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Purification and characterization of an anticonvulsant-induced human cytochrome *P*-450 catalysing cyclosporin metabolism

Peter M. SHAW,* Tristan S. BARNES,† Denise CAMERON,* Jetmund ENGESET,‡ William T. MELVIN,† Ghada OMAR,* James C. PETRIE,§ W. Roger RUSH,¶ Carolyn P. SNYDER,* Paul H. WHITING,‖ C. Roland WOLF** and M. Danny BURKE,*††

Departments of *Pharmacology, †Biochemistry, ‡Surgery, §Medicine & Therapeutics and ‖Clinical Biochemistry, University of Aberdeen, Aberdeen AB9 1AS, ¶Syntex Pharmaceuticals Ltd., Riccarton, Edinburgh EH14 4AS, and **ICRF Laboratory of Molecular Pharmacology, University of Edinburgh, Edinburgh EH8 9XD, U.K.

A form of human hepatic microsomal cytochrome *P*-450 (P450hA7) with subunit M_r 50400 has been purified from an epileptic who had been receiving long-term treatment with anticonvulsant drugs. P450hA7 metabolized the immunosuppressant drug cyclosporin A and the dihydropyridine calcium channel antagonist nifedipine, but did not metabolize a similar dihydropyridine drug, nicardipine, nor a series of alkoxyresorufin model substrates. The hepatic microsomal concentration of P450hA7 was higher in five individuals who had been receiving long-term anticonvulsant treatment than in any of 21 individuals who had not been similarly treated. The mean P450hA7 concentration in the treated individuals was 5-fold higher than the mean concentration in the untreated individuals. It is concluded that P450hA7 is a member of the cytochrome P450III family which is induced by anticonvulsant drugs in man.

INTRODUCTION

The cytochromes *P*-450 are a superfamily of haemoprotein enzymes, coded for by several genes (Nebert *et al.*, 1987; Guengerich, 1987), which are responsible for the oxidative metabolism of a wide variety of xenobiotics and endogenous compounds, including drugs, carcinogens and other toxic chemicals, steroids, eicosanoids and proteins (Burke, 1981; Fucci *et al.*, 1983; Ko *et al.*, 1987; Romano *et al.*, 1987; Sonderfan *et al.*, 1987; Weiss *et al.*, 1987). Amongst humans, differences in the complement of *P*-450 forms present in various tissues, especially in the liver, is a major factor responsible for interindividual variation in the metabolism and effects of drugs and, by inference, of certain physiological and numerous toxic chemicals (Kalow, 1987). Several forms of human hepatic *P*-450, some of which are probably identical with each other, have been purified (Wang *et al.*, 1983; Distlerath *et al.*, 1985; Watkins *et al.*, 1985; Guengerich *et al.*, 1986*a*; Gut *et al.*, 1986; Shimada *et al.*, 1986; Wrighton *et al.*, 1986; Kawano *et al.*, 1987). A characteristic feature of *P*-450 in experimental animals is the selective induction of certain forms by drugs and other chemicals (Nebert *et al.*, 1987; Guengerich, 1987). Induction of *P*-450 also occurs in man and can result in important clinical drug interactions (Sotaniemi & Pelkonen, 1987). We report here the purification and characterization of an apparently anticonvulsant-induced form of human hepatic *P*-450.

MATERIALS AND METHODS

Materials

The following items were generous gifts: Emulgen 911 from the Kao Atlas Corporation, Tokyo, Japan; benzphetamine from the Upjohn Co. Ltd., Crawley; nicardipine from Syntex Research, Edinburgh; nifedipine from Bayer U.K. Ltd., Haywards Heath; [^{3}H]cyclosporin A (9.3 mCi/μmol) from Sandoz Ltd., Basle, Switzerland and phenanthrene 9,10-oxide and 9,10-diol from Dr. P. Dansette, Paris, France. All other materials were from the usual commercial suppliers.

Hepatic microsome preparation

Human livers were obtained from renal transplant donors within 30 min of circulatory arrest, placed immediately into ice-cold iso-osmotic saline and frozen within 1 h as approx. 1 cm cubes at −80 °C until used. Clinical histories of the individuals from whom liver was obtained are included in Table 2 as appropriate. Rat livers were from 220–260 g male Sprague–Dawley rats bred in the University of Aberdeen, unless stated otherwise. Liver microsomes were prepared as described previously (Burke *et al.*, 1983), except that the fibrous nature of the human livers necessitated their homogenization using an Ultra Turrax Homogeniser.

Purification of human cytochrome P-450

All microsome solubilization and *P*-450 purification procedures were at 4 °C. Purification was by column chromatography and fractions were analysed by SDS/PAGE: those showing identical protein band patterns and the highest purity of proteins in the molecular mass range 45–60 kDa were pooled for spectroscopic measurement of total *P*-450 and further purification. Fractions were concentrated or dialysed using Amicon stirred cells fitted with PM10 membranes. All percentage concentrations are w/v unless shown otherwise.

Abbreviations used: CBZ, carbamazepine; CsA, cyclosporin A; DTT, dithiothreitol; PB, phenobarbitone; PBS, phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM-Na_2HPO_4, pH 7.2); PCN, pregnenolone 16α-carbonitrile; PHT, phenytoin; PMSF, phenylmethanesulphonyl fluoride; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

†† To whom correspondence and reprint requests should be addressed.

Hepatic microsomes from individual no. 7, containing 5410 nmol of total *P*-450 (0.58 nmol of total *P*-450/mg total protein), were diluted to 20 mg of protein/ml in buffer A [100 mM-sodium/potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1 mM-EDTA and 1 mM-DTT] and solubilized for 30 min with 0.61% CHAPS. The 100000 *g* × 1 h supernatant (4430 nmol of *P*-450, 0.64 nmol of *P*-450/mg of protein) was applied to an 80 cm × 4.5 cm 8-amino-octyl–Sepharose CL4B column, synthesized according to Cuatrecasas (1970), equilibrated with buffer A containing 0.35% sodium cholate and eluted at 70 ml/h. The column was washed with equilibration buffer, then developed with a stepwise gradient of, sequentially, 0.06%, 0.08%, 0.1% and 0.5% Emulgen 911 in the same buffer. Of the *P*-450 applied to the column, 1% was eluted during washing with equilibration buffer, a minor *P*-450 fraction (2% of that applied) was eluted with 0.06% Emulgen 911 and the major fraction (70% of the *P*-450 applied, with a specific content of 4.2 nmol of *P*-450/mg of protein) was eluted with 0.08% Emulgen 911. The column was then developed with a stepwise gradient of 0.5%, 1% and 2% Emulgen 911 in buffer A containing 0.35% cholate and 0.15% sodium deoxycholate. A minor *P*-450 fraction (4% of the total applied) was eluted with 1% Emulgen 911. Finally the column was developed with 2% Emulgen 911 in buffer A containing 0.5% cholate and 0.15% deoxycholate, but no more *P*-450 was eluted. Of the *P*-450 applied to this column, 77% was recovered in total.

The major *P*-450 fraction from the octylamine column was concentrated, dialysed against buffer B [10 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.1 mM-DTT, 0.2% cholate and 0.1% Emulgen 911] and applied to a 48 cm × 2.5 cm DEAE-Trisacryl column equilibrated with buffer B and eluted at 60 ml/h. The major *P*-450 fraction (61% of the total applied to this column, with a specific content of 10.5 nmol of *P*-450/mg of protein) was eluted in the wash with buffer B. A linear gradient of 0–0.25 M-NaCl in buffer B eluted four successive broad *P*-450 fractions between 0 and 0.12 M-NaCl, containing respectively 13%, 6%, 5% and 2% of the *P*-450 applied. Of the *P*-450 applied to this column, 89% was recovered.

The major *P*-450 fraction from the DEAE column was concentrated, dialysed against buffer C [5 mM-potassium phosphate buffer, pH 7.0, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.1 mM-DTT and 0.1% Emulgen 911] and applied to a 30 cm × 2.5 cm CM-Trisacryl column equilibrated with buffer C and eluted at 25 ml/h. Of the *P*-450 loaded onto this column, 8% was eluted in the wash with buffer C. The major *P*-450 fraction (35% of the total *P*-450 applied to this column, with a specific content of 15.4 nmol of *P*-450/mg of protein) was eluted during a linear gradient of 0–0.5 M-NaCl in buffer C at approx. 0.25 M-NaCl. *P*-450 fractions eluted in the wash or subsequently in the gradient had specific contents less than 5 nmol/mg of protein. Total recovery of *P*-450 from this column was 80%.

The major *P*-450 fraction from the CM column was dialysed against buffer D [5 mM-potassium phosphate, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.1 mM-DTT] and applied to a 5.5 cm × 2.5 cm hydroxyapatite column (Bio-Gel HTP) equilibrated with buffer D and eluted at 25 ml/h. After removing Emulgen 911 from the bound sample by washing with approx. 12 column vol. of buffer D until the eluate absorbance at 280 nm was undetectable, *P*-450 was eluted with 500 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.1 mM-DTT, then dialysed against 10 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.1 mM-DTT, stored at −80 °C and named P450hA7.

Purification of human NADPH: cytochrome *P*-450 reductase

Human NADPH:*P*-450 reductase was purified by a modification of the method of Yasukochi & Masters (1976). A sample of solubilized hepatic microsomes from individual no. 7 (specific activity of NADPH:*P*-450 reductase 0.37 μmol/min per mg of protein) was applied to an 8-amino-octyl–Sepharose CL4B column as described above. After washing the column with buffer A containing 0.35% cholate and 0.08% Lubrol PX, NADPH:*P*-450 reductase was eluted using buffer A containing 0.35% cholate, 0.15% deoxycholate and 0.5% Lubrol PX (specific activity of NADPH:*P*-450 reductase 0.71 μmol/min per mg of protein). This fraction was concentrated, dialysed against buffer E [10 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.1 mM-DTT, 0.2% cholate and 0.1% Lubrol PX] and applied to a 45 cm × 2.5 cm DEAE-Trisacryl column equilibrated with buffer E and run at 60 ml/h. After washing the column with buffer E, NADPH:*P*-450 reductase was eluted using a linear gradient of 0–0.25 M-NaCl in buffer E at 0.18 M-NaCl (specific activity 6.54 μmol/min per mg of protein). This fraction was concentrated, supplemented with 2 μM-FMN and applied to a 1.8 cm × 1.6 cm 2′,5′-ADP–Sepharose 4B column equilibrated with buffer F [10 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.02 mM-EDTA, 0.2 mM-DTT, 2 μM-FMN and 0.1% Lubrol PX]. The column was washed with 250 ml of buffer G [300 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.02 mM-EDTA, 0.2 mM-DTT, 2 μM-FMN and 0.1% Lubrol PX] followed by 250 ml of buffer H [30 mM-potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 2 μM-FMN and 0.15% deoxycholate], and then NADPH: *P*-450 reductase was eluted with buffer H containing 0.4 mM-PMSF and 5 mM-2′-AMP. Fractions showing a ratio of A_{417} to A_{380} greater than 1.05 were pooled, dialysed against buffer I [10 mM-sodium/potassium phosphate buffer, pH 7.7, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.1 mM-DTT] and stored at −80 °C. The purified human NADPH:*P*-450 reductase had a specific activity of 77.1 μmol/min per mg of protein and showed a single protein band on SDS/PAGE of M_r 78000.

Enzyme assays

Total *P*-450 was measured by the CO-binding method of Omura & Sato (1964), using a Cary 219 split-beam spectrophotometer. Protein was measured by the Naphthalene Black 12B dye-binding method of Schaffner & Weissman (1973), with bovine serum albumin as standard, having first established that the assay was not affected by the concentrations of glycerol, Emulgen, Lubrol, CHAPS and other chemicals present in the samples. All enzyme activities were assayed at 37 °C. Cytochrome b_5, NADPH:*P*-450 reductase (measured as NADPH:cytochrome *c* reductase, using 0.6 mM-

NADPH), NADH:cytochrome b_5 reductase (measured as NADH:ferricyanide reductase, using 2 mM-NADH) and epoxide hydrolase (using 20 μM-phenanthrene 9,10-oxide as substrate and excitation and emission wavelengths of 270 nm and 320 nm respectively) were assayed by spectrophotometry or fluorimetry as described previously (Falzon *et al.*, 1984) with the modifications indicated above. Mono-oxygenase activities were measured using 50 μl of either a hepatic microsomal suspension (20 mg of protein/ml) or a reconstituted system prepared thus: 0.1 nmol of P450hA7 and 0.1 nmol of purified human NADPH:*P*-450 reductase were added to 0.05 mg of lecithin [10 μl of a 5 mg/ml solution in chloroform/ethanol (3:1, v/v), evaporated to dryness in a test tube], diluted with 100 mM-Tris/HCl buffer, pH 7.7, containing 0.1 mM-EDTA to a total volume of 50 μl and gently mixed for 5 min at room temperature. All reaction volumes were 1 ml total. Alkoxyresorufin *O*-dealkylations were measured by the continuous fluorimetric method of Burke *et al.* (1985). All other reactions were assayed in the presence of an NADPH-generating system as described previously (Burke *et al.*, 1983) and were measured at intervals up to 15 min to allow calculation of initial reaction rates. Control incubations lacking NADP were included. *N*-Demethylation was measured using 5 mM-aminopyrine, 1 mM-ethylmorphine, 1 mM-benzphetamine or 0.4 mM-erythromycin (Burke *et al.*, 1983). The combined 11- and 12-hydroxylation of lauric acid was measured using 0.1 mM-[1-^{14}C]lauric acid (1 μCi/μmol) (Orton & Parker, 1982) and cholesterol 7α-hydroxylation was measured using 9 μM-[4-^{14}C]cholesterol (59 μCi/μmol) (Rush *et al.*, 1981), with t.l.c. separation of metabolites followed by scintillation counting. Nifedipine oxidation was conducted under yellow light, using 0.2 mM-nifedipine. For analysis, 200 μl of incubation mixture was removed into 0.8 ml of ice-cold methanol, protein was precipitated at 2000 ***g*** for 10 min, then 20 μl of supernatant was assayed for non-metabolized nifedipine by h.p.l.c., using a 25 cm × 0.46 cm Partisil 5 μ ODS3 column eluted isocratically at 1 ml/min with 0.02 M-KH_2PO_4/acetonitrile (2:3, v/v), and detection by A_{320}. Nifedipine was eluted at 7 min, free of interfering compounds and was measured by reference to a series of nifedipine standards (0–220 μM) put through the same extraction and h.p.l.c. procedure. Nicardipine metabolism was measured as for nifedipine, but using 0.1 mM-nicardipine and detection by A_{254}. Cyclosporin A metabolism was measured using 50 μM-[^{3}H]CsA (1.6 mCi/μmol), whilst the reaction time was increased to 30 min and the amount of microsomal protein and the total incubation volume were decreased to 0.3 mg and 0.1 ml respectively. For analysis, the reaction was stopped with 0.1 ml of ice-cold acetonitrile, the tubes vortex-mixed, protein precipitated at 2000 ***g*** for 20 min and the supernatants filtered at 0.45 μm. Overall recovery of ^{3}H in the assay to this stage was greater than 95%. A portion (75 μl) of the filtrate was analysed by h.p.l.c. at 70 °C, using a 25 cm × 0.46 cm Hypersil 5 μ SAS column eluted at 1 ml/min with an initially isocratic mobile phase of water/acetonitrile (4:1, v/v) for 5 min followed by a linear gradient of water/acetonitrile increasing from 3:2 to 2:3 (v/v) over the succeeding 55 min. CsA and its metabolites were detected by flow-through ^{3}H detection, using a Reeve Analytical flow-through ^{3}H detector operating in heterogeneous counting mode. Immunoinhibition of nifedipine and CsA metabolism by a polyclonal antibody against P450hA7 was carried out as described below.

N-Terminal amino acid sequence analysis

N-Terminal amino acid liquid-phase sequencing of a lyophilized sample of P450hA7, previously dialysed against 0.1% cholate and finally redissolved in 0.1% trifluoroacetic acid, was carried out as described elsewhere (Russell *et al.*, 1986).

SDS/PAGE, immunoblotting, immunoinhibition and immunoquantification

Liver microsomal proteins and purified P450hA7 were resolved by discontinuous SDS/PAGE (Laemmli, 1970), using a 7–10% polyacrylamide linear gradient resolving gel, as described previously (Burke *et al.*, 1983). Proteins separated by SDS/PAGE were electroblotted onto nitrocellulose by the method of Towbin *et al.* (1979) and immunostained, as described previously (Barnes *et al.*, 1987), but substituting 2% 'Marvel' skimmed milk powder for 10% new-born-calf serum in the blot washing buffer. Three different preparations of primary antibodies were used: (i) murine monoclonal antibodies against P450hA7, prepared as 45% $(NH_4)_2SO_4$ fractions dissolved in 5% of the original volume in PBS as described previously (Barnes *et al.*, 1987); (ii) polyclonal antibodies against P450hA7, prepared as the lyophilized 50% $(NH_4)_2SO_4$ fraction of serum taken from New Zealand White rabbits that had been immunized with 0.2 mg of purified P450hA7 in 0.5 ml of complete Freund's adjuvant diluted equally with PBS, followed by a boost after 4 weeks with 0.1 mg of pure P450hA7 in incomplete Freund's adjuvant and exsanguination after a further 11 days; (iii) polyclonal antibodies against purified forms of rat liver *P*-450, prepared as described previously (Wolf *et al.*, 1986). For immunostaining, the monoclonal antibodies were diluted 80-fold in PBS, whilst the polyclonal antibodies were diluted to 4 μg/ml in PBS. For immunoinhibition studies, stock solutions of polyclonal antibodies against P450hA7 (80 mg/ml in PBS), or equivalent preparations of pre-immune serum, prepared as described above, were incubated with human liver microsomal suspensions for 20 min on ice with occasional swirling, prior to starting the reaction. For immunoquantification of P450hA7, 1–15 μg of human liver microsomes, plus calibration standards of 5–30 ng of purified P450hA7 on the same gel, were immunoblotted and immunostained as described above and the staining intensity was quantified using a Bio-Rad model 620 video densitometer in reflectance mode. The curves of absorbance versus amount of microsomal or purified P450hA7 protein were linear and overlapping in the range used.

Proteolytic cleavage

Samples of human liver microsomes (100 μg of protein from individual no. 7, the epileptic, and 400 μg of protein from a mixture of microsomes from individuals nos. 6, 9 and 10, who had not received drug therapy) and of purified P450hA7 (4 μg of protein) were diluted to 80 μl in 250 mM-Tris/HCl, pH 6.8, containing 0.1% SDS, 10% glycerol and 0.001% Bromophenol Blue and digested for 8 min at 37 °C with 5 μg of V8 protease, followed by the addition of 10 μl of 50% 2-mercaptoethanol containing 10% SDS and incubation at 100 °C for 3 min. A portion (15 μl) of each digest was then analysed by SDS/PAGE and immunoblotting as above.

Treatment of rats with inducing agents

Rats were treated once daily for 5 days with phenobarbitone (PB; 1% solution of the sodium salt in water) at a dose of 20, 40 or 80 mg/kg intraperitoneally, or alternatively with phenytoin (PHT; 1% in water) 80 mg/kg intraperitoneally or with carbamazepine (CBZ; 2% suspension in 10% ethanol in olive oil) 80 mg/kg orally. Other treatments were as described previously (Barnes *et al.*, 1987). Control treatments with appropriate drug vehicles were also carried out.

Purification of rat cytochromes *P*-450

Various forms of *P*-450 were purified from liver microsomes of 250 g male Sprague–Dawley rats as described elsewhere (Wolf *et al.*, 1986).

RESULTS

Purification

Individual no. 7, from whose liver the *P*-450 form P450hA7 was purified as described above, was a 33-year-old, non-smoking epileptic who drank alcohol 'occasionally' and who had been receiving anticonvulsant drug therapy for several years until his death from an unspecified injury. The presence of anticonvulsant drugs in this individual was confirmed by their blood levels measured 3 days before his death (mg/l, with normal therapeutic ranges shown in parentheses): phenobarbitone, 33 (15–40); phenytoin, 10 (10–20); carbamazepine, 4 (4–12) and valproate, 43 (trough > 100). P450hA7 showed a single protein band on SDS/PAGE of M_r 50400 (Fig. 1), gave a single *N*-terminal amino acid sequence (see below) and had a specific content of 15.5 nmol of *P*-450/mg of protein, all of which suggests that the protein was of high purity. Of the initial total microsomal *P*-450, 11% was recovered as purified P450hA7.

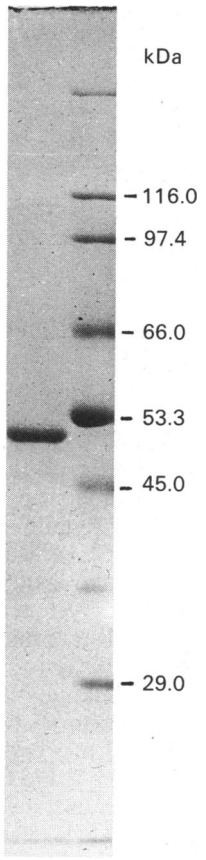

Fig. 1. Purified cytochrome P450hA7 electrophoresed on SDS/PAGE

Purified cytochrome P450hA7 (1.5 μg) was electrophoresed in the left-hand lane as described in the Materials and methods section. The right-hand lane shows the molecular mass markers.

Characterization of cytochrome P450hA7

The *N*-terminal amino acid sequence was obtained for 30 residues as follows:

A L I P D L A M E T W L L L A V S L V L L Y L Y G T S X E G

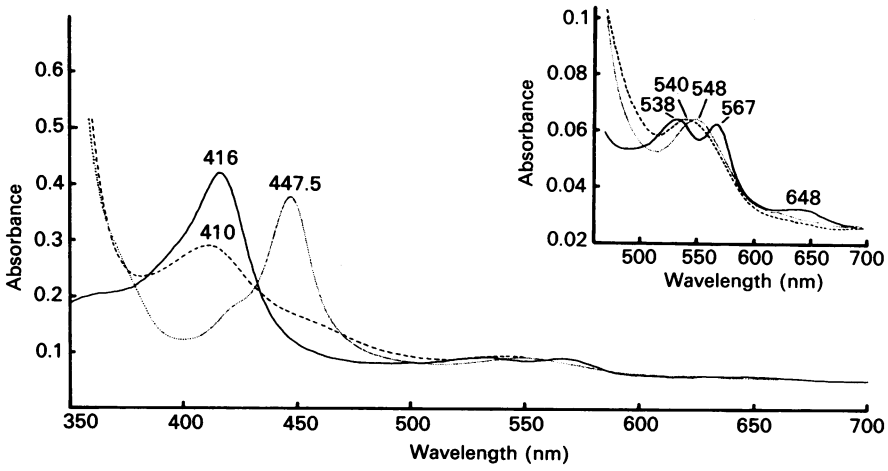

Fig. 2. Absolute optical absorption spectra of cytochrome P450hA7

The spectra of buffer solutions of oxidized (——), dithionite-reduced (----) and dithionite-reduced – CO-complexed (·····) purified cytochrome P450hA7 were measured against a reference solution of buffer, as described in the Materials and methods section.

The absolute absorption spectra of oxidized, dithionite-reduced and dithionite-reduced – CO-complexed P450hA7 are shown in Fig. 2. The 648 nm absorption peak indicates that a proportion of P450hA7 was in the high-spin state.

The activity of P450hA7 in a reconstituted monooxygenase system was tested with a number of substrates and compared with the activity of the hepatic microsomal suspension from which it was purified (Table 1). P450hA7 showed relatively high activity for nifedipine oxidation and the *N*-demethylation of benzphetamine, ethylmorphine, erythromycin and aminopyrine, but had very low lauric acid hydroxylation activity. P450hA7 showed no nicardipine oxidation or cholesterol 7α-hydroxylation activity, was unable to 7-*O*-dealkylate any alkoxyresorufin in the homologous series from methoxy- to octoxyresorufin plus benzyloxyresorufin, and was devoid of 7-hydroxylation activity toward the unsubstituted parent compound of the alkoxyresorufins, phenoxazone.

A polyclonal antibody preparation against purified P450hA7, which recognized purified P450hA7 on immunoblots (see Fig. 6) and gave a single stained band at 50.4 kDa on immunoblots of human liver microsomes (results not shown), failed to inhibit nifedipine oxidation by the hepatic microsomes from which P450hA7 had been purified (individual no. 7). Moreover, the same antibody stimulated ethylmorphine and erythromycin *N*-dealkylation activities in the same microsomes approx. 2-fold (preimmune serum preparations had no effect; results not shown). These effects were observed at antibody concentrations up to 20 mg of immunoglobulin protein per nmol of total microsomal *P*-450. In contrast, this concentration of the same antibody preparation inhibited cyclosporin A metabolism by liver microsomes from individuals nos. 11, 20, 29 and 31 to the extent of 91%, 90%, 73% and 74% respectively (Fig. 3).

Table. 1. Metabolic activities of purified cytochrome P450hA7

Total cytochrome *P*-450 was measured spectrophotometrically as its reduced ferrous-carbonyl complex, as described in the Materials and methods section. Reactions were measured using either liver microsomes from individual no. 7 or cytochrome P450hA7 purified from the same microsomes and reconstituted with NADPH:cytochrome *P*-450 reductase, as described in the Materials and methods section.

Reaction	Activity (nmol/min per nmol of total cytochrome *P*-450)	
	P450hA7	Microsomes
Aminopyrine *N*-demethylation	5.4	7.2
Benzphetamine *N*-demethylation	8.0	8.1
Benzyloxyresorufin *O*-dealkylation*	0	0.2
Cholesterol 7α-hydroxylation	0	0
Erythromycin *N*-demethylation	5.75	2.6
Ethoxyresorufin *O*-de-ethylation	0	0.05
Ethylmorphine *N*-demethylation	7.5	9.6
Laurate 11- and 12-hydroxylation	0.1	1.6
Methoxyresorufin *O*-demethylation	0	0.05
Nicardipine oxidation	0	7.3
Nifedipine oxidation	17.7	4.0

* Activities with alkoxyresorufins in the series methoxy to octoxy not shown here ranged between 0.002 and 0.009 in the microsomes but were zero with purified cytochrome P450hA7.

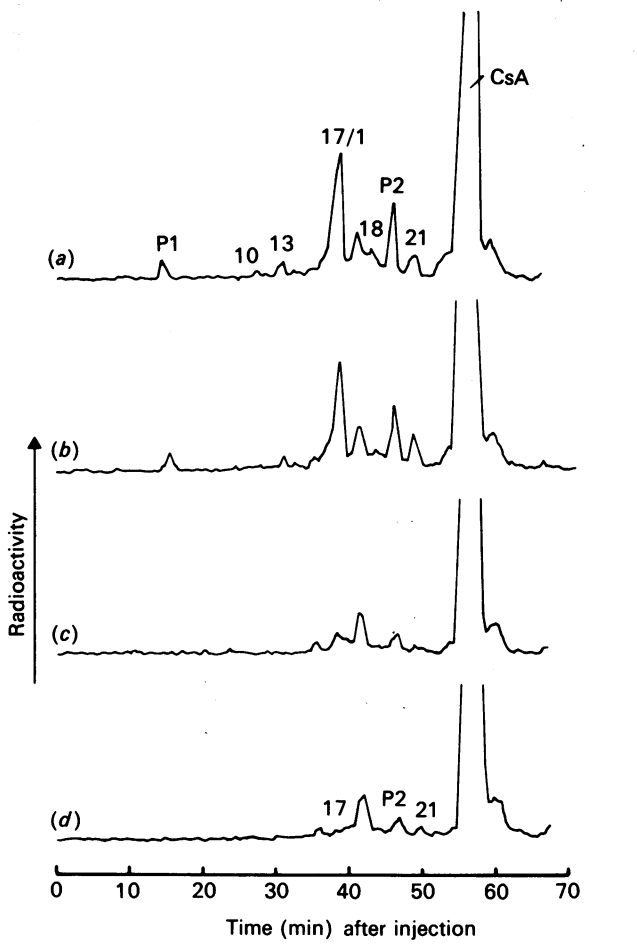

Fig. 3. Inhibition of human hepatic microsomal metabolism of cyclosporin A by an antibody against P450hA7

Hepatic microsomes from individual no. 11 were incubated with [^{3}H]cyclosporin A and analysed by h.p.l.c. with on-line radioactivity detection as described in the Materials and methods section. The Figures show the h.p.l.c. traces from four 30 min incubations, with in each case 75 μl of extract injected into the h.p.l.c. and a detector sensitivity of 32 c.p.s.: (*a*) the complete reaction mixture; (*b*) the complete reaction mixture preincubated for 20 min with pre-immune IgG (20 mg of IgG/mg of microsomal protein); (*c*) the complete reaction mixture preincubated for 20 min with polyclonal antibody IgG to P450hA7 (20 mg/mg of microsomal protein); (*d*) reaction blank (microsomes + CsA without the NADPH-generating system). The identities of the cyclosporin A metabolites (shown by numbers against the peaks) are based on a comparison with a sample of authentic metabolites provided by Sandoz Ltd. Metabolites 1 and 17 were eluted together as a combined peak. The relatively large unnumbered peak eluted in all four traces between the combined peak for metabolites 17 and 1 and the peak for metabolite 18 is probably a breakdown product of CsA and not a metabolite. Similar chromatograms were obtained with liver microsomes from individuals no. 20, 29 and 31 (not shown).

Immunoquantification of cytochrome P450hA7 in human liver microsomes

Six monoclonal antibodies against P450hA7 each recognized a single 50.4 kDa protein band in immunoblots of liver microsomes from different individuals (Barnes *et al.*, 1987). P450hA7 was immunoquantified in liver microsomes of 26 individuals, using one of these monoclonal antibodies, HL4 (Table 2). Almost identical results were obtained using monoclonal antibodies HL3 and HL5 (results not shown). The mean concentration of P450hA7 (5.5 nmol/mg of microsomal protein) was 5-fold higher in the long-term anticonvulsant-treated individuals (nos. 7 and 23–26 in Table 2) than in the others (0.11 nmol/mg of protein), whilst the individual P450hA7 levels in the long-term anticonvulsant-treated patients were 3.3–6.5-fold higher than the mean for all the others. In the five long-term anticonvulsant-treated individuals P450hA7 comprised 60–193 % (mean 109 %) of the total hepatic microsomal *P*-450, compared with 17–51 % (mean 35 %) in the 12 individuals measured who had not been so treated. In these 12 individuals who had not been treated with anticonvulsants there was a significant correlation between the hepatic microsomal

Table. 2. Immunoquantification of P450hA7 in human liver microsomes

Cytochrome P450hA7 was immunoquantified in the hepatic microsomes of 26 individuals, using a monoclonal antibody against cytochrome P450hA7 as described in the Materials and methods section. The data are arranged in order of increasing P450hA7 concentration. n.d., not determined.

Individual	P450hA7 (nmol/mg*)	Sex	Age	Smoking	Alcohol	Drug therapy and other information†
29	n.d.	F	63	Unknown	Unknown	None
31	n.d.	F	45	10/day	Social	Tranxene (1 tablet/day)
4	0.047	M	33	20/day	Social	None
5	0.047	M	21	Unknown	Unknown	None
16	0.058	F	68	Unknown	Unknown	PHT (1 g) once 4 days pre-death
22	0.066 (29)	M	54	Unknown	Heavy	None
9	0.070 (17)	M	25	Unknown	Unknown	None
11	0.070 (25)	M	51	< 5/day	Unknown	None
15	0.072 (27)	F	41	Unknown	Unknown	None
1	0.074	F	15	None	None	None
10	0.092 (29)	M	19	None	None	None
17	0.098	M	42	Unknown	Unknown	PHT (300 mg) and dexamethasone (24 mg) for 2 days pre-death
20	0.098 (33)	F	62	None	None	Papaveratrum
3	0.102	M	49	None	Social	None
6	0.135 (31)	M	51	40/day	Heavy	None; nodular liver
8	0.135	M	66	10/day	None	None
21	0.138 (51)	F	48	Unknown	Unknown	None
14	0.147 (51)	F	29	Unknown	Unknown	Terminal betamethasone and methylprednisolone
19	0.151 (44)	M	62	6/day	Social	None
12	0.167 (45)	M	34	Unknown	Unknown	None
2	0.169	M	40	Unknown	None	Terminal chlorpromazine
18	0.174	M	34	Unknown	Unknown	PHT (300 mg) and dexamethasone (18 mg) for 3 days pre-death
13	0.220 (44)	F	49	Unknown	Unknown	Long-term amitriptyline (100 mg) and trifluoperazine (15 mg)
23	0.363 (60)	M	61	20/day	Heavy	Long-term PB (30 mg) and primidone (750 mg); Hodgkin's disease
26	0.377 (80)	F	55	None	Social	Long-term PHT (325 mg)
25	0.636 (193)	F	41	5/day	None	Long-term PHT (450 mg) prednisolone (5 mg) and theophylline (600 mg)
24	0.654 (88)	F	75	None	None	Long-term PHT (50 mg); gastric carcinoma
7	0.712 (123)	M	33	None	Social	Long-term PB, PHT, CBZ and valproate

* Values in parentheses are the P450hA7 concentration as a percentage of the total concentration of CO-binding *P*-450 where obtained.

† Drug doses are total daily amounts unless stated otherwise.

concentrations of P450hA7 and total *P*-450 ($r = 0.65$, $P < 0.05$), whereas no such correlation existed for the five long-term anticonvulsant-treated patients.

There was no correlation ($r = 0.34$) between nifedipine oxidase activity and P450hA7 concentration in hepatic microsomes from the seven individuals measured who had not received anticonvulsant therapy (P450hA7 was immunoquantified using monoclonal antibody HL4 against P450hA7; nifedipine oxidase activities for these individuals, nos. 6, 9, 10 and 12–15, were 0.40, 0.15, 0.62, 0.24, 0.78, 0.25 and 0.44 nmol/min per mg of protein respectively). The nifedipine oxidase activity was much higher in the anticonvulsant-treated individual, no. 7 (2.58 nmol/min per mg of protein).

In contrast, CsA oxidase activity did correlate ($r = 0.91$, $P < 0.05$) with the P450hA7 concentration in liver microsomes from another five individuals who had not undergone drug therapy (CsA oxidase activities for these individuals, nos. 11, 15 and 20–22, were 19.5, 16.1, 23.2, 26.1 and 14.8 pmol/min per mg of protein respectively). The anticonvulsant-treated individual, no. 7, had a 1.7-fold higher microsomal CsA oxidase activity (43.7 pmol/min per mg of protein) and a 5.2-fold higher P450hA7 concentration (0.712 nmol/mg of protein) than the most active of the non-treated individuals (no. 21) and the correlation was maintained when individual no. 7 was included in the comparison ($r = 0.95$, $P < 0.05$).

Immunoblotted peptide map

Purified P450hA7, liver microsomes from an anticonvulsant-treated individual (no. 7, from whom P450HA7 had been purified) and a mixture of liver microsomes from three individuals who had not been receiving drug treatment (nos. 6, 9 and 10) were subjected to partial proteolysis with V8 protease and the resulting peptides were separated on SDS/PAGE and immunoblotted with monoclonal antibody HL3. The immunostained peptide map was similar for each sample (Fig. 4). There were no bands in either of the microsomal samples (middle and left-hand lanes) that were not also in the purified P450hA7 (the right-hand lane), while bands present only in the purified *P*-450 sample were visible to the eye in the microsomal samples but too faint to photograph. There were no stained bands in the middle lane (microsomes from non-drug-treated individuals) that were not also present in the left-hand lane (microsomes from an anticonvulsant-treated individual), while the bands that were present in the left-hand but not the middle lane were, again, visible but too faint to photograph.

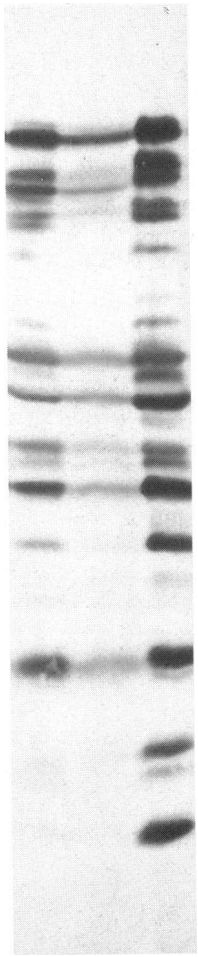

Fig. 4. Immunoblotted partial proteolysis peptide map of purified cytochrome P450hA7 and human liver microsomes

Samples were partially proteolysed with V8 protease, subjected to SDS/PAGE and immunoblotted with a monoclonal antibody against cytochrome P450hA7, as described in the Materials and methods section. Three lanes are shown: the left-hand lane is liver microsomes from the anticonvulsant-treated individual no. 7 (100 μg of protein); the middle lane is a mixture of equal amounts of liver microsomes from individuals no. 6, 9 and 10, who had not received drug treatment (400 μg of protein, the maximum that could be loaded); the right-hand lane is purified cytochrome P450hA7 (4 μg of protein).

Immunological cross-reactivity between human P450hA7 and rat *P*-450 forms

A polyclonal antibody preparation to human P450hA7 was immunoblotted against a range of highly purified rat liver *P*-450 forms which, from their *N*-terminal sequences, monomeric molecular masses and induction characteristics, were equivalent to Levin's forms a, b, c, d, e and k and to Guengerich's form *P*-450$_{PCN\text{-}E}$ (Wolf *et al.*, 1986; Guengerich, 1987); only the major PCN-inducible form, *P*-450$_{PCN\text{-}E}$, was recognized even when the other forms were present in 10-fold higher amounts (results not shown). When polyclonal antibodies to each of these rat *P*-450 forms were immunoblotted against purified human P450hA7, recognition was given only by the antibody against P450$_{PCN\text{-}E}$ (results not shown). A polyclonal antibody to human P450hA7 was immunoblotted against liver microsomes from rats pretreated *in vivo* with one of various different inducers: in each rat sample a single protein band was recognized, with the same M_r (50400) as purified human P450hA7 and showing a similar pattern of induction to P450$_{PCN\text{-}E}$, i.e. it was induced by PB, PCN, isosafrole and Aroclor 1254 (Guengerich, 1987) (Fig. 5).

The six monoclonal antibodies against human P450hA7 have been classified, according to their recognition of induced and constitutive rat liver microsomal *P*-450, into four groups: HL3, HL4 = HP16, HP10 = HL5, and HP3 (Barnes *et al.*, 1987). Liver microsomes from rats pretreated *in vivo* with PB, PHT, CBZ or PCN were immunoblotted against each of the four groups of monoclonal antibodies (Fig. 6). PCN was the most

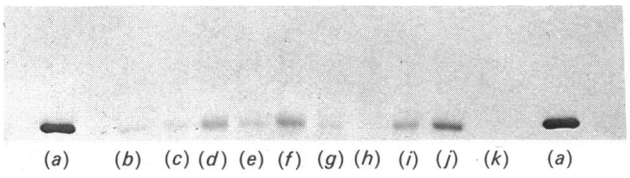

Fig. 5. Immunoblot of liver microsomes from rats treated with various inducing agents using a polyclonal antibody against cytochrome P450hA7

The inducer treatments and the immunoblotting were carried out as described in the Materials and methods section. Lanes (*a*) contained purified cytochrome P450hA7. The other lanes contained microsomes (5 μg of protein per lane) from rats treated with different inducers, as follows: (*b*) saline-treated control for clofibrate, phenobarbitone and imidazole treatments; (*c*) clofibrate; (*d*) phenobarbitone; (*e*) 2.5% Tween 80-treated control for pregnenolone 16α-carbonitrile treatment; (*f*) pregnenolone 16α-carbonitrile; (*g*) imidazole; (*h*) olive oil-treated control for isosafrole, Aroclor 1254 and 3-methylcholanthrene treatments; (*i*) isosafrole; (*j*) Aroclor 1254; (*k*) 3-methylcholanthrene. Microsomes were pooled from three rats receiving the same treatment.

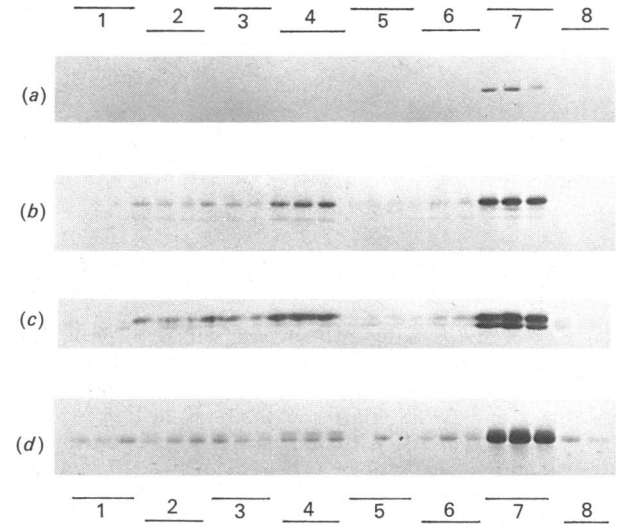

Fig. 6. Immunoblot of liver microsomes from rats treated with anticonvulsant drugs or PCN using four monoclonal antibodies against human cytochrome P450hA7

The inducer treatments and the immunoblotting were carried out as described in the Materials and methods section. The stained segments of immunoblots with each of four different monoclonal antibodies are shown: (*a*) antibody HL4; (*b*) antibody HP3; (*c*) antibody (HL3); (*d*) antibody HP10. The same series of microsome samples was run on each immunoblot (1 μg of protein per lane). All but two of the samples are arranged in groups of three. Groups 1–7 are microsomes from three individual rats, each of the same drug treatment group, while group 8 is microsomes from two individual rats. The treatments corresponding to the groupings are as follows: (1) untreated controls for all groups except group 6; (2), (3) and (4) phenobarbitone at doses of 20, 40 and 80 mg/kg respectively; (5) phenytoin (80 mg/kg); (6) carbamazepine (80 mg/kg); (7) pregnenolone 16α-carbonitrile (50 mg/kg); (8) 10% ethanol in olive oil control for group 6. Saline and 2.5% Tween 80 (the vehicle for PCN) gave identical results to the untreated group.

effective inducer of the rat protein(s) recognized by each of the monoclonal antibodies to human P450hA7. The proteins recognized by antibodies HL3 and HP3 were detected only weakly in untreated rats, were strongly induced by PB in a dose-dependent manner, but were only minimally induced by CBZ and were not induced by PHT. The protein recognized by antibody HL4 would appear to be a hitherto unrecognized form of *P*-450, being present only in microsomes from PCN-induced rats. Antibody HP10 recognized two proteins in microsomes from PB-treated rats: the higher-M_r protein appeared to be absent from control rats and to be induced by PB but not by PHT or CBZ, whereas the lower-M_r protein was constitutive in control rats and was not induced by PB, PHT or CBZ. Among the three rats in some of the treatment groups there were distinct interindividual differences in the intensity of staining of the recognized protein(s), suggesting inter-individual differences in the levels of the recognized protein(s) and in the inducing effects of the drugs. This was most clearly evident in the PCN-treatment group blotted with HL4 and in the non-PCN treatment groups blotted with HP10.

DISCUSSION

In hepatic microsomes from phenobarbitone-treated rats the majority of the *P*-450 is composed of the major PB-induced form(s) (Adesnik & Atchison, 1985). Consequently our strategy for purifying an anticonvulsant-induced form of human *P*-450 was to isolate the major *P*-450 fraction from the liver of an epileptic (individual no. 7) who had been receiving long-term anticonvulsant drug therapy. At each stage of the purification the fraction containing P450hA7 was clearly the largest single *P*-450 fraction and never constituted less than 35% of the total *P*-450 applied to the column. Immunoquantification using a monoclonal antibody subsequently showed that P450hA7 comprised 123% of the total hepatic microsomal *P*-450 in this patient, indicating that P450hA7 had indeed been the single most prevalent form of *P*-450 present.

Immunoquantification in the hepatic microsomes of 26 individuals showed that P450hA7 is a major constitutive *P*-450 form and provided the main evidence that it is inducible by anticonvulsant therapy. Both the concentration of P450hA7 and the percentage that it comprised of the total *P*-450 were higher in the five individuals who had been receiving long-term anticonvulsant therapy than in 21 individuals who had not. Since the spectrophotometric assay used for total *P*-450 measures only holo-haemoprotein, whereas immunoquantification measures both haemo- and apoprotein, the finding that P450hA7 comprised more than 100% of the total *P*-450 in two of the long-term anticonvulsant-treated individuals raises the question of whether the 'excess' represents P450hA7 apoprotein and, if so, whether haem incorporation is a limiting factor in the induction of holo-cytochrome *P*-450 in man. This is important, since only holo-*P*-450 would be metabolically active. The idea that a proportion of the anticonvulsant-induced P450hA7 may be metabolically inactive is supported by our observation that, among the six individuals measured, the apparent turnover number for CsA metabolism was lower in the person (no. 7) who had been receiving long-term anticonvulsant treatment

(61 pmol of CsA metabolized/min per nmol of P450hA7) than in the five non-treated individuals (ranging between 189 and 279 pmol/min per nmol of P450hA7).

P450hA7 was not significantly increased in three individuals (nos. 16–18) who had received very short-term phenytoin treatment immediately prior to death, in accordance with observations that induction by anticonvulsant drugs in man requires several weeks of continuous treatment to reach a maximum effect (Hildebrandt *et al.*, 1979; Park & Breckenridge, 1981). It is possible that the immunoquantification study measured a form of *P*-450 in the anticonvulsant-treated individuals that was different from, but shared an epitope with, the form measured in non-drug-treated individuals. The fact, however, that a monoclonal antibody recognized virtually identical peptide maps with purified P450hA7 and microsomes from an anticonvulsant-treated individual and three individuals who had not been receiving drug therapy, suggests that the form which was induced in the anticonvulsant-treated individuals was the same as the form that was present at lower concentrations in the non-drug-treated individuals and that in both cases this was P450hA7.

The hepatic microsomal concentration of P450hA7 did not appear to correlate with either the age or sex of the individual. Levels of P450hA7 were not increased in those individuals who were known to be cigarette smokers but who had not received anticonvulsant therapy (nos. 4, 6, 8 and 19), whilst among the five individuals who had received such therapy the level was lowest in the heavy smoker (no. 23). This agrees with a view that smoking induces in human liver microsomes primarily a form of *P*-450 related to the rat forms *P*-450c and *P*-450d (members of the P450IA family) (Sesardic *et al.*, 1988), which our results have shown not to be closely related to P450hA7 (probably a member of the P450III family; see below). It is also in accordance with a concept that the induction of one family of *P*-450 forms is sometimes accompanied by the concomitant suppression of other *P*-450 families (Adesnik & Atchison, 1985). It is interesting that the P450hA7 level in an individual who had received antidepressant drug therapy (no. 13) was higher than in all the patients except the five who had received long-term anticonvulsant therapy, suggesting the possibility that antidepressants induce P450hA7.

In view of the range of different anticonvulsant drugs that had been taken by the five individuals in whom P450hA7 had apparently been induced, it is not possible to decide which anticonvulsants were primarily responsible for the induction. However, the results for individuals no. 23 and 24 suggest that phenobarbitone (or its close analogue and prodrug, primidone) and phenytoin were inducers of P450hA7 in man; similarly, in rats phenobarbitone induced a form of *P*-450 related to P450hA7. Park & Breckenridge (1981) reported that in man phenytoin is a weaker inducer of drug metabolism than is phenobarbitone, but Perucca *et al.* (1984) claim that phenobarbitone, phenytoin, primidone and carbamazepine induce to similar extents when given in doses that are therapeutically equipotent. Valproate does not appear to induce *P*-450 in either man (Perucca *et al.*, 1984) or rats (Walker *et al.*, 1986). There is a major limitation, however, in current attempts to compare clinical induction measured in patients *in vivo* with the results from studies *in vitro* such as this, in that the induction parameters generally measured *in vivo*, for example antipyrine half-life, may have little relationship with the specific form(s) of *P*-450 studied *in vitro*.

The results presented here consistently identify P450hA7 as being a member of the P450III family, being closely related to the human *P*-450 forms $P450_{NF}$, P450HLp and *P*-450 human-1 and to the PCN-inducible family of rat *P*-450. The 30-residue *N*-terminal amino acid sequence of P450hA7 is identical over its first part with the 21-residue sequence published for P450HLp (Watkins *et al.*, 1985), the 18-residue sequence for *P*-450 human-1 (Kawano *et al.*, 1987) and the 21-residue sequence predicted from cDNA for $P450_{NF}$ (Beaune *et al.*, 1986*a*), and is 57 % identical to the cDNA-predicted *N*-terminal sequence for rat P450PCN1 and P450PCN2 (Gonzalez *et al.*, 1986). In addition, the metabolic capabilities of P450hA7 are also similar to P450HLp and $P450_{NF}$ (Watkins *et al.*, 1985; Guengerich *et al.*, 1986*a*). Furthermore, antibodies against P450hA7 selectively recognized PCN-induced rat liver microsomal proteins and a purified PCN-induced rat *P*-450, equivalent to $P450_{PCN-E}$. The wavelength of the Soret absorption peak of the ferrous-carbonyl complex of P450hA7 was the same (447.5 nm) as reported for *P*-450 human-1 but lower than that for $P450_{NF}$ (449 nm). However, the 648 nm absorption peak of ferric P450hA7, which is indicative of a proportion of P450hA7 being in the high-spin state, was reportedly absent from both $P450_{NF}$ and P450 human-1.

Watkins *et al.* (1985) reported that P450HLp was induced in the liver microsomes of two patients who had been administered phenobarbitone, phenytoin and dexamethasone for 2 or 3 days prior to death, but they concluded that dexamethasone was the major inducer. Since only one of the five long-term anticonvulsant-treated individuals in our study had received glucocorticoids, our results strongly indicate that anticonvulsants are major inducers of this type of human *P*-450. It has long been known that anticonvulsant therapy increases the renal clearance of 6β-hydroxycortisol in man and this has been suggested as a practical indicator of *P*-450 induction (Hildebrandt *et al.*, 1979; Park & Breckenridge, 1981; Desager *et al.*, 1987). Since the human *P*-450 forms $P450_{NF}$, $P450_5$ and *P*-450 human-1, which are probably closely related to each other and to P450hA7, catalyse the 6β-hydroxylation of several steroids, including cortisol (Guengerich *et al.*, 1986*a*,*b*; Beaune *et al.*, 1986*b*; Kawano *et al.*, 1987), it may be that anticonvulsant-induced increases in human urinary 6β-hydroxycortisol reflect primarily the induction of hepatic P450hA7 or its equivalent forms.

In view of the report by Guengerich *et al.* (1986*a*) that polyclonal antibodies to human $P450_{NF}$ inhibited nifedipine oxidation by human liver microsomes, the failure of polyclonal antibodies against P450hA7 to inhibit nifedipine oxidation, despite the purified enzyme being able to carry out this reaction, suggests that P450hA7 may not be the sole form responsible for nifedipine oxidation in human liver microsomes. This conclusion is supported by the lack of correlation between the nifedipine oxidase activity and P450hA7 concentration of human liver microsomes. It is noteworthy that P450hA7 did not metabolize nicardipine, a structural analogue of nifedipine that was not included in Bocker & Guengerich's (1986) study of the ability of $P450_{NF}$ to oxidize 18 analogues of nifedipine. Nicardipine is extensively metabolized in man, however (Rush *et al.*,

1986), suggesting that the bulky aryl substituent on the pyridine ring of nicardipine directs it as a substrate to a different form of human *P*-450.

Cyclosporin A, an important immunosuppressive agent, is extensively metabolized by man *in vivo* and this metabolism is increased by anticonvulsant therapy (Maurer & Lemaire, 1986). Kronbach *et al.* (1988) have presented indirect evidence that $P450_{NF}$ catalyses cyclosporin A metabolism in human liver microsomes. Our results indicate that P450hA7 has a major role in cyclosporin A metabolism and that induction of this form of *P*-450 may be the main cause of increased cyclosporin A metabolism in anticonvulsant-treated patients. The fact that cyclosporin A and erythromycin are alternative substrates for P450hA7 provides a rational explanation for clinical observations that erythromycin inhibits cyclosporin A metabolism (Maurer & Lemaire, 1986; Vereerstraeten *et al.*, 1987).

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