Characterization of pig kidney microsomal cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_3 and C_{27} steroids

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The cytochrome *P*-450 enzyme which catalyses 25-hydroxylation of vitamin D_3 (cytochrome *P*-450₂₅) from pig kidney microsomes [Postlind & Wikvall (1988) *Biochem. J.* **253**, 549–552] has been further purified. The specific content of cytochrome *P*-450 was 15.0 nmol·mg of protein⁻¹, and the protein showed a single spot with an apparent isoelectric point of 7.4 and an M_r of 50 500 upon two-dimensional isoelectric-focusing/SDS/PAGE. The 25-hydroxylase activity towards vitamin D_3 was 124 pmol·min⁻¹·nmol of cytochrome *P*-450⁻¹ and towards 1 α -hydroxyvitamin D_3 it was 1375 pmol·min⁻¹·nmol⁻¹. The preparation also catalysed the 25-hydroxylation of 5 β -cholestane-3 α ,7 α -diol at a rate of 1000 pmol·min⁻¹·nmol of cytochrome *P*-450⁻¹ and ω – 1 hydroxylation of lauric acid at a rate of 200 pmol·min⁻¹·nmol of cytochrome *P*-450⁻¹. A monoclonal antibody raised against the 25-hydroxylating cytochrome *P*-450, designated mAb 25E5, was prepared. After coupling to Sepharose, the antibody was able to bind to cytochrome *P*-450₂₅ from kidney as well as from pig liver microsomes, and to immunoprecipitate the activity for 25-hydroxylation of vitamin D_3 and 5 β -cholestane-3 α ,7 α -diol when assayed in a reconstituted system. The hydroxylase activity towards lauric acid was not inhibited by the antibody. By SDS/PAGE and immunoblotting with mAb 25E5, cytochrome *P*-450₂₅ was detected in both pig kidney and pig liver microsomes. These results indicate a similar or the same species of cytochrome *P*-450 in pig kidney and liver microsomes catalysing 25-hydroxylation of vitamin D_3 and C_{27} steroids. The *N*-terminal amino acid sequence of the purified cytochrome *P*-450₂₅ from pig kidney microsomes differed from those of hitherto isolated mammalian cytochromes *P*-450.

INTRODUCTION

25-Hydroxylation is involved in the bioactivation of vitamin D and in bile acid metabolism [1,2]. The 25-hydroxylation of vitamin D_3 is the first step in the bioactivation of vitamin D_3 into its hormonal form, and microsomal 25-hydroxylation is involved in an alternative pathway for bile acid synthesis [2]. So far, no extrahepatic enzyme systems able to catalyse 25-hydroxylation of bile acid intermediates have been described.

In a previous study [3], the isolation of a cytochrome *P*-450 (cytochrome *P*-450₂₅) from pig kidney microsomes which is active in the 25-hydroxylation of vitamin D_3 was described. In the present study the enzyme is further characterized with a monoclonal antibody and by determination of the *N*-terminal amino acid sequence. The properties of the enzyme are compared with those of liver microsomal 25-hydroxylase.

EXPERIMENTAL

Materials

TSK DEAE-5PW and ampholines were from LKB, and Mono S HR 10/10 and Protein A-Sepharose were from Pharmacia. Freund's complete adjuvant was from Difco. Standards for IgG subclass determination were kindly donated by Pharmacia. Preparations and sources for steroid analogues have been described previously [4].

Enzyme purification

Cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_3 and C_{27} steroids from pig kidney microsomes was purified to electrophoretic homogeneity with some modifications of the previously reported procedure [3]. After the octylamine-Sepharose

and hydroxyapatite chromatography steps, the DEAE- and CM-chromatographies were performed using high-performance ion-exchange chromatography as follows. The cytochrome *P*-450 fraction was dialysed overnight against 10 mM-potassium phosphate buffer, pH 7.6, containing 20 % glycerol, 0.1 % sodium cholate and 0.05 % Lubrol PX. The sample was injected into a TSK DEAE-5PW column (21.5 mm × 150 mm) equilibrated with the same buffer as used for dialysis. The flow rate was 4 ml/min. The non-bound material was pooled and injected into a Mono S HR 10/10 column equilibrated in the same buffer as used for TSK DEAE-5PW chromatography. The flow rate was 2 ml/min. The 25-hydroxylating cytochrome *P*-450 fraction was eluted with 0.5 M-sodium acetate in the equilibrating buffer. Detergents were removed as described previously [5].

Partially purified 25-hydroxylating pig liver microsomal cytochrome *P*-450 was prepared essentially as described for pig kidney with minor modifications. The hydroxyapatite column was washed with 80 mM-potassium phosphate buffer, pH 7.4, containing 20 % glycerol, 0.1 mM-EDTA and 0.2 % Lubrol PX. The cytochrome *P*-450 was eluted with 300 mM-potassium phosphate buffer, pH 7.4, containing 20 % glycerol, 0.1 mM-EDTA and 0.2 % Lubrol PX. It was then concentrated and dialysed against 10 mM-potassium phosphate buffer, pH 7.6, containing 20 % glycerol, 0.1 % sodium cholate and 0.05 % Lubrol PX. The sample was injected on to a TSK DEAE-5PW column (21.5 mm × 150 mm) equilibrated in the same buffer as used for dialysis. The flow rate was 4 ml/min. The cytochrome *P*-450 was eluted with the equilibrating buffer and detergent was then removed [5].

NADPH-cytochrome *P*-450 reductase was prepared from rat liver microsomes as described by Yasukochi & Masters [6]. Cytochrome *b*₅ was isolated from rat liver and pig kidney

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microsomes by octylamine-Sepharose chromatography [7] and was further purified to apparent homogeneity as described by Strittmatter *et al.* [8].

Production of monoclonal antibodies

An 8-week-old female mouse of the Balb/c strain was used. Purified cytochrome *P*-450 from pig kidney microsomes was emulsified in an equal volume of Freund's complete adjuvant. Enzyme (75 μ g) was injected subcutaneously and intraperitoneally. At 1 month later, another 75 μ g of the same antigen in 0.9% NaCl was injected intraperitoneally. After a further 3 days the mouse was killed and the spleen was used as source of antibody-producing cells. Fusions and the establishment of antibody-producing hybridomas were performed essentially as described by Köhler & Milstein [9]. Spleen cells were mixed 1:1 with sp 2/0 [10] myeloma cells and fused using 50% poly(ethylene glycol) 1500 [11]. The hybridoma cells were screened by e.l.i.s.a. after hypoxanthine/aminopterin/thymidine selection. Of 2100 microwells resulting from one single fusion, 70 produced antibodies which recognized the cytochrome *P*-450. Four hybridomas of the 70 that produced antibodies were monospecific for the antigen. Cloning of positive cultures was carried out as described previously [12].

Purification of monoclonal antibodies

Immunoglobulins were purified on a Protein A-Sepharose column (1.5 cm $\times$ 6 cm) equilibrated in 1.5 M-glycine buffer, pH 9.0, containing 3 M-NaCl. The immunoglobulins were eluted in 0.1 M-glycine buffer, pH 3.0. The purified immunoglobulins were dialysed against phosphate-buffered saline (10 mM-potassium phosphate buffer, pH 7.4, containing 150 mM-KCl), and coupled to CNBr-activated Sepharose as described by the manufacturer. The subclass of each monoclonal antibody was determined by the Ouchterlony immunodiffusion technique.

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

One-dimensional gel electrophoresis

One-dimensional electrophoresis was performed according to Laemmli [13] with 15% polyacrylamide/0.09% bisacrylamide slab gels, containing 0.1% (w/v) SDS. Electrophoresis was carried out at 30 mA/slab gel and the gels were stained as described by Wray *et al.* [14].

Two-dimensional gel electrophoresis

Two-dimensional isoelectric-focusing/SDS/PAGE was performed essentially as described [15], except that the isoelectric focusing gel was not pre-run. Isoelectric focusing was carried out at 300 V for 16 h followed by 1000 V for 3 h. The second dimension, i.e. SDS/PAGE, was performed as described above.

Incubation procedures

Incubations were carried out for 7 min with kidney cytochrome *P*-450 and for 20 min with liver cytochrome *P*-450 at 37 °C. Vitamin D₃, 1 α -hydroxyvitamin D₃, 5 β -cholestane-3 α ,7 α -diol, 5 β -cholestane-3 α ,7 α ,12 α -triol, lauric acid (40 μ g of each) or cholesterol (10 μ g), dissolved in 25 μ l of acetone, were incubated with 0.1 nmol of cytochrome *P*-450, 2 units of microsomal NADPH-cytochrome *P*-450 reductase and 1 μ mol of NADPH, in a total volume of 1 ml of 50 mM-Tris-acetate buffer, pH 7.4. In experiments for assay of cholesterol 7 α -hydroxylase, cholesterol incubations contained, in addition, 9 μ mol of dithiothreitol and 0.06% Triton X-100. Incubations with whole microsomes (1 mg) were performed as above, except that microsomal NADPH-cytochrome *P*-450 reductase was omitted and the incubation time was 20 min. Vitamin D₃ and 1 α -hydroxyvitamin D₃

incubations were terminated, extracted and analysed as described previously [3]. 5 β -Cholestane-3 α ,7 α -diol, 5 β -cholestane-3 α ,7 α ,12 α -triol, lauric acid and cholesterol incubations were terminated by addition of 5 ml of ethanol (95%, v/v). 5 β -Cholestane-3 α ,7 α -diol and 5 β -cholestane-3 α ,7 α ,12 α -triol incubations were extracted as described by Wikvall [5], lauric acid incubations as described by Dahlbäck & Wikvall [16] and cholesterol incubations as described by Boström *et al.* [17]. Incubations were quantified by h.p.l.c. using a LiChrosorb RP-18 column (150 mm $\times$ 3 mm internal diam., 5 μ m, Merck), and a Berthold LB 503 D h.p.l.c. radioactivity monitor with a 100 μ l flow-through cell (solid glass scintillator). The analyses were performed with aqueous solutions, and the columns were eluted isocratically. Flow rate was 0.5 ml/min. Mobile phases were 85% methanol for 5 β -cholestane-3 α ,7 α -diol incubations, 80% methanol for 5 β -cholestane-3 α ,7 α ,12 α -triol, 62% methanol/0.2% acetic acid for laurate and 88% methanol for detection of 7 α -hydroxycholesterol. For on-line radioactivity measurement, the substrate was eluted with methanol. Detection of 25-hydroxycholesterol was performed by straight-phase h.p.l.c. as described by Pedersen *et al.* [18].

Incubations with antibody-coupled Sepharose

Incubations with cytochrome *P*-450 and antibody-coupled Sepharose were performed as follows. Cytochrome *P*-450 (0.1 nmol in kidney and 0.2 nmol in liver incubations) was incubated with the indicated amounts of Sepharose-bound monoclonal antibody (mAb) 25E5 or mAb 25-6 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. 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 once with the same buffer without CHAPS and pooled. The reaction mixture was incubated for 20 min at 37 °C, terminated, extracted and analysed as described above.

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 a Beckman 121 M amino acid analyser.

N-Terminal sequence analysis

Gas-phase sequencer analysis was performed with an Applied Biosystems 470A instrument, and phenylthiohydantoin derivatives were identified by reverse-phase h.p.l.c. essentially as described [19]. Samples were applied directly in solution or via electroblotting after SDS/PAGE [20].

Other methods

Protein was determined as described by Lowry *et al.* [21]. Cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase activities were determined as described [22,23].

RESULTS

Cytochrome *P*-450, active in the 25-hydroxylation of vitamin D₃ and C₂₇ steroids, was isolated from pig kidney microsomes by solubilization with sodium cholate and chromatography on octylamine-Sepharose, hydroxyapatite, TSK DEAE-5PW and Mono S. Cytochrome *P*-450 from pig liver microsomes was purified in the same way except that the Mono S chromatography step was excluded. The final enzyme fraction had a specific content of 15 nmol of cytochrome *P*-450 $\cdot$ mg of protein⁻¹ for kidney microsomes and 7 nmol of cytochrome *P*-450 $\cdot$ mg of protein⁻¹ for liver microsomes. To further examine the purity of

Fig. 1. Two-dimensional isoelectric-focusing/SDS/PAGE electrophoresis of microsomal cytochrome *P*-450 from pig kidney

A sample containing 2 μ g of purified microsomal cytochrome *P*-450 from pig kidney was subjected to two-dimensional electrophoresis as described in the Experimental section. Indicated are the electrophoretic migrations of proteins of known M_r (cytochrome *P*-450 LM₄, M_r 55000 and cytochrome *P*-450 LM₂, M_r 48000, both from rabbit liver microsomal fraction). 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 5 mm slices that were incubated in degassed water overnight at 4 °C before pH measurement.

Fig. 2. Absolute spectra of purified microsomal pig kidney cytochrome *P*-450

The concentration of the cytochrome *P*-450 was 0.5 nmol/ml in 50 mM-potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. —, Oxidized cytochrome *P*-450; ----, reduced cytochrome *P*-450; ·····, reduced cytochrome *P*-450-CO complex.

the preparation from kidney microsomes, experiments were performed in which the cytochrome *P*-450 was submitted to two-dimensional isoelectric-focusing/SDS/PAGE. A single spot with an apparent isoelectric point of 7.4 and an M_r of 50500 was observed (Fig. 1). The spectral properties of kidney microsomal cytochrome *P*-450 are shown in Fig. 2. The fraction showed a major absorbance maximum in the oxidized state at 420 nm and minor maxima at 470, 520, 535, 570, 600 and 630 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 [24].

The catalytic activities of the original microsomes and the purified cytochrome *P*-450 from pig kidney are summarized in Table 1. The hydroxylations studied were the 25-hydroxylation of vitamin D₃, 1 α -hydroxyvitamin D₃, 5 β -cholestane-3 α ,7 α -diol, 5 β -cholestane-3 α ,7 α ,12 α -triol and cholesterol, the 7 α -hydroxylation of cholesterol, and the ω - and ω -1-hydroxylations of lauric acid. The original microsomes catalysed all of these reactions with the exception of the 25-hydroxylation and the 7 α -hydroxylation of cholesterol. The 25-hydroxylase activity, expressed as pmol of product formed $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹, was about 3500 times higher with the purified cytochrome *P*-450 than with microsomes for vitamin D₃, and about 20000 times higher for 1 α -hydroxyvitamin D₃. The 25-hydroxylase activity towards C₂₇ steroids was 600–900 times higher, whereas the ω -1-hydroxylase activity towards lauric acid was 10 times higher with purified enzyme. No ω -hydroxylation could be detected with the purified cytochrome *P*-450 fraction. In Table 2, the catalytic activities of the partially purified cytochrome *P*-450 from liver microsomes as well as the original microsomes are summarized. The 25-hydroxylase activity towards vitamin D₃ and 1 α -hydroxyvitamin D₃ was 300–350 times higher than in the microsomes. The 25-hydroxylase activity towards C₂₇ steroids was 200–400 times higher, whereas the ω - and ω -1-hydroxylations of lauric acid were 20–30 times higher and the α -hydroxylation of cholesterol was 40 times higher than in the original microsomes. No 25-hydroxylation of cholesterol could be detected in microsomes or in the purified cytochrome *P*-450 fraction from pig liver.

The reconstitution conditions for both kidney and liver microsomal cytochrome *P*-450 showed an absolute requirement for cytochrome *P*-450, NADPH-cytochrome *P*-450 reductase and NADPH. However, addition of phospholipid was not

Table 1. Hydroxylase activities in microsomes and purified cytochrome *P*-450 from pig kidney

Incubations were performed at 37 °C for 7–20 min with 0.1 nmol of cytochrome *P*-450, 2 units of microsomal cytochrome *P*-450 reductase, 1 μ mol of NADPH and 40 μ g of vitamin D₃, 1 α -hydroxyvitamin D₃, 5 β -cholestane-3 α ,7 α -diol, 5 β -cholestane-3 α ,7 α ,12 α -triol or lauric acid, or 10 μ g of cholesterol, in a total volume of 1 ml of 50 mM-Tris-acetate buffer, pH 7.4. Incubations with intact microsomes were performed as described in the Experimental section.

Reaction measured	Microsomal activity (pmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	Cytochrome <i>P</i> -450 activity		Purification (fold)
		(pmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	(pmol $\cdot$ min ⁻¹ $\cdot$ nmol of cyt. <i>P</i> -450 ⁻¹)	
Vitamin D ₃ 25-hydroxylation	0.5	1860	124	3720
1 α -Hydroxyvitamin D ₃ 25-hydroxylation	1.0	20625	1375	20625
5 β -Cholestane-3 α ,7 α -diol 25-hydroxylation	17	15000	1000	882
5 β -Cholestane-3 α ,7 α ,12 α -triol 25-hydroxylation	35	21450	1430	612
Lauric acid ω -hydroxylation	420	< 10	< 10	< 10
Lauric acid ω -1-hydroxylation	250	3000	200	12
Cholesterol 25-hydroxylation	< 1	< 10	< 10	< 10
Cholesterol 7 α -hydroxylation	< 1	< 10	< 10	< 10

Table 2. Hydroxylase activities in microsomes and partially purified cytochrome *P*-450 from pig liver

Incubations were performed at 37 °C for 20 min as described in the legend to Table 1.

Reaction measured	Microsomal activity (pmol · min ⁻¹ · mg of protein ⁻¹)	Cytochrome <i>P</i> -450 activity		Purification (fold)
		(pmol · min ⁻¹ · mg of protein ⁻¹)	(pmol · min ⁻¹ · nmol of cyt. <i>P</i> -450 ⁻¹)	
Vitamin D ₃ 25-hydroxylation	2.2	616	88	280
1 α -Hydroxyvitamin D ₃ 25-hydroxylation	8.5	2961	423	348
5 β -Cholestane-3 α ,7 α -diol 25-hydroxylation	25	10325	1475	413
5 β -Cholestane-3 α ,7 α ,12 α -triol 25-hydroxylation	70	15400	2200	220
Lauric acid ω -hydroxylation	165	5600	800	34
Lauric acid ω -1-hydroxylation	350	5600	800	16
Cholesterol 25-hydroxylation	< 1	< 10	< 10	< 10
Cholesterol 7 α -hydroxylation	15	609	87	40

Fig. 3. Immunoprecipitation of hydroxylase activities of cytochrome *P*-450₂₅ by anti-(cytochrome *P*-450₂₅) monoclonal antibody (mAb 25E5)

25-Hydroxylation in kidney (a) and in liver (b), and hydroxylations of lauric acid in kidney (c) and in liver (d) are shown. Cytochrome *P*-450₂₅ (0.1–0.2 nmol) was incubated with the indicated amounts of Sepharose-bound mAb 25E5 or mAb 25-6 and assayed for hydroxylase activity as described in the Experimental section. In the kidney, the 100% control values for hydroxylase activity in tubes containing no antibody were: for 25-hydroxylation of vitamin D₃ (●), 58 pmol · min⁻¹ · nmol of *P*-450⁻¹; for 5 β -cholestane-3 α ,7 α -diol (○), 550 pmol · min⁻¹ · nmol of *P*-450⁻¹; for ω -1-hydroxylation of lauric acid (■), 100 pmol · min⁻¹ · nmol of *P*-450⁻¹. In the liver, the 100% of control values were: for 25-hydroxylation of vitamin D₃ (●), 25 pmol · min⁻¹ · nmol of *P*-450⁻¹; for 5 β -cholestane-3 α ,7 α -diol (○), 730 pmol · min⁻¹ · nmol of *P*-450⁻¹; for ω -hydroxylation of lauric acid (□), 320 pmol · min⁻¹ · nmol of *P*-450⁻¹ and for ω -1-hydroxylation of lauric acid (■), 280 pmol · min⁻¹ · nmol of *P*-450⁻¹.

required for 25-hydroxylation; rather, it inhibited the reaction by 15–20%. The inclusion of an equimolar amount of cytochrome *b*₅ in the reconstitution system had no stimulatory effect on the rate of 25-hydroxylation of any of the substrates.

A monoclonal antibody against cytochrome *P*-450₂₅ in kidney

Fig. 4. SDS/PAGE and immunoblotting of cytochrome *P*-450₂₅ in kidney and liver microsomes from male and female pigs

Samples containing the indicated amounts of proteins were subjected to SDS/PAGE, electrophoretic transfer and immunoblotting with mAb 25E5, as described in the Experimental section. Migration was from top to bottom. Lane 1, cytochrome *P*-450₂₅ from kidney microsomes (1 μg); lane 2, kidney microsomes from male pig (80 μg); lane 3, kidney microsomes from female pig (80 μg); lane 4, liver microsomes from male pig (80 μg); lane 5, liver microsomes from female pig (80 μg). *M_r* standards are indicated as described in the legend to Fig. 1.

microsomes, designated mAb 25E5, was prepared by immunization of mice with purified cytochrome *P*-450₂₅. mAb 25E5 was found to belong to the IgG 2a subclass. To demonstrate the reactivity of this antibody with purified cytochrome *P*-450₂₅ from both kidney and liver microsomes, it was coupled covalently to Sepharose and incubated with cytochrome *P*-450₂₅. After 1 h incubation, the antibody-Sepharose was removed by centrifugation and the supernatant was assayed in a reconstituted system for 25-hydroxylase activity toward vitamin D₃ and 5 β -cholestane-3 α ,7 α -diol as well as for ω - and ω -1-hydroxylase activity towards lauric acid. As a control, Sepharose was coupled to an irrelevant monoclonal antibody, mAb 25-6, directed against the male-specific form of rat liver microsomal 25-hydroxylase [12]. Figs. 3(a) and 3(b) show that increasing amounts of mAb 25E5 but not mAb 25-6 were able to bind up to 80% of the 25-hydroxylase activity when incubated with purified cytochrome *P*-450₂₅ from both kidney and liver microsomes. The monoclonal antibody did not inhibit the ω - or ω -1-hydroxylation of lauric acid (Figs. 3c and 3d).

Previous work on 25-hydroxylation of vitamin D₃ and 5 β -

cholestane-3 α ,7 α ,12 α -triol in rat liver microsomes showed that the reaction was catalysed by different isoenzymes of cytochrome *P*-450 in male and female rats [12,25]. Therefore experiments were performed with both male and female pigs. Fig. 4 shows SDS/PAGE and immunoblotting analyses with mAb 25E5 of cytochrome *P*-450₂₅ in kidney and liver microsomes. The antibody recognized purified cytochrome *P*-450₂₅ from kidney microsomes (lane 1) as well as a protein of the same *M_r* in both male and female kidneys (lanes 2 and 3) and liver (lanes 4 and 5). These results show that cytochrome *P*-450₂₅ is not sex-specific in either pig kidney or liver.

The amino acid composition of cytochrome *P*-450₂₅ was similar to that of other purified mammalian cytochromes *P*-450 [26]. *N*-Terminal amino acid sequence analysis of purified cytochrome *P*-450₂₅ (900 pmol) revealed the following sequence of the first 20 amino acids (98 % repetitive yield): Gly-Leu-Leu-Thr-Gly-Leu-Leu-Leu-Ile-Leu-Ala-Leu-Ala-Met-Val-Val-X-Leu-Leu.

DISCUSSION

In a previous report [3] the purification of a microsomal cytochrome *P*-450 from pig kidney which catalysed the 25-hydroxylation of vitamin D₃ was presented. In the present work, this enzyme was further purified, which gave an enzyme fraction with a higher content of cytochrome *P*-450 (15.0 nmol/mg of protein). The amino acid composition was similar to that of other cytochromes *P*-450 [26]. However, the *N*-terminal amino acid sequences of cytochrome *P*-450₂₅ differed from those of hitherto isolated mammalian cytochromes *P*-450 [26].

For comparison, a 25-hydroxylating microsomal cytochrome *P*-450 fraction from the liver was prepared, by using essentially the same purification procedure. This fraction contained 7 nmol of cytochrome *P*-450 · mg of protein⁻¹. Both cytochrome *P*-450 fractions show the same catalytic properties.

Cytochrome *b*₅ has been reported to stimulate certain hydroxylations catalysed by purified cytochrome *P*-450 [27,28]. Noshiro *et al.* [29] have reported that antibodies to proteinase-solubilized cytochrome *b*₅ inhibit 25-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol catalysed by crude rat liver microsomes. However, cytochrome *b*₅ was found to have no stimulatory effect on the 25-hydroxylation of vitamin D₃ or 5 β -cholestane-3 α ,7 α -diol catalysed by the purified cytochrome *P*-450 fractions used in the present investigation.

The finding of cytochrome *P*-450-dependent 25-hydroxylation of bile acid intermediates in kidney microsomes was somewhat unexpected. Bile acid biosynthesis is considered to take place exclusively in the liver, and 5 β -cholestane-3 α ,7 α ,12 α -triol is an intermediate in the biosynthesis of cholic acid, which is not synthesized in the pig [30]. A plausible explanation is that the microsomal 25-hydroxylase might not be very specific with respect to the structure of the steroid nucleus of C₂₇ steroids.

The low 25-hydroxylase activity towards vitamin D₃ compared with 5 β -cholestane-3 α ,7 α -diol and 5 β -cholestane-3 α ,7 α ,12 α -triol could be due to the hydrophobic nature of vitamin D₃. In the reconstituted system, less hydrophobic substrates such as 5 β -cholestane-3 α ,7 α -diol and 5 β -cholestane-3 α ,7 α ,12 α -triol may be more readily hydroxylated than they are *in vivo*. In this connection, it might be mentioned that cholesterol is not 25-hydroxylated by microsomal enzyme systems. The 25-hydroxylation of cholesterol occurs in mitochondrial systems [31].

In the present report, no attempts were made to study the possibility of vitamin D₃ being a competitive inhibitor of the 25-hydroxylation of C₂₇ steroids. The reason for this was that previous reports as well as accumulated experience in this laboratory have shown that no accurate conclusions can be drawn from inhibition experiments with these hydrophobic

substrates. Björkhem *et al.* [32] studied the mitochondrial 26- and 25-hydroxylation of C₂₇ steroids and vitamin D₃ respectively in rat liver. They obtained an inhibition with vitamin D₃ that was obviously not of a competitive but rather of an uncompetitive nature. When the C₂₇ steroid was used as an inhibitor it was not possible to obtain straight lines in a Lineweaver-Burk plot.

Immunoprecipitation of hydroxylase activities of cytochrome *P*-450 by the mAb shows that the 25-hydroxylase activities towards vitamin D₃ and 5 β -cholestane-3 α ,7 α -diol were decreased by about 80 %, whereas the hydroxylase activity towards lauric acid was not decreased to any greater degree than by the irrelevant antibody. These results indicate that different species of microsomal cytochrome *P*-450 are involved in 25-hydroxylation of vitamin D₃ and C₂₇ steroids on the one hand and the ω -, ω -1-hydroxylations of lauric acid on the other. The monoclonal antibody recognized the cytochrome *P*-450 in both kidney and liver microsomes from male as well as female pigs, indicating that similar isoenzymes of cytochrome *P*-450 are involved in the 25-hydroxylation of vitamin D₃ and C₂₇ steroids in pig kidney and liver and that there are no sex differences. The lack of sex difference is in contrast with rat liver microsomal cytochrome *P*-450₂₅, which is male-specific [12,25]. The mAb directed against the male-specific form in rat liver microsomes, mAb 25-6, did not inhibit the 25-hydroxylase activity in pig kidney or liver, showing that there are important structural differences between the 25-hydroxylases in pig and rat liver.

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