Purification and characterization of a vitamin D₃ 25-hydroxylase from pig liver microsomes

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A cytochrome *P*-450 which catalyses 25-hydroxylation of vitamin D₃ has been purified to apparent homogeneity from pig liver microsomes. The specific content of cytochrome *P*-450 was 12 nmol·mg of protein⁻¹, and the preparation showed a single band with an apparent *M_r* of 50 500 upon SDS/PAGE. A monoclonal antibody raised against the vitamin D₃ 25-hydroxylase reacted strongly with the purified 25-hydroxylating cytochrome *P*-450 from pig kidney microsomes [Bergman & Postlind (1990) *Biochem. J.* 270, 345–350]. The liver enzyme showed structural and functional properties very similar to those of the kidney enzyme. The two enzymes differed with respect to only one of the first 16 *N*-terminal amino acids. The vitamin D₃ 25-hydroxylase in pig liver microsomes exhibited a turnover and an apparent *K_m* for 25-hydroxylation of vitamin D₃ which were of the same order of magnitude as those of a well-characterized male-specific 25-hydroxylating cytochrome *P*-450 in rat liver microsomes. The two enzymes differed structurally. The pig liver enzyme was, in contrast to the rat liver enzyme, not sex-specific, and did not catalyse 16 α -hydroxylation of testosterone. These properties of the 25-hydroxylase in rat liver microsomes have led to questions on the role of microsomal 25-hydroxylation of vitamin D₃. It is concluded that studies on microsomal 25-hydroxylation with the rat may be misleading. The results of the present study show that the pig appears to be a representative species for evaluation of vitamin D₃ hydroxylases in other mammals, including man.

INTRODUCTION

A cytochrome *P*-450-dependent 25-hydroxylation is the first step in the transformation of vitamin D₃ (cholecalciferol) into its active hormonal form, 1 α ,25-dihydroxyvitamin D₃ [1–3]. It was initially suggested that the physiologically important 25-hydroxylase is located in the microsomal fraction of liver [4]. Cytochromes *P*-450 catalysing 25-hydroxylation have been subsequently purified, not only from liver microsomes [5,6] but also from liver mitochondria [7–9] and kidney microsomes [10]. Whereas the liver appears to be the major source of 25-hydroxyvitamin D₃ under normal conditions [1], the role of the liver microsomal 25-hydroxylase in the bioactivation process has been questioned [11–14]. In particular, some properties of highly purified 25-hydroxylating cytochrome *P*-450 from liver microsomes of the male rat appear to be incompatible with the concept of an important role for microsomal 25-hydroxylation. Thus the purified rat enzyme also catalyses 16 α -hydroxylation of testosterone, is male-specific and is not present in significant amounts in the female rat [6,12,15]. In fact, it has been suggested that the major microsomal vitamin D₃ 25-hydroxylase in the male rat might be identical with the testosterone 16 α -hydroxylating male-specific CYP2C11 [6,12,16]. On the other hand, results have also been reported suggesting that CYP2C11 and vitamin D₃ 25-hydroxylase are different gene products, although they are immunologically indistinguishable [17].

The obvious confusion concerning the identity, properties and importance of the microsomal vitamin D₃ 25-hydroxylase may depend on the fact that rat liver has been the only source for studies of purified 25-hydroxylating enzymes. Species differences may exist and the rat may not be a representative animal for evaluation of vitamin D hydroxylases in other mammals, including man. Recently, this laboratory reported the purification from pig kidney of a microsomal cytochrome *P*-450 active in the 25-hydroxylation of vitamin D₃ [10]. Studies with a monoclonal

antibody indicated that a similar 25-hydroxylase is present in the liver of both male and female pigs [10]. The present paper reports the purification and characterization of the 25-hydroxylating cytochrome *P*-450 in pig liver microsomes.

EXPERIMENTAL

Materials

25-Hydroxy[23,24(n)-³H]vitamin D₃ (105.5 Ci/mmol), 1 α , 25-dihydroxy[23,24(n)-³H]vitamin D₃ (91.4 Ci/mmol) and [4-¹⁴C]testosterone (8.7 mCi/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). 5 β -([7 β -³H]Cholestane-3 α ,7 α -diol (400 mCi/mmol) was prepared as described previously [18,19]. Unlabelled vitamin D₃ was obtained from Sigma Chemical Co. Unlabelled 1 α -hydroxyvitamin D₃ was a gift from Lövens, Copenhagen, Denmark. Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad. Hydroxyapatite was mixed with an equal amount (w/w) of Whatman CF-1 cellulose powder before chromatography. Octylamine-Sepharose 4B was prepared by coupling 1,8-diamino-octane to CNBr-Sepharose 4B (Pharmacia). Emulgen 913 was obtained from Kao Chemicals, Tokyo, Japan. Q-Sepharose fast flow and S-Sepharose fast flow were obtained from Pharmacia. The remaining chemicals were reagent grade.

Enzyme purification

Liver microsomes from castrated pigs were prepared, cholate-solubilized and fractionated with poly(ethylene glycol) 6000 according to the procedures described by Andersson *et al.* [20]. The fraction used was that precipitated by 8–15% poly(ethylene glycol). The poly(ethylene glycol) precipitate was dissolved in 0.1 M-potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM-EDTA and 0.7% (w/v) sodium cholate. All buffers in the following purification procedure contained 20% glycerol and 0.1 mM-EDTA. The solubilized proteins were ap-

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plied to an octylamine-Sepharose column (3 cm × 40 cm) equilibrated in 0.1 M-phosphate buffer, pH 7.4, containing 0.7% (w/v) sodium cholate. After washing with 0.1 M-phosphate buffer, pH 7.4, containing 0.46% sodium cholate, cytochrome *P*-450 active in the 25-hydroxylation of vitamin D₃ was eluted as one peak with 0.1 M-phosphate buffer, pH 7.4, containing 0.37% sodium cholate and 0.08% (w/v) Emulgen 913. Cytochrome *P*-450 fractions were pooled, diluted 1:4 with 20% glycerol/0.1 mM-EDTA, and applied to a hydroxyapatite column (5 cm × 40 cm), equilibrated in 10 mM-phosphate buffer, pH 7.4. The hydroxyapatite column was washed with equilibrating buffer containing 0.2% Emulgen 913, and cytochrome *P*-450 was eluted with 35, 120, and 300 mM-phosphate buffers containing 0.2% Emulgen 913. The 120 mM-phosphate eluate was concentrated using an Amicon Diaflo PM 30 membrane and dialysed against 10 mM-phosphate buffer, pH 8.0, containing 0.1% sodium cholate and 0.4% Emulgen 913. The sample was injected into a Q-Sepharose column (4 cm × 30 cm) equilibrated in the same buffer as used for dialysis. The flow rate was 2 ml/min. The vitamin D₃ 25-hydroxylase activity was eluted with the equilibrating buffer. These non-bound cytochrome *P*-450 fractions were pooled, concentrated and dialysed against 10 mM-phosphate buffer, pH 7.4, containing 0.1% sodium cholate and 0.4% Emulgen 913. The dialysate was applied to an S-Sepharose column (2 cm × 30 cm) equilibrated in the same buffer as used for dialysis. The flow rate was 2 ml/min. After washing with equilibrating buffer, cytochrome *P*-450 was eluted stepwise with 25, 150 and 500 mM-sodium acetate in the buffer. The fractions with the highest vitamin D₃ 25-hydroxylase activity were eluted with 25 mM-sodium acetate. The Q- and S-Sepharose chromatographies were performed at room temperature. All other purification steps were performed at +4 °C. Detergents were removed from the cytochrome *P*-450 preparations as described previously [20].

The kidney microsomal cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D₃ was purified from pig kidney as described previously [10]. NADPH-cytochrome *P*-450 reductase was prepared from liver microsomes of phenobarbital-treated rats as described by Yasukochi & Masters [21].

Microsomes for immunoblotting experiments were prepared from various tissues of castrated male and female pigs obtained from the local slaughterhouse. Human liver microsomes, obtained from a 26-year-old female donor who had died of an intracranial bleeding secondary to trauma, were generously supplied by Drs. J. Shoda, A. Toll, M. Axelsson, F. Pieper, K. Wikvall and J. Sjövall (unpublished work).

Production and purification of monoclonal antibodies

An 8-week-old female mouse of the Balb/c strain was used. Purified cytochrome *P*-450 from pig liver microsomes was emulsified in an equal volume of Freund's complete adjuvant. A 40 µg portion of enzyme was injected intraperitoneally; 8 weeks later, another 100 µg of the same antigen in 0.9% NaCl was injected intraperitoneally. After a further 4 days, the mouse was killed and the spleen was used as source of antibody-producing cells, as described by Andersson & Jörnvall [12]. Of 1920 microwells resulting from one single fusion, 69 produced antibodies which recognized the cytochrome *P*-450. Twenty-one hybridomas of the 80 that produced antibodies were monospecific for the antigen. It should be mentioned that antibodies produced by all these hybridomas also recognized the 25-hydroxylating cytochrome *P*-450 in pig kidney microsomes [10]. Cloning of positive cultures, purification of immunoglobulins and coupling to Sepharose were performed as described previously [12]. The monoclonal antibody was designated mAb 25H6. Immuno-

adsorbent preparation, e.l.i.s.a. and immunoblotting were performed as described by Andersson & Jörnvall [12].

The monoclonal antibody directed against the 25-hydroxylating cytochrome *P*-450 in pig kidney microsomes was the mAb 25E5 obtained by Bergman & Postlind [10]. The monoclonal antibody directed against the male-specific cytochrome *P*-450₂₅ in rat liver microsomes was the mAb 25-6 obtained by Andersson & Jörnvall [12].

Incubation procedures

Incubations were performed at 37 °C for 10 min. Vitamin D₃, 1α-hydroxyvitamin D₃, 5β-cholestane-3α,7α-diol and testosterone (40 µg of each) were incubated with 0.1 nmol of cytochrome *P*-450, 2 units of microsomal NADPH-cytochrome *P*-450 reductase and 1.2 µmol of NADPH in a total volume of 1 ml of buffer. The incubation buffer was 50 mM-Tris/acetate buffer, pH 7.8, containing 20% glycerol and 0.1 mM-EDTA for vitamin D₃ and 1α-hydroxyvitamin D₃ incubations, and 150 mM-phosphate buffer, pH 7.8, containing 20% glycerol and 0.1 mM-EDTA for testosterone and 5β-cholestane-3α,7α-diol incubations. Incubations with intact microsomes (1 mg) were performed as above, except the addition of microsomal NADPH-cytochrome *P*-450 reductase was omitted and the incubation time was 20 min. Incubations with cholesterol (5 µg) and lauric acid (40 µg) were performed as described above for vitamin D₃, except that 0.5 nmol of cytochrome *P*-450 and 6 units of NADPH-cytochrome *P*-450 reductase were used and the incubation time was 30 min. Vitamin D₃, 1α-hydroxyvitamin D₃ and cholesterol incubations were terminated by addition of 5 ml of trichloroethane/methanol (2:1, v/v). Testosterone, lauric acid and 5β-cholestane-3α,7α-diol incubations were terminated by addition of 5 ml of ethanol. To incubations with vitamin D₃ and 1α-hydroxyvitamin D₃, ³H-labelled 25-hydroxyvitamin D₃ (10000 d.p.m.) and 1α,25-dihydroxyvitamin D₃ (10000 d.p.m.) respectively, were added immediately after termination to correct for recovery during the extraction and analysis procedures. These incubations were extracted twice with 5 ml of trichloroethane/methanol (2:1, v/v) and the organic phases were dried with N₂, dissolved in 100 µl of mobile phase and submitted to straight-phase h.p.l.c.. An LKB 2150 h.p.l.c. pump, LKB 2151 variable wavelength monitor, LKB 2210 1-channel recorder and a LiChrosorb Si 60 column (150 mm × 3 mm internal diam; 5 µm; Merck) were used for the straight-phase analysis. The mobile phase was hexane/propanol-2-ol (24:1, v/v) for 25-hydroxyvitamin D₃ analysis and hexane/propanol-2-ol (9:1, v/v) for 1α,25-dihydroxyvitamin D₃ analysis. The flow rate was 0.7 ml/min. The retention time for 25-hydroxyvitamin D₃ was 6.2 min, and for 1α,25-dihydroxyvitamin D₃ it was 9.8 min. The fractions corresponding to authentic 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃ were collected and dried with N₂, dissolved in 100 µl of the mobile phase and submitted to reverse-phase h.p.l.c. A Merck Hitachi L-6000 liquid chromatography pump, a Merck Hitachi 655 A Wavelength U.V. Monitor, a Merck Hitachi D-2000 Chromatointegrator and a LiChrosorb RP-18 column (150 mm × 3 mm internal diam; 5 µm; Merck) were used for reverse-phase analysis. The mobile phase was methanol/water (23:2, v/v) for 25-hydroxyvitamin D₃ and methanol/water (17:3, v/v) for 1α,25-dihydroxyvitamin D₃. The flow rate was 0.5 ml/min, the retention time for 25-hydroxyvitamin D₃ was 6 min and for 1α,25-dihydroxyvitamin D₃ it was 7 min. The two vitamin D₃ metabolites were detected at 265 nm. The total recovery was about 60%. 5β-Cholestane-3α,7α-diol and testosterone incubations were acidified and extracted twice with ether. The organic phase was washed until neutral with water, the solvent was evaporated and the residue subjected to t.l.c. The developing solvent was ethyl acetate for

5 β -cholestane-3 α ,7 α -diol and ethyl acetate/hexane/acetic acid (70:20:5, by vol.) for testosterone. In some experiments the residue was dissolved in 100 μ l of mobile phase and submitted to reverse-phase h.p.l.c. using Berthold HPLC radioactivity Monitor LB 503. The mobile phase for 5 β -cholestane-3 α ,7 α -diol was methanol/water (17:3, v/v) and the 25-hydroxylated product had a retention time of 11.3 min. The mobile phase for testosterone was methanol/acetonitrile/water (35:10:55, by vol.) and the 16 α -hydroxylated product had a retention time of 12.4 min. Incubations with cholesterol and lauric acid were analysed as described in previous reports from this laboratory [7,20].

Incubations with antibody-coupled Sepharose

Incubations with cytochrome *P*-450 and antibody-coupled Sepharose were performed as follows. Cytochrome *P*-450 (0.2 nmol) from liver or kidney was incubated with the indicated amounts of Sepharose-bound monoclonal antibody (mAb) 25H6 or 26C5 in 0.4 ml of 50 mM-Tris/acetate buffer, pH 7.4, containing 20% glycerol, 0.1 mM-EDTA and 0.5% (w/v) CHAPS. Sepharose without antibody was used as a control. After incubation for 1 h at room temperature on a rotating mixer followed by centrifugation (4000 *g*, 10 min at 4 °C), the supernatant was used for assay of hydroxylase activity. The Sepharose pellet was washed twice with the same buffer without CHAPS and pooled. The reaction mixture was incubated for 10 min at 37 °C, terminated, extracted and analysed as described above.

Structural analysis

Cytochrome *P*-450 from liver microsomes was precipitated with acetone and the precipitate was used for amino acid analysis and *N*-terminal sequence determination.

For amino acid analysis, samples were hydrolysed in 6 M-HCl containing 0.5% (w/v) phenol in evacuated tubes at 110 °C for 24 h and analysed on an LKB 4151 Alpha Plus amino acid analyser. *N*-Terminal sequence analysis was performed with an Applied Biosystems 470A instrument. Phenylthiohydantoin derivatives were identified by reverse-phase h.p.l.c. essentially as described [22].

Other methods

SDS/PAGE was performed according to Laemmli [23] with 15% acrylamide and 0.09% bisacrylamide slab gels (15 cm $\times$ 10 cm $\times$ 0.1 cm) containing 0.1% (w/v) SDS. The gels

were polymerized by addition of 0.1% (v/v) tetramethylethylenediamine and 0.1% (w/v) ammonium hydrogen sulphate. Electrophoresis was carried out at 45 mA/slab gel and the gels were stained as described by Wray *et al.* [24]. Isoelectric focusing and immunoblotting were performed as described by Andersson & Jörnvall [12]. Protein and cytochrome *P*-450 were determined as described by Lowry *et al.* [25] and Omura & Sato [26] respectively.

RESULTS

Purification of 25-hydroxylating cytochrome *P*-450 from pig liver microsomes

A liver microsomal cytochrome *P*-450 catalysing the 25-hydroxylation of vitamin D₃ was purified from castrated male pigs. The purification procedure included solubilization of microsomes with sodium cholate, precipitation with poly(ethylene glycol) 6000 and chromatography on octylamine-Sepharose, hydroxyapatite, Q-Sepharose and S-Sepharose. The 25-hydroxylation of vitamin D₃ was analysed at the different steps in the purification procedure (Table 1). After hydroxyapatite chromatography, 25-hydroxylase activity was found in the 35, 120 and 300 mM-phosphate eluates. The major part of the 25-hydroxylase activity and the highest specific activity were found in the 120 mM-phosphate eluate. In the Q-Sepharose chromatography essentially all 25-hydroxylase activity appeared in the non-bound fraction. The final Sepharose step yielded four peaks of cytochrome *P*-450. The highest specific activity and the highest cytochrome *P*-450 content were in the 25 mM-sodium acetate fraction and this fraction was used as the final vitamin D₃ 25-hydroxylase. The enzyme preparation had a specific content of 11.7 nmol of cytochrome *P*-450 $\cdot$ mg of protein⁻¹. The specific 25-hydroxylase activity expressed as pmol of product formed $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ was about 1800 times higher in the purified enzyme than in the microsomes. The yield of cytochrome *P*-450 represented about 2.5% of all cytochrome *P*-450 present in liver microsomes. It is notable that the yield of 25-hydroxylase activity represented twice as much as the total activity present in microsomes. Thus, as can be calculated from Table 1, the total 25-hydroxylase activity in the microsomes was 33.1 nmol of product formed $\cdot$ min⁻¹, whereas the total activity in the cytochrome *P*-450 recovered after purification was 62.4 nmol $\cdot$ min⁻¹. The apparent suppression of microsomal 25-

Table 1. Purification from the livers of untreated castrated male pigs of microsomal cytochrome *P*-450 catalysing the 25-hydroxylation of vitamin D₃

Details of the purification and incubation procedures are given in the Experimental section.

	Protein (mg)	Cytochrome <i>P</i> -450		Vitamin D ₃ 25-hydroxylation		Testosterone 16 α -hydroxylation	
		(nmol)	(nmol/mg of protein)	(pmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	(pmol $\cdot$ min ⁻¹ $\cdot$ nmol of <i>P</i> -450 ⁻¹)	(pmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	(pmol $\cdot$ min ⁻¹ $\cdot$ nmol of <i>P</i> -450 ⁻¹)
Microsomes	15255	8280	0.5	2	4	287	574
Poly(ethylene glycol) (8–15%) precipitate	3965	3900	1.0	29	29	1655	1655
Octylamine-Sepharose	796	1340	1.7	99	59	2069	1241
Hydroxyapatite (120 mM-phosphate eluate)	290	943	3.3	283	87	< 10	< 10
Q-Sepharose (non-bound fraction)	84	521	6.2	606	97	< 10	< 10
S-Sepharose (25 mM-sodium acetate eluate)	17	200	11.7	3650	312	< 10	< 10

Fig. 1. PAGE of purified 25-hydroxylating microsomal cytochrome *P*-450 from pig liver

The protein samples were pretreated with SDS and mercaptoethanol at 100 °C for 2 min and subjected to PAGE in the presence of SDS. Migration was from top to bottom. Gel electrophoresis was performed according to Laemmli [23] with modifications as described in the Experimental section. Lane 1, liver microsomes (40 µg); lane 2, purified 25-hydroxylating cytochrome *P*-450 (1 µg); lane 3, a mixture of cytochromes *P*-450 1A2 and cytochrome *P*-450 IIB4 from rabbit liver microsomal fraction, prepared in this laboratory as described by Haugen & Coon [29] (1 µg of each).

hydroxylase may be due to the presence of inhibiting factor(s) in the microsomes [5,6,27]. In fact, an inhibiting protein fraction has been partially purified from rat liver microsomes [27]. An alternative explanation for the lower total activity of the 25-hydroxylase in microsomes compared with that of the purified enzyme could be that there are differences in accessibility to the enzyme of the hydrophobic vitamin *D*₃ substrate. The much higher activity with the more hydrophilic 1α-hydroxyvitamin *D*₃ as substrate supports this contention. It is conceivable that both factors could be responsible for the observed difference in total catalytic activity.

Testosterone 16α-hydroxylase activity co-purified with the vitamin *D*₃ 25-hydroxylase activity up to the hydroxyapatite chromatography step. This purification step separated the 16α-hydroxylase from the 25-hydroxylase; the 16α-hydroxylase was found in the 300 mM-phosphate eluate. Fig. 1 shows the result of SDS/PAGE of the 25-hydroxylating cytochrome *P*-450. The preparation showed only one protein band, corresponding to an apparent *M_r* of 50 500.

Structural characterization

The purified microsomal 25-hydroxylating cytochrome *P*-450 from pig liver was analysed with respect to amino acid composition and *N*-terminal sequence. The amino acid composition was similar to that of other purified mammalian cytochromes *P*-450, including that of the kidney microsomal 25-hydroxylase [10,28]. *N*-Terminal amino acid sequence analysis of purified liver microsomal 25-hydroxylase (1 nmol) revealed the following

Fig. 2. Absolute spectra of purified 25-hydroxylating microsomal cytochrome *P*-450 from pig liver

The concentration of the cytochrome *P*-450 was 0.91 nmol/ml in 50 mM-potassium phosphate buffer, pH 7.8, containing 20 % glycerol and 0.1 mM-EDTA. —, Oxidized; ----, reduced; ·····, reduced-CO complex.

sequence of the first 16 amino acids (97 % repetitive yield): Gly-Leu-Leu-Thr-Gly-Asp-Leu-Leu-Gly-Ile-Leu-Ala-Leu-Ala-Met-Val. This sequence only differs from that reported for the kidney enzyme [10] with respect to position 6 (aspartic acid instead of leucine). The sequence differs, however, from that of the rat liver 25-hydroxylase [12] (cf. Table 2).

Absorption spectra

The spectral properties of the enzyme are shown in Fig. 2. The fraction showed a major absorbance maximum in the oxidized state at 420 nm and minor maxima at 470, 505, 530 and 560 nm. No absorbance maximum was observed in the reduced state, and the absorbance maximum for the reduced CO complex was at 450 nm. The spectral properties indicate a low-spin form of cytochrome *P*-450 [29].

Isoelectric focusing

Since the apparent *M_r* of the 25-hydroxylase in liver is identical with that in kidney microsomes [10], it was considered of interest to perform isoelectric focusing of the two enzymes. Fig. 3 shows the result of such an experiment, in which a mixture of equal amounts of the purified 25-hydroxylases from liver and kidney was subjected to isoelectric focusing. Only one protein band was detected, at a pI of approx. 7.8.

Catalytic properties

Table 3 summarizes the catalytic properties of the original microsomes and the purified 25-hydroxylase from pig liver. The hydroxylations studied were the 25-hydroxylation of vitamin *D*₃, 1α-hydroxyvitamin *D*₃, cholesterol and 5β-cholestane-3α,7α-diol, the ω- and (ω-1)-hydroxylations of lauric acid, and the

Table 2. *N*-Terminal amino acid sequences of microsomal 25-hydroxylating cytochromes *P*-450 from pig liver, pig kidney and rat liver

Source	Residue...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Pig liver		G	L	L	T	G	D	L	L	G	I	L	A	L	A	M	V
Pig kidney		G	L	L	T	G	L	L	L	G	I	L	A	L	A	M	V
Rat liver		M	D	P	V	L	V	L	V	L	T	L	S	S	L	L	L

Fig. 3. Isoelectric focusing of the 25-hydroxylating microsomal cytochromes *P*-450 from pig kidney and liver

A mixture of equal amounts of purified 25-hydroxylases (2 μ g of each) was subjected to isoelectric focusing as described in the Experimental section. The pH gradient in the tube gel was determined by subjecting a parallel gel to isoelectric focusing, after which the gel was cut into consecutive 7 mm slices that were incubated in degassed water overnight at 4 $^{\circ}$ C before measurement.

16 α -hydroxylation of testosterone. The 16 α -hydroxylation of testosterone and the hydroxylations of lauric acid were by far the most efficient hydroxylations in microsomes. The 25-hydroxylating cytochrome *P*-450 catalysed the 25-hydroxylation of vitamin D₃, 1 α -hydroxyvitamin D₃ and 5 β -cholestane-3 α ,7 α -diol much more efficiently than did the microsomes. No hydroxylase activities towards cholesterol, lauric acid or testosterone were detectable in the purified 25-hydroxylase.

Fig. 4 shows that the conversion of vitamin D₃ into 25-hydroxyvitamin D₃ was approximately linear with the amount of cytochrome *P*-450 up to about 0.2 nmol and increased with time, although not linearly, up to 20 min. The system was saturated with 2 units of NADPH-cytochrome *P*-450 reductase and with 30 μ M-vitamin D₃. The rate of hydroxylation showed an optimum at pH 7.8 (results not shown). The apparent K_m for the 25-hydroxylation of vitamin D₃ in the reconstituted system was measured in separate experiments and was found to be 6 μ M (Fig. 4). The K_m value and the turnover are of the same order of magnitude as those reported for the microsomal 25-hydroxylase in rat liver [5,6].

Immunological studies

A monoclonal antibody against the 25-hydroxylating

Table 3. Hydroxylase activities in microsomes and the purified cytochrome *P*-450 from pig liver

Incubations with vitamin D₃ and 1 α -hydroxyvitamin D₃ were performed at 37 $^{\circ}$ C for 10 min. The incubation mixture contained 0.1 nmol of cytochrome *P*-450, 2 units of microsomal NADPH-cytochrome *P*-450 reductase, 1.2 μ mol of NADPH and 40 μ g of substrate. The total volume was 1 ml of 50 mM-Tris/acetate buffer, pH 7.8, containing 20% glycerol and 0.1 mM-EDTA. Cholesterol and lauric acid were incubated in the same way, except that 5 μ g of cholesterol, 40 μ g of lauric acid, 0.5 nmol of cytochrome *P*-450 and 6 units of NADPH-cytochrome *P*-450 reductase were used and the incubation time was 30 min. Incubations with testosterone and 5 β -cholestane-3 α ,7 α -diol were performed as with vitamin D₃ except that the incubation buffer was 150 mM-potassium phosphate, pH 7.8, containing 20% glycerol and 0.1 mM-EDTA. Incubations with intact microsomes (1 mg of protein) were performed with vitamin D₃, 1 α -hydroxyvitamin D₃, 5 β -cholestane-3 α ,7 α -diol and testosterone (80 μ g of each) for 20 min and with cholesterol (5 μ g) and lauric acid (40 μ g) for 30 min. No NADPH-cytochrome *P*-450 reductase was added to incubations with microsomes. The results from five experiments are given as the means $\pm$ S.D.

Reaction measured	Microsomal activity (pmol $\cdot$ min ⁻¹ $\cdot$ nmol of cytochrome <i>P</i> -450 ⁻¹)	Cytochrome <i>P</i> -450 activity (pmol $\cdot$ min ⁻¹ $\cdot$ nmol of cytochrome <i>P</i> -450 ⁻¹)
Vitamin D ₃ 25-hydroxylation	4 $\pm$ 3	300 $\pm$ 21
1 α -Hydroxyvitamin D ₃ 25-hydroxylation	7 $\pm$ 2	1604 $\pm$ 49
5 β -Cholestane-3 α ,7 α -diol 25-hydroxylation	$\leq$ 15	2795 $\pm$ 529
Testosterone 16 α -hydroxylation	412 $\pm$ 170	< 10
Lauric acid ω + (ω - 1)-hydroxylations	213 $\pm$ 39	< 10
Cholesterol 25-hydroxylation	< 1	< 1
7 α -hydroxylation	1	< 1

Fig. 4. Effects of cytochrome *P*-450 concentration (a), time (b), NADPH-cytochrome *P*-450 reductase concentration (c) and vitamin D₃ concentration (d and e) on the rate of 25-hydroxylation of vitamin D₃

Incubations were performed as described in the Experimental section, except when the concentration of a component was varied. *v* is given in nmol $\cdot$ min⁻¹ $\cdot$ nmol of cytochrome *P*-450⁻¹.

Fig. 5. Effect of mAb 25H6 (against the pig liver microsomal 25-hydroxylase) on 25-hydroxylation of 1α -hydroxyvitamin D_3 by the 25-hydroxylating microsomal cytochromes *P*-450 from pig liver (a) and kidney (b)

Cytochrome *P*-450 (0.2 nmol) was incubated with the indicated amounts of Sepharose-bound mAb 25H6 (●) or mAb 26C5 (○, against a mitochondrial 26-hydroxylase and used as an irrelevant antibody) and assayed for hydroxylase activity as described in the Experimental section. Inactivated CNBr-Sepharose without antibody was used as control. Results are given as percentage of control values for 25-hydroxylase activity in incubations without antibody. The 100 % control value was $760 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{nmol}$ of cytochrome *P*-450⁻¹ with the liver enzyme and $763 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{nmol}$ of cytochrome *P*-450⁻¹ with the kidney enzyme.

cytochrome *P*-450 in liver microsomes, designated mAb 25H6, was prepared by immunization of mice with the purified enzyme. The antibody was coupled to Sepharose and incubated with purified 25-hydroxylating cytochrome *P*-450 from both liver and kidney. After incubation, the antibody-Sepharose was removed and the supernatant was assayed for 25-hydroxylase activity in a reconstituted system. As a control, Sepharose was coupled to an irrelevant monoclonal antibody, directed against a mitochondrial 26-hydroxylase from pig liver. As shown in Fig. 5, increasing amounts of mAb 25H6 but not the irrelevant antibody was able to bind and decrease 25-hydroxylase activity from both organs. Fig. 6(a) shows SDS/PAGE and immunoblotting analyses with the antibody. It reacted strongly with the purified 25-hydroxylase as well as with a protein of the same apparent M_r in liver microsomes. The antibody also recognized purified 25-hydroxylase from kidney microsomes. The monoclonal antibody was used to further examine the tissue distribution of the microsomal 25-hydroxylase in the castrated male pig. The antibody detected a protein with an apparent M_r of 50 500 in microsomes from kidney and liver, but not from adrenal gland, heart, intestine, lung, muscle, spleen or thymus (Fig. 6c). In separate experiments (results not shown) the monoclonal antibody against the kidney microsomal cytochrome *P*-450₂₅, designated mAb 25E5 [10], was found to react strongly with the purified liver enzyme and revealed the same tissue distribution.

Previous work on the 25-hydroxylation of vitamin D_3 in rat liver microsomes showed a sex difference with respect to the amount of 25-hydroxylase [12]. Furthermore, an antibody against the rat liver enzyme was not able to recognize protein in pig or human liver microsomes (results not shown). Therefore experiments were performed with both male and female pigs and with human liver. Fig. 6(b) shows SDS/PAGE and immunoblotting analyses with mAb 25H6 of the 25-hydroxylase in liver microsomes. The antibody recognized purified 25-hydroxylase as well as a protein of the same apparent M_r in livers of both male and female pigs. There was no difference between the sexes in the apparent concentration. In addition, the antibody reacted with a

Fig. 6. SDS/PAGE and immunoblotting of the microsomal 25-hydroxylating cytochrome *P*-450

Samples containing the indicated amounts of proteins were subjected to SDS/PAGE, electrophoretic transfer and immunoblotting with mAb 25H6 as described in the Experimental section. (a) Lane 1, purified 25-hydroxylase from liver microsomes (1 μg); lane 2, liver microsomes from male pigs (50 μg); lane 3, purified 25-hydroxylase from kidney microsomes (1 μg). M_r standards are indicated as described in Fig. 1. (b) Microsomal protein (50 μg) from liver of male and female pig and a human female. Lane 1, male pig; lane 2, female pig; lane 3, human. (c) Microsomal protein (50 μg) from different tissues of castrated male pigs. Lane 1, liver; lane 2, intestine; lane 3, thymus; lane 4, spleen; lane 5, muscle; lane 6, heart; lane 7, adrenal; lane 8, lung; lane 9, kidney; lane 10, liver.

protein of similar M_r in human liver. It should be mentioned that 25-hydroxylase activity towards 1α -hydroxyvitamin D_3 and 5β -cholestane- $3\alpha,7\alpha$ -diol was present in the human liver microsomes (cf. also ref. [30]).

DISCUSSION

In previous work, male rats have been the source for purification and characterization of liver microsomal cytochrome *P*-450 active in the 25-hydroxylation of vitamin D_3 [5,6,12,15]. The 25-hydroxylating cytochrome *P*-450 (cytochrome *P*-450₂₅/cytochrome *P*-450_{cc25}) in rat liver microsomes is male-specific and shows testosterone 16α -hydroxylase activity, with *N*-terminal amino acid sequence and immunochemical properties indistinguishable from those of the male-specific CYP2C11 [5,6,12,15,16,31]. The present paper describes the purification and characterization of a 25-hydroxylating cytochrome *P*-450 in pig liver microsomes. The results clearly demonstrate that the 25-hydroxylation of vitamin D_3 and the 16α -hydroxylation of testosterone are catalysed by different cytochromes *P*-450 in pig liver. The 25-hydroxylase is present in approximately the same amount in both male and female pigs and is structurally different from the rat liver enzyme. These differences in properties between the rat and pig liver microsomal 25-hydroxylases are important for several reasons. Firstly, the role of microsomal 25-hydroxyl-

ation of vitamin D₃ in the bioactivation process has been questioned because of the properties of the rat liver enzyme [12–14]. Secondly, the present results in fact suggest that studies on cytochromes *P*-450 in vitamin D₃ metabolism with the rat as a model may be misleading. Indeed, the finding in the present work of a vitamin D₃ 25-hydroxylase in pig liver microsomes, which is without testosterone 16 α -hydroxylase activity and present in both sexes, demonstrates that the rat is not a representative animal for studies on microsomal 25-hydroxylation of vitamin D₃. This is perhaps not surprising, since it is known that the rat, in contrast to most other mammals including man, exhibits sex differences between certain cytochrome *P*-450 isoenzymes in liver [32,33]. It is of interest in this connection that a monoclonal antibody raised against the purified 25-hydroxylase from pig liver, but not an antibody against the 25-hydroxylase from rat liver, was found to recognize protein of similar apparent *M_r* in liver microsomes of a human female. It thus appears that the pig may be a representative animal for evaluation of 25-hydroxylation in human liver.

The tissue distribution of microsomal 25-hydroxylase in pig was examined using the monoclonal antibody raised against the purified liver enzyme. A 25-hydroxylase similar to that in liver was found only in kidney. The properties of the 25-hydroxylating cytochrome *P*-450 in pig liver were therefore compared with those of the 25-hydroxylating cytochrome *P*-450 purified from pig kidney microsomes in this laboratory [10]. The two 25-hydroxylases show the same apparent *M_r*, spectral properties and isoelectric point, and are immunologically indistinguishable. The turnover of 25-hydroxylation by the liver and kidney enzymes is also of the same order of magnitude. The 25-hydroxylase in pig liver, however, did not catalyse (ω -1)-hydroxylation of lauric acid, an activity found for the purified 25-hydroxylase from pig kidney [10]. The liver enzyme differs from the kidney enzyme with respect to only one of the first 16 *N*-terminal amino acids. These results show that the 25-hydroxylases in liver and kidney are structurally and functionally closely related, although not identical.

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