) as judged spectroscopically [10] were quantitative for all metal ions except Cd^{2+} and Fe^{2+}. Prebleeding (see below) Cd^{2+}- and Fe^{2+}-charged columns increased the total recovery of spectroscopically intact P450 but did not result in binding of this protein.

Cu^{2+}- and Ni^{2+}-charged columns were reproducibly found to be capable of binding P450, and the degrees of this were dependent on both the choice of elution buffer and the use of prebleeding. In all instances, Cu^{2+}-charged columns permitted tighter binding, in agreement with Porath *et al.* [11]. This was evidenced in this work by the finding that the total yields for elutions from Cu^{2+}-charged columns were consistently less than those from Ni^{2+}-charged columns. Remaining bound protein in such instances was nonetheless retrieved quantitatively and spectroscopically intact with a stepwise gradient of 50 m*M* EDTA or, with substantial denaturation, using a descending pH gradient as described below.

Elution buffers

Commonly used buffers in IMAC for the desorption of bound P450 were first inspected for their ability to retain enzyme activity. Concentrations in m*M* were chosen for gradients found appropriate for the elution of P450. In addition, the buffer solutions contained 50 m*M* potassium phosphate (pH 7.5), 500 m*M* NaCl, 20% glycerol and 0.4% Lubrol PX. Judgment of intact P450 and P420 (denatured P450) contents recovered in the presence of these buffer solutions prior to any chromatography was based on spectrophotometric measurements [10] after contact for 15 min at 25°C. Assessment of real catalytic activities based on substrate reactions is considered below. The recoveries of P450 and P420 shown in Table I are each given as a percentage of the total P450 originally present. Imidazole and tryptamine each gave in our opinion unacceptable degrees of denaturation even after having been recrystallized twice before use. Loss of P450 seen here appeared to be over and above that seen to cause the type II spectral changes which are known for these kinds of compounds [12]. These two buffers will not be considered further here.

TABLE I

EFFECT OF ELUTION BUFFERS ON P450 STABILITY

Contents of intact P450 recovered in the presence of various elution buffers were determined spectrophotometrically [10]. The recoveries of P450 and P420 are each given as a percentage of the total P450 originally present. Values shown represent means of triplicate measurements at 25°C. Sample buffer includes 50 m*M* potassium phosphate and 0.4% Lubrol. For further details, see text.

Elution buffer	Concentration (m*M*)	Recovery (%)		
		P450	P420	Total
None		100	0	100
Glycine	200	100	0	100
Ammonium chloride	500	99	0	99
Cysteine	50	97	3	100
Tryptophan	50	93	0	93
Histidine	50	92	3	95
Histamine	50	83	17	100
Imidazole[a]	50	43	20	63
Tryptamine[a]	50	28	0	28
EDTA	50	100	0	100
pH	pH 4.5	34	18	52

[a] Recrystallized as described under Experimental.

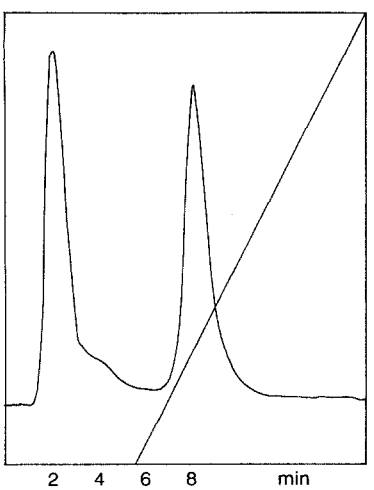

Fig. 1. Elution profile of FPLC purification of P450 on Chelating Superose. Sample, 3.9 nmol of P450 (1.13 mg of protein); column size, 20 mm × 10 mm I.D. (1.6 ml); metal ion, Ni^{2+}; flow-rate, 2.0 ml/min; sample buffer, 50 m*M* potassium phosphate (pH 7.5)–20% glycerol–500 m*M* sodium chloride–0.4% Emulgen 911; gradient, 0–200 m*M* glycine over 20 ml; detection, 405 nm at 0.05 a.u.f.s. Prior to application of the sample, the column was prebled with 2 ml of 200 m*M* glycine.

EDTA and decreased pH values were also found to be capable of eluting P450 bound to IMAC columns. However, as EDTA resulted in complete stripping of coordinated metal ion, and as low pH values resulted in considerable denaturation, these two modes of elution were not investigated further. Interestingly, the non-ionic detergent Emulgen 911 in the start buffer allowed more elution of spectroscopically intact P450 and at a lower pH than did Lubrol PX. After a pH gradient of 7.7–3.0, the highest recovery of P450 in the gradient using 0.4% Emulgen 911 was 55% (at pH 4.5), whereas using 0.4% Lubrol PX the highest recovery was only 34% (at pH 5.5).

In all instances after the application of a continuous gradient of any elution buffer, there appeared only a single, narrow, symmetrical peak. A typical chromatogram is shown in Fig. 1. This appearance of the chromatographic profile has also been reported for the IMAC purifications of α_1-antitrypsin and α_1-macroglobulin [13] and platelet-derived growth factor [14].

Bleeding and prebleeding

Bleeding (the loss or leakage of metal ions from the gel's complexing groups during application of the sample and/or elution buffer gradient) is common for many metal ion–elution buffer pairs and often of no significance [1] other than the fact that the eluents containing the proteins of interest may also contain metal ions. In such instances, this can be obviated by deliberately charging the column under non-saturating concentrations of metal ion, by subsequent batch treatment of the eluents with uncharged gel (such as Chelating Sepharose Fast Flow) or by attaching a second, uncharged, column in tandem as employed by Roos [4].

Bleeding during initial application of elution buffer may, however, adversely affect the adsorption and stability of the applied protein owing to a type of metal contact denaturation. This minimum, "threshold" concentration of elutable metal ion causing protein denaturation can be removed by prebleeding the charged column with elution buffer before applying the sample.

We found that using the six elution buffers described above, metal ions were routinely bled from both Ni^{2+}- and Cu^{2+}-charged columns (see Table II, where the values given represent contents of

P450 recovered after chromatography as a percentage of the applied load). The effects of this can now be summarized into three categories.

First, bleeding from Ni^{2+}-charged columns in most instances had no great effect on the total yield (Table II, except for elution buffers cysteine and histidine which are discussed below). That is, for elution buffers such as glycine or histamine and to a lesser extent tryptophan and ammonium chloride with Ni^{2+}-charged columns, there is no dramatic difference between the total recoveries of P450 for "non-prebled" and "prebled" columns. Prebleeding these columns therefore appears to be unnecessary. It appears, in fact, only to lead to less binding and more pass-through material. This finding is in accordance with that of Porath and Olin [9], who omitted prebleeding in such a system. The amount of charged Ni^{2+} ion left on the column as a function of the volume of prebleeding with the elution buffer glycine is discussed below. Ni^{2+} ions in the eluents can be eliminated with uncharged gel as described above or by subsequent hydroxyapatite chromatography, *e.g.*, in conjunction with removal of excess detergent.

Second, prebleeding Cu^{2+}-charged columns appeared to be necessary. If these were not prebled, almost no P450 protein was recovered spectrophotometrically intact (Table II). Note that this denaturation also applies to the pass-through fraction (*i.e.*, before the application of any elution buffer). In this instance, subsequent treatment with uncharged gel was of no use. Prebleeding Cu^{2+}-charged col-

TABLE II

EFFECTS OF BLEEDING AND PREBLEEDING ON TOTAL RECOVERY

Recoveries of P450 contents as a percentage of the applied load were detected spectrophotometrically [10] in pass-through and gradient fractions subsequent to chromatography on Cu^{2+}- or Ni^{2+}-charged columns, each with six different elution buffers. The start buffer included 0.4% Lubrol PX (pH 7.5) (see also text). In each instance, values are given for non-prebled and prebled columns. For Ni^{2+}-charged columns, values in parentheses indicate recoveries of P450 plus P420. It is these values for the gradient fractions in parentheses which were used to deduce a relative order of elution buffer strength.

Elution buffer	Fraction	Metal ion				
		Ni^{2+}-charged columns			Cu^{2+}-charged columns	
		Non-bled	(P450 + P420)	Prebled	Non-bled	Prebled
Cysteine	Pass-through	16	(14)	94	0	95
	Gradient	49	(84)	3	0	0
	Total	65	(98)	97	0	95
Histidine	Pass-through	16	(13)	89	0	91
	Gradient	71	(82)	4	7	0
	Total	87	(95)	93	7	91
Glycine	Pass-through	14	(15)	46	0	6
	Gradient	78	(78)	50	0	13
	Total	92	(93)	96	0	19
Histamine	Pass-through	16	(15)	45	0	5
	Gradient	57	(69)	36	3	40
	Total	73	(84)	81	3	45
Tryptophan	Pass-through	15	(16)	33	0	4
	Gradient	34	(45)	39	0	23
	Total	49	(61)	72	0	27
Ammonium chloride	Pass-through	16	(15)	32	0	5
	Gradient	13	(13)	13	0	2
	Total	29	(28)	45	0	7

umns, however, markedly and reproducibly increased the yield of intact P450, especially in the gradient fractions where the elution buffers glycine, histamine and tryptophan were used.

Third, with Ni^{2+}- or Cu^{2+}-charged columns and elution buffers cysteine or histidine, prebleeding appeared to be impossible as these buffers not only desorbed protein (from non-prebled columns) but also removed all of the charged metal ion from the gel matrix prior to loading of any protein. This was evidenced by the bluish colours of the gradient eluents and the subsequent quantitative yields of (spectroscopically still intact) P450 in the pass-through eluents.

In Table II, the values given in parentheses for Ni^{2+}-charged, non-prebled columns represent the total amount of P450 and P420 recovered as a percentage of the total P450 applied to the column. Using these values, and given the premise that cysteine and histidine are judged as "stronger" buffers where P450 and P420 are taken together, the decreasing order of elution strength is then cysteine $\approx$ histidine > glycine > histamine > tryptophan > ammonium chloride. Judging qualitatively, of course, glycine is a "better" elution buffer for high recoveries of intact P450.

An attempt to summarize qualitatively the results on bleeding and prebleeding according to the three categories described above is presented in Table III.

Table IV illustrates the effect of the degree of prebleeding on sample binding and homogeneity of the gradient fraction. As might be expected, it was found that more prebleeding results in less capacity (for any or all forms of P450). At the same time, however, more prebleeding also seems to result in less apparent electrophoretic homogeneity of bound P450, suggesting that the binding of Ni^{2+}-charged columns is specific for only one (or a few) forms of P450. With regard to Table IV, the gradient recovery (of up to 86%) and apparent homogeneity (of up to 85%) actually imply a yield of homogeneous P450 of up to 70%. Evidence for the presence of more than one P450 form in this fraction is discussed below under *Characterization of the purified P450 form(s)*.

Suitable metal ion–elution buffer pairs

Regarding the protein sample and elution conditions present, it is obvious from Table II that some pairs are more suitable than others for preparative chromatographic separation. Cysteine and histidine are less suited because they strip too much metal ion. On the other hand, ammonium chloride was found to elute very little bound P450. Increasing the concentration of this elution buffer to 2 *M* had no further effect. Up to 100% of the P450 load was, however, retrieved spectroscopically intact after elution with 50 m*M* EDTA. The final criterion thus involves (a) a good overall yield and (b) a reasonable amount of binding for subsequent elution.

TABLE III

BLEEDING AND PREBLEEDING IN IMAC: QUALITATIVE SUMMARY OF RESULTS

Chelating Superose columns with various metal ion–elution buffer combinations were used to purify P450. For chromatographic conditions, see Table II.

Bleeding occurs during application of	Result	Example	Remedy	Prebleeding category
Elution buffer	Metal ions in eluent	Ni^{2+}–glycine	Metal ions capture methods (see text)	Unnecessary
Sample	Denaturation of pass-through and gradient	Cu^{2+}–NH_4Cl, glycine, tryptophan, histamine	Prebleeding	Necessary
Elution buffer (Ni^{2+}), sample and elution buffer (Cu^{2+})	Total stripping of metal ions	Ni^{2+}, Cu^{2+}–cysteine, histidine	None	Impossible

TABLE IV

EFFECT OF DEGREE OF PREBLEEDING ON SAMPLE BINDING AND HOMOGENEITY OF THE GRADIENT FRACTION

A Ni^{2+}-charged column of Chelating Superose was prebled with various volumes of the elution buffer (200 m*M* glycine) and then equilibrated further with start buffer which included 0.4% Lubrol PX (for other constituents, see Experimental) prior to sample loading. The sample load in each instance was 6.8 nmol of P450. Values depict the recoveries of spectroscopically intact P450 as a percentage of the load. Values for homogeneity indicate the apparent homogeneity of the protein(s) eluted in the gradient peak and are in percent as defined under Experimental

Parameter	Volume (ml) of elution buffer used for prebleeding					
	0	1	2	4	8	15
Recovery of pass-through (%)	14	34	46	72	86	99
Recovery of gradient (%)	86	61	50	23	5	0
Total recovery (%)	100	95	96	95	91	99
Homogeneity of gradient fraction (%)	85	79	74	70	65	n.d.[a]

[a] Not determined.

Four pairs which meet these requirements are Ni^{2+} and glycine, histamine or tryptophan, and Cu^{2+} and histamine.

For the sake of simplicity of comparison, only Ni^{2+}–glycine, Ni^{2+}–histamine and Cu^{2+}–histamine systems were investigated further. The yields in the gradient portions of such chromatographic separations using prebleeding (in Table II 50, 36 and 40%, respectively), are considered acceptable for preparative purposes and were reproducible to within ± 10% of those mean values from three runs each.

Judging by the overall yields and apparent electrophoretic homogeneity of the gradients in these three systems (Fig. 2) the Ni^{2+}–glycine system seemed to be the most suitable, and was chosen for further optimization.

As among such combinations of metal ions and elution buffers the elution strengths of the buffers necessary to elute substantial amounts of bound P450 are also high enough to elute some amounts of metal ion, this means that metal ions must be recharged prior to each run. This is in contrast, for example, to a combination involving the separation of standard marker proteins using Cu^{2+} (a "strong binder") and ammonium chloride (a "weak eluter") [1]. In this instance, the proteins could be loaded and eluted more than twenty times without having to recharge the column with Cu^{2+} ions.

Start buffers

The use of potassium or sodium phosphate as start (or "equilibration" or "sample") buffer resulted in very narrow, symmetrical gradient peaks. Tris–HCl as start buffer gave similar profiles where the gradient peaks were much smaller and contained up to 70% less P450, indicative of a reduction in binding as reported elsewhere for this buffer [15]. Sodium acetate gave even smaller, very round and tailing peaks.

pH values

When runs were made at pH 7.0–8.0, higher values resulted in gradient peaks being narrower and having decreased retention volumes and lower yields. This is summarized in Table V, where it is noticeable that recoveries in the gradient fraction decreased by 30%. This is in agreement with the results of Kato *et al.* [16], who used a Zn^{2+}-charged column and glycine to separate a mixture of transferrin and carbonic anhydrase. Here, too, proteins were more strongly retained at lower pH, although as originally postulated by Porath *et al.* [11] for IMAC, the affinity of proteins for these metal ions at alkaline pH should be more effective but less specific. We observed with increasing pH, however, more electrophoretic homogeneity (Table V). A reasonable pH optimum for the sample in this work therefore seems to be between 7.75 and 8.0.

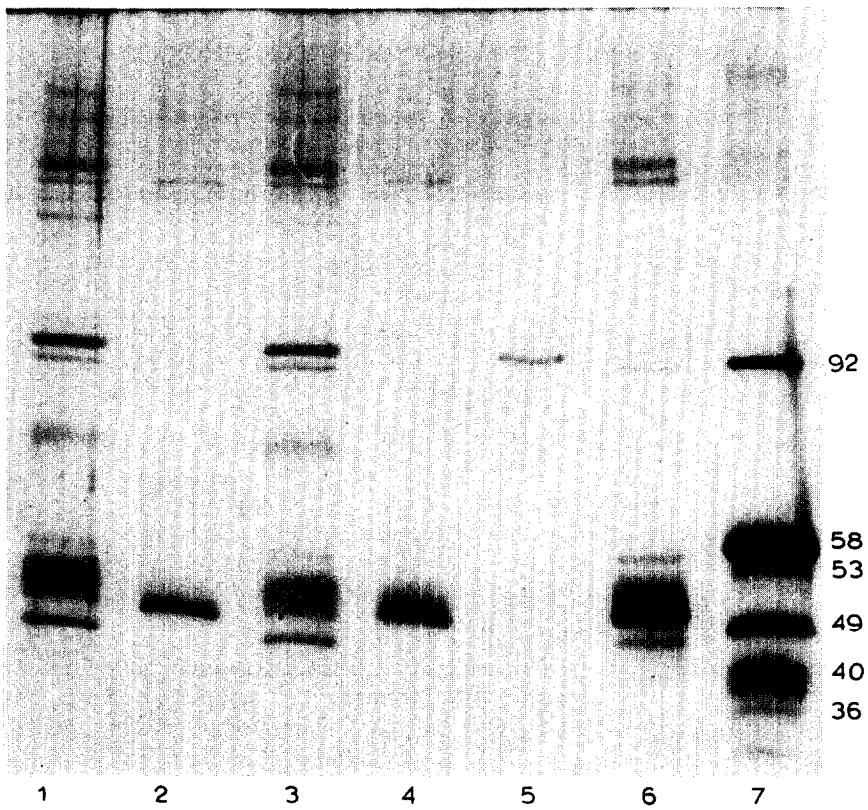

Fig. 2. SDS-PAGE of P450 as purified using Chelating Superose. Start buffer includes 0.4% Lubrol PX (pH 7.5) (see also Experimental). Metal ion and elution buffer, Ni^{2+} or Cu^{2+} and glycine or histamine as depicted. Load in each instance, 8-aminooctyl-Sepharose pool of solubilized phenobarbital-induced rat liver microsomes. The homogeneous 7.5% gel was stained with silver [7] and the anode is at the bottom. Lanes: 1 and 2 = pass-through and gradient fraction of a Ni^{2+}-charged column eluted with glycine; 3 and 4 = pass-through and gradient fraction of a Ni^{2+}-charged column eluted with histamine; 5 and 6 = pass-through and gradient fraction of a Cu^{2+}-charged column eluted with histamine; 7 = marker proteins (with molecular weights in kilodalton): phosphorylase *a* (92), catalase (58), glutamate dehydrogenase (53), fumarase (49), aldolase (40) and lactate dehydrogenase (36).

TABLE V

EFFECT OF pH ON RETENTION TIME, PEAK WIDTH, RELATIVE P450 RECOVERY, AND HOMOGENEITY OF THE GRADIENT FRACTIONS

The relative P450 recoveries in the pass-through fractions were not determined here; total P450 recoveries of gradient and pass-through fractions together for all pH values were consistently 85–95%. For definitions, see Experimental. Metal ion, Ni^{2+}; elution buffer, glycine; detergent, 0.4% Lubrol; sample load, 3.6 nmol.

Parameter	pH				
	7.0	7.25	7.5	7.75	8.0
Retention time (min)	3.2	4.0	2.7	2.6	2.7
Peak width (ml)	9.8	9.4	7.3	6.8	5.3
Relative P450 recovery (%)	63	64	55	44	33
Homogeneity (%)	79	83	84	88	91

Detergents

Chromatographic separations incorporating 0.4% (w/v) concentrations of five of the most commonly used non-ionic detergents in P450 purification schemes were compared: Emulgen 911, Emulgen 913, Lubrol PX, Nonidet P40 and Renex 690 [17,18].

As far as apparent SDS-PAGE homogeneity, P450 recovery, peak volume (area under the curve, AUC), peak width and retention time were concerned, Emulgen 911, Emulgen 913, Lubrol PX and Renex 690 all gave similar results. Emulgen 911 was slightly more advantageous with regard to P450 recovery and peak width. As mentioned above, during descending pH gradients, Emulgen 911 also had a higher stabilizing effect on P450 than did Lubrol PX.

Nonidet P40, on the other hand, gave very flat profiles and poor yields, and it was noticed that during chromatography the column back-pressure increased strongly. This was also found, albeit to a lesser extent, when the anionic detergent sodium cholate was used, presumably owing to detergent binding to the gel matrix, as has been shown elsewhere for the strong anion exchanger Mono Q [17].

In contrast to Kato *et al.* [19], who used the detergent octaethylene glycol dodecyl ether (polyoxyethylene 8 lauryl ether, $C_{12}E_8$) with IMAC (where the adsorbent was also IDA) to fractionate human placenta mitochondrial membranes, we could not demonstrate that this detergent was in any way superior to Emulgen 911. It should be pointed out that for the chromatogram illustrated there, the gradient was started concomitant with sample application.

Flow-rates

A flow-rate of 2.0 ml/min (2.53 cm/min) was used for routine screenings, allowing a complete run with a continuous gradient (see below) in 15 min. Higher values were avoided as this resulted in more than the 1.0 MPa back-pressure reported by the manufacturer to be deleterious to this gel matrix. Flow-rates of down to 0.5 ml/min did not resolve gradient peak profiles into more peaks or shoulders as judged optically and did not have any effect on the separation of proteins as judged by SDS-PAGE of individual fractions. There was also no noticeable negative effect on the total recovery with prolonged contact with the gel at 25°C.

Gradients

Fast chromatographic runs incorporated continuous gradients over 20 ml. Using a Ni^{2+}-charged column with 0–200 m*M* glycine at a flow-rate of 2 ml/min, fractionation of this P450 sample routinely yielded, as mentioned above, a single symmetrical gradient peak between 20 and 60 m*M* glycine. Increasing the gradient size to 30 ml or more brought about no apparent improvement in resolution. Using a flow-rate of 0.5 ml/min reduced the concentration at which P450 eluted with this buffer; a stepwise gradient of 6–10 m*M* (in place of 20–60 m*M*) glycine at this flow-rate resulted in a gradient peak shoulder. Again, however, this caused no apparent heterogeneity with regard to molecular weight as revealed by SDS-PAGE. Use of such segmented gradients may, of course, be helpful in analytical fractionations of P450 derived from a variety of other inducing agents, as was recently reported by Roos [5].

Temperature

Chromatographic runs at 25°C using the conditions described above routinely yielded 80–100% recoveries of P450. Maintaining the column in a 4°C ice-bath during chromatography in fact reduced the recovery and resolution, as has been reported in ion-exchange chromatography [20]. In the present work, the basic chromatographic profile of separations at 0–4°C remained unaltered, but the peak width of the gradient fraction was more than doubled and the total recovery here was decreased by 40%.

Sample size

Varying the sample load from 5 to 100 nmol demonstrated that for this column size of 1.6 ml, 20–50 nmol of this type of P450 (5–15 mg of protein) could be accommodated for fractionation. Hence, this column appears to be suitable for both preparative and analytical needs. When more than 20–50 nmol of the P450 sample under study were loaded, lower relative protein recoveries and apparent homogeneity of bound material were obtained (Table VI).

Characterization of the purified P450 form(s)

The specific contents of the gradient fractions derived from optimized conditions (Tables IV–VI)

TABLE VI

EFFECTS OF SAMPLE LOAD (IN NMOL P450) ON RELATIVE P450 RECOVERY, RELATIVE RECOVERY OF PROTEIN (AUC) AND HOMOGENEITY OF THE GRADIENT FRACTIONS

For definitions see Experimental. Metal ion, Ni^{2+}; elution buffer, glycine; pH, 7.75; detergent, 0.4% Lubrol.

Parameter	Sample load (nmol)				
	5	10	20	50	100
Relative P450 recovery (%)	46	44	48	46	32
Relative protein recovery (%)	38	37	35	28	24
Homogeneity (%)	85	86	80	79	63

ranged from 14.3 to 15.1 nmol of P450 per milligram of protein. Hence, in comparison with the values given for homogeneity in Tables IV–VI, the loss of haeme from P450 may possibly amount to as much as 10%.

Guengerich *et al.* [21] have described in detail the purification and characterization of several P450 forms present in liver microsomes of rats induced with phenobarbital (and other substances). Using this procedure in our laboratory, we obtained these forms for use as authentic samples for comparison purposes. P450 isolated in the gradient fraction of the Chelating Superose column was freed from residual non-ionic detergent as described [7], and preliminary analyses may now allow us to make a tentative allocation.

The apparent molecular weight of 50–51 kilodalton as judged by SDS-PAGE is consistent with those found for P450 forms 2B1 [22] (Guengerich *et al.*'s designation PB-B), 2B2 (PB-D) and 2C11 (UT-A). Fig. 3 illustrates that in SDS-PAGE the Chelating Superose fraction co-migrates with 2B1. The iron(II)–CO difference spectrum maximum of 450 nm is consistent with those seen for 2B1, 2B2 and 2C6 (PB-C).

More important, however, is the finding that the present P450 form gave a very strong ELISA reaction with monoclonal antibodies prepared against 2B1, some reaction with anti-2B2 (known to cross-react to some extent with 2B1 [21]), only minimum reaction with anti-2C6 and no reaction with anti-2C11. Finally, a reconstituted system of P450 taken from the Chelating Superose gradient fraction was also capable of dealkylating pentoxyresorufin at the relatively high rate of 1.9 nmol of product per minute per nanomole of P450, which is similar to that found by us previously for 2B1 [7]. As has been shown elsewhere [23] for seven different forms of P450 induced either mainly or marginally by phenobarbital, only 2B1 demonstrated such high specificity towards this substrate.

Taken together, these results suggest that the Chelating Superose fraction of P450 may contain large amounts of 2B1. Roos [5] has pointed out recently that phenobarbital-induced P450 fractions eluting in IMAC gradients may also contain certain amounts of 3A1 and/or 3A2 (PB/PCN-E). At present, we have no evidence for or against this finding.

CONCLUSION

The aim of this work was to investigate the general usefulness of IMAC in purifying P450 for preparative purposes. Under suitably chosen conditions, and with respect to the P450 sample load used here, this type of chromatography is apparently capable of yielding high recoveries of virtually homogeneous protein in one step. In light of the fact that several different P450 forms have virtually identical mobilities as judged by SDS-PAGE, we cannot yet assume that the P450 form encountered here is indeed only a single form.

The number of steps in preparing each IMAC run is unusually large, as is the number of parameters affecting this chromatographic method itself, but this can be mitigated by taking advantage of fully programmable automation. Consequent care in cleaning and recharging the column as described under Experimental ensured excellent reproducibility of chromatography, with no increase in back-

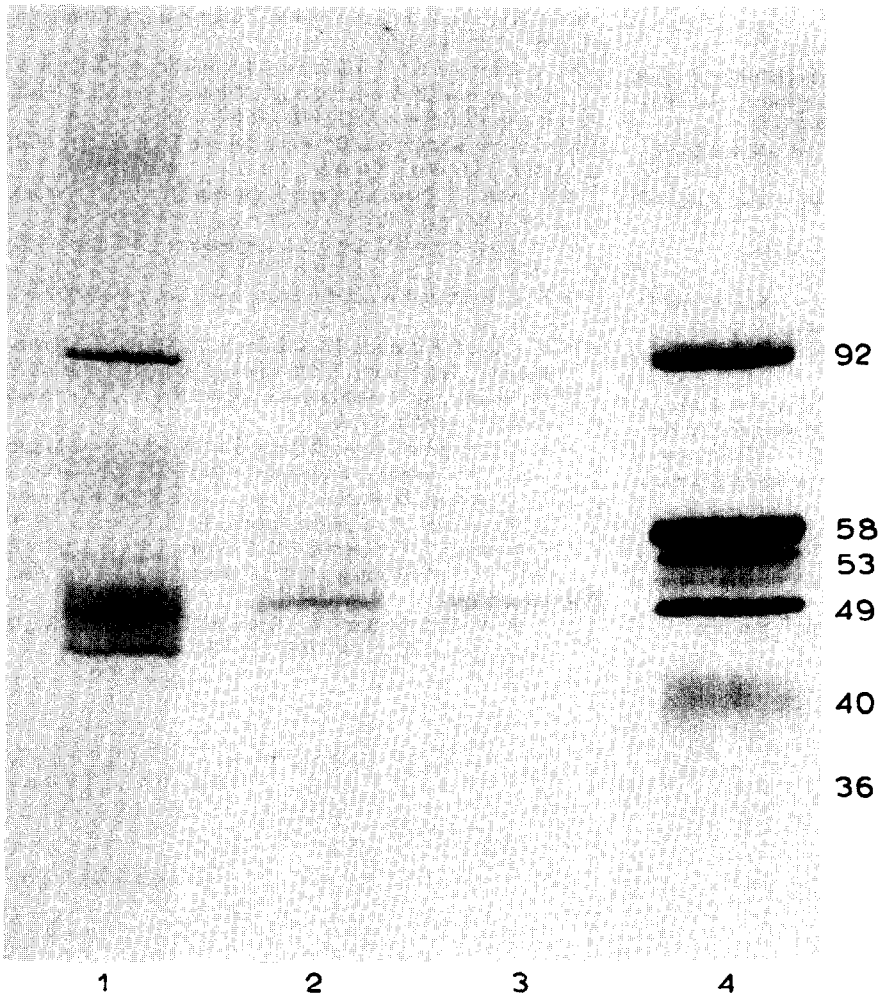

Fig. 3. SDS-PAGE of P450: purification by IMAC and co-migration of an authentic sample. Start buffer includes 0.4% Emulgen 911 (pH 7.75). Metal ion and elution buffer, Ni^{2+} and glycine. The 4–15% gradient gel was stained with silver. For details, see Fig. 2. Lanes: 1 = load; 2 = gradient fraction; 3 = P450 2B1 [22] (P450 PB-B) prepared according to Guengerich *et al.* [21]; 4 = marker proteins as in Fig. 2.

pressure, even after 200 runs. The type of set-up incorporated here also lends itself well, of course, to general routine column hygiene of any other types of columns.

IMAC may prove to be a useful method in supplementing conventional types of chromatography such as ion-exchange and hydroxyapatite for the purification of a larger number of constitutive and inducible P450 forms.

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