Characterization of mitochondrial cytochromes *P*-450 from pig kidney and liver catalysing 26-hydroxylation of 25-hydroxyvitamin D₃ and C₂₇ steroids

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The properties of cytochrome *P*-450 from pig kidney mitochondria, catalysing 26-hydroxylation of 25-hydroxyvitamin D₃ and C₂₇ steroids [Postlind & Wikvall (1989) *Biochem. Biophys. Res. Commun.* **159**, 1135–1140; Postlind (1990) *Biochem. Biophys. Res. Commun.* **168**, 261–266], were compared with those of a 26-hydroxylating cytochrome *P*-450 from pig liver mitochondria. The liver enzyme was purified to a cytochrome *P*-450 content of 7.4 nmol/mg of protein and showed only one protein band with an apparent *M_r* of 53 000 upon SDS/PAGE. The cytochrome *P*-450 catalysed 26-hydroxylation of 25-hydroxyvitamin D₃, cholesterol and 5 β -cholestane-3 α ,7 α -diol at rates of 361, 1090 and 2065 pmol/min per nmol of cytochrome *P*-450. A monoclonal antibody against the purified liver mitochondrial cytochrome *P*-450 26-hydroxylase (cytochrome *P*-450₂₆) was prepared. After coupling to Sepharose, the antibody was able to bind to cytochrome *P*-450₂₆ from liver as well as from kidney mitochondria and to immunoprecipitate the 26-hydroxylase activity towards 25-hydroxyvitamin D₃ and cholesterol when assayed in a reconstituted system. After SDS/PAGE and immunoblotting with the antibody, the cytochrome *P*-450₂₆ was detected in the purified liver and kidney preparations. These results indicate that similar species of cytochrome *P*-450 catalyse 26-hydroxylation of 25-hydroxyvitamin D₃ and C₂₇ steroids in liver and kidney mitochondria. The results with the monoclonal antibody together with the finding that cholesterol competitively inhibits the 26-hydroxylation of 25-hydroxyvitamin D₃ further indicate that 26-hydroxylation of 25-hydroxyvitamin D₃ and cholesterol is catalysed by the same species of cytochrome *P*-450 in each tissue. The *N*-terminal amino acid sequence of cytochrome *P*-450₂₆ in kidney mitochondria resembled that of pig kidney microsomal 25-hydroxylase active in 25-hydroxylation of vitamin D₃ and C₂₇ steroids, whereas the sequence of pig liver mitochondrial cytochrome *P*-450₂₆ differed from those of rabbit and rat liver mitochondrial 26-hydroxylases as well as from those of other hitherto isolated mammalian cytochromes *P*-450.

INTRODUCTION

26-Hydroxylation of 25-hydroxyvitamin D₃ has been recently shown to be catalysed by a mitochondrial cytochrome *P*-450 in the kidney [1,2]. This hydroxylation is considered to be involved in the production of biologically inert compounds, but the possibility exists that they provide some special function of vitamin D not yet discovered [3]. The finding that plasma concentrations of 25,26-dihydroxyvitamin D₃ are only slightly lowered or even unaffected in anephric patients indicates the existence of extrarenal production of 25,26-dihydroxyvitamin D₃ [4–7]. However, no extrarenal enzyme system able to form 25,26-dihydroxyvitamin D₃ has hitherto been described.

The present paper reports the isolation of a mitochondrial 26-hydroxylase from pig liver active in 26-hydroxylation of 25-hydroxyvitamin D₃ and C₂₇ steroids. Further, the properties of the liver enzyme are compared with those of the mitochondrial 26-hydroxylase from pig kidney.

EXPERIMENTAL

Materials

Q-Sepharose and S-Sepharose were from Pharmacia and 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 [1,8].

Enzyme purifications

26-Hydroxylating pig liver mitochondrial cytochrome *P*-450

(cytochrome *P*-450₂₆) was purified to electrophoretic homogeneity with some modifications of the procedure reported by Dahlbäck for rabbit liver mitochondrial 26-hydroxylase [9]. The hydroxyapatite column was washed with 50 mM-phosphate buffer, pH 7.25, containing 0.2% (w/v) Lubrol PX. The cytochrome *P*-450 was eluted with 150 mM-phosphate buffer, pH 7.25, containing 0.2% (w/v) Lubrol PX. It was concentrated and dialysed against 20 mM-Tris/acetate buffer, pH 8.0, containing 0.1% (w/v) sodium cholate and 0.05% (w/v) Lubrol PX. The sample was injected into a Q-Sepharose column (1.6 cm × 20 cm) equilibrated in the same buffer as used for dialysis. The flow rate was 4 ml/min and the cytochrome *P*-450 was eluted with linear gradients of sodium acetate in the equilibrating buffer (0–10 min, 0 M; 10–40 min, 0–0.15 M; 40–60 min, 0.15–0.3 M; 60–80 min, 0.3 M; 80–120 min, 0.3–0.5 M). The cytochrome *P*-450 was eluted at 0.18 M-sodium acetate in the gradient. The fractions with highest content of cytochrome *P*-450/mg of protein were pooled, concentrated and dialysed against 10 mM-phosphate buffer, pH 7.8, containing 0.1% (w/v) sodium cholate and 0.05% (w/v) Lubrol PX. The dialysis residue was applied to an S-Sepharose column (1.6 cm × 20 cm) equilibrated in the same buffer as used for dialysis. The column was eluted with linear gradients of sodium acetate in the equilibrating buffer (0–30 min, 0–0.05 M; 30–55 min, 0.05–0.15 M; 55–75 min, 0.15 M; 75–110 min, 0.15–0.5 M). The flow rate was 2 ml/min and the cytochrome *P*-450 was eluted at 0.15 M-sodium acetate in the gradient. The fractions were pooled, concentrated and dialysed against 10 mM-phosphate buffer, pH 7.0, containing 0.1% (w/v) sodium cholate

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and 0.05 % (w/v) Lubrol PX. The dialysis residue was injected into a second Q-Sepharose column (1.6 cm $\times$ 15 cm) equilibrated in the same buffer as used for dialysis. The flow rate was 2 ml/min. The column was eluted with a stepwise gradient of sodium acetate in the equilibrating buffer. The 26-hydroxylating cytochrome *P*-450 was eluted with 0.2 M-sodium acetate. Detergents were removed and the final enzyme fraction was dialysed as described previously [10]. Phosphate was used as the potassium salt and all buffers contained 20 % (v/v) glycerol and 0.1 mM-EDTA.

Pig kidney mitochondrial cytochrome *P*-450 catalysing 26-hydroxylation of 25-hydroxyvitamin D₃ and C₂₇ steroids was purified as described previously [2].

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 mitochondria was emulsified in an equal volume of Freund's complete adjuvant. A 75 μ g portion of enzyme was injected intraperitoneally. Then 3 weeks 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 for antibody-producing cells as described by Andersson & Jörnvall [11]. Of 3100 microwells resulting from one single fusion, 80 produced antibodies that recognized the cytochrome *P*-450. Eight hybridomas of the 80 that produced antibodies were monospecific for the antigen. Cloning of positive cultures, purification of immunoglobulins and coupling to Sepharose were performed as described previously [11]. The monoclonal antibody was designated MAb 26C5. Subclass of the monoclonal antibody was determined by the Ouchterlony immunodiffusion technique. Immunoabsorbent preparation, e.i.s.a. and immunoblotting were performed as described by Andersson & Jörnvall [11].

Incubation procedures

Incubations with purified cytochrome *P*-450 from the liver were carried out for 5 min and with the cytochrome *P*-450 preparation from kidney for 20 min at 37 °C. The substrates, dissolved in 25 μ l of acetone, were added to the incubation mixture at saturating concentrations. 25-Hydroxyvitamin D₃ (25 μ g), cholesterol (10 μ g) or 5 β -cholestane-3 α ,7 α -diol (25 μ g) was incubated with 0.1 nmol of the purified liver preparation or with 0.2 nmol of the purified kidney preparation and 2 nmol of adrenodoxin, 0.2 nmol of adrenodoxin reductase and 1 μ mol of NADPH in a total volume of 1 ml of 50 mM-Tris/acetate buffer, pH 7.4. Incubations with intact mitochondria were performed with saturating concentrations of substrate (40 μ g dissolved in 25 μ l of acetone), 20 mg of mitochondrial protein, 45 μ mol of isocitrate and 10 mM-MgCl₂ in a total volume of 5 ml of 50 mM-Tris/acetate buffer, pH 7.4.

Incubations with 25-hydroxyvitamin D₃ were terminated by addition of 5 ml of trichloroethane/methanol (2:1, v/v). The incubations were extracted twice with trichloroethane/methanol (2:1, v/v) and the organic phases were dried down with N₂, dissolved in 100 μ l of mobile phase and submitted to straight-phase h.p.l.c. analysis. The mobile phase was hexane/propan-2-ol (9:1, v/v) and the solvent flow was 0.7 ml/min. The fractions corresponding to authentic 25,26-dihydroxyvitamin D₃ were collected and evaporated to dryness with N₂. The residue was dissolved in 100 μ l of the mobile phase, [methanol/water (17:3, v/v)] and submitted to reverse-phase h.p.l.c. with a solvent flow of 0.5 ml/min. The amount of 25,26-dihydroxyvitamin D₃ was quantified and corrections were made for recovery as described [12].

Cholesterol and 5 β -cholestane-3 α ,7 α -diol incubations were terminated by addition of 5 ml of 95 % (v/v) ethanol and

extracted as described by Wikvall [10]. The incubations were quantified by h.p.l.c. as reported previously [13,14].

Incubations with antibody-coupled Sepharose and cytochrome *P*-450 (0.1 nmol in the liver and 0.2 nmol in the kidney incubations) were performed as described in ref. [13].

Amino acid and N-terminal sequence analyses

MAb 26 C5-Sepharose-bound cytochromes *P*-450₂₆ from liver and kidney were eluted from the columns with 0.1 M-sodium citrate buffer, pH 3, and precipitated with acetone. The precipitate was used for amino acid and N-terminal sequence analyses.

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 a Beckman 121 M amino acid analyser. N-Terminal sequences were determined by gas-phase sequencer analysis with an Applied Biosystems 470A instrument. Phenylthiohydantoin derivatives were identified by reverse-phase h.p.l.c. as described in ref. [15].

Additional methods

Adrenodoxin and adrenodoxin reductase from bovine adrenal mitochondria were prepared as described by Wikvall [10]. Protein was determined by the method of Lowry *et al.* [16], with BSA as standard, and cytochrome *P*-450 as described by Omura & Sato [17]. Gel electrophoresis was performed according to the procedure of Laemmli [18] with 15 % polyacrylamide (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.* [19].

RESULTS AND DISCUSSION

26-Hydroxylating cytochrome *P*-450 was isolated from pig kidney mitochondria as described previously [2]. The preparation had a specific cytochrome *P*-450 content of 2.8 nmol/mg of

Fig. 1. SDS/PAGE of purified mitochondrial cytochrome *P*-450₂₆ from pig liver

The protein samples were pretreated with SDS and mercaptoethanol at 100 °C for 2 min and subjected to PAGE. Migration was from top to bottom. Gel electrophoresis was performed as described in the Experimental section. A mixture of cytochrome *P*-450 IA2 and cytochrome *P*-450 IIB4 from rabbit liver microsomal fraction, prepared in this laboratory as described by Haugen & Coon [24] (0.5 μ g of each) in lane 1; liver mitochondrial cytochrome *P*-450 (1 μ g) in lane 2.

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

Incubations were performed at 37 °C for 20 min with 0.2 nmol of cytochrome P-450, 2 nmol of adrenodoxin, 0.2 nmol of adrenodoxin reductase, 1 μ mol of NADPH and 25-hydroxyvitamin D₃ (25 μ g), cholesterol (10 μ g) or 5 β -cholestane-3 α ,7 α -diol (25 μ g) in a total volume of 1 ml of 50 mM-Tris/acetate buffer, pH 7.4. Incubations with original mitochondria were performed as described in the Experimental section. All incubations were performed with saturating substrate concentrations. The results from three experiments with intact mitochondria and from five experiments with purified cytochrome P-450 are given as means $\pm$ S.D.

Reaction measured	Hydroxylase activity		
	Mitochondria	Cytochrome P-450	
	(pmol/min per mg of protein)	(pmol/min per mg of protein)	(pmol/min per nmol of P-450)
25-Hydroxyvitamin D ₃ 26-hydroxylation	0.32 $\pm$ 0.15	309 $\pm$ 56	110 $\pm$ 20
Cholesterol 26-hydroxylation	6.3 $\pm$ 2.1	633 $\pm$ 86	229 $\pm$ 35
5 β -Cholestane-3 α ,7 α -diol 26-hydroxylation	7.3 $\pm$ 2.5	688 $\pm$ 85	246 $\pm$ 30

protein. Attempts to purify the 26-hydroxylase further with various chromatographic methods were unsuccessful.

A 26-hydroxylating liver mitochondrial cytochrome P-450 fraction was prepared by solubilization with sodium cholate and chromatography on octylamine-Sepharose, hydroxyapatite, Q-Sepharose, S-Sepharose and an additional Q-Sepharose. The final enzyme fraction had a specific content of 7.4 nmol of

Table 2. Hydroxylase activities in mitochondria and purified cytochrome P-450 from pig liver

Incubations were performed at 37 °C for 5 min with 0.1 nmol of cytochrome P-450 in the same way as described in Table 1. The results from three experiments with intact mitochondria and from five experiments with purified cytochrome P-450 are given as means $\pm$ S.D.

Reaction measured	Hydroxylase activity		
	Mitochondria	Cytochrome P-450	
	(pmol/min per mg of protein)	(pmol/min per mg of protein)	(pmol/min per nmol of P-450)
25-Hydroxyvitamin D ₃ 26-hydroxylation	2.7 $\pm$ 0.4	2671 $\pm$ 668	361 $\pm$ 90
Cholesterol 26-hydroxylation	9.8 $\pm$ 3.1	8066 $\pm$ 1394	1090 $\pm$ 188
5 β -Cholestane-3 α ,7 α -diol 26-hydroxylation	33 $\pm$ 5	15284 $\pm$ 1075	2065 $\pm$ 145

cytochrome P-450/mg of protein and showed only one protein band corresponding to an apparent M_r of 53000 upon SDS/PAGE (Fig. 1).

The haem content of the mitochondrial cytochrome P-450₂₆ preparations was less than maximal, 2.8 nmol/mg of protein for the kidney preparation and 7.4 nmol/mg of protein for the liver preparation instead of approx. 19 nmol/mg of protein. None of the preparations contained cytochrome P-420. The cytochromes P-450 in kidney mitochondria are extremely difficult to purify owing to the small amount of cytochrome P-450 in the mitochondria [20–22]. Ohyama *et al.* [23] recently reported the

Fig. 2. Effect of various amounts of substrate and inhibitory effects of cholesterol on the 26-hydroxylation of 25-hydroxyvitamin D₃ in (a) the kidney and (b) liver preparation

Incubations were performed at 37 °C for 20 min with cytochrome P-450 (0.1 nmol in the liver and 0.2 nmol in the kidney incubations), 2 nmol of adrenodoxin, 0.2 nmol of adrenodoxin reductase, 1 μ mol of NADPH and various amounts of substrate in a total volume of 1 ml of 50 mM-Tris/acetate buffer, pH 7.4. In inhibition experiments a constant amount of cholesterol (10 μ g) was added to the incubation mixture. v , velocity (pmol/min per nmol of cytochrome P-450); s , concentration of substrate (μ M). ○, Without addition of cholesterol; ●, in the presence of cholesterol (10 μ g).

Fig. 3. Immunoprecipitation of hydroxylase activities of cytochrome $P-450_{26}$ by anti-(cytochrome $P-450_{26}$) monoclonal antibody (MAb 26C5): 26-hydroxylation of 25-hydroxyvitamin D_3 in (a) kidney and (b) liver; 26-hydroxylation of cholesterol in (c) kidney and (d) liver

Cytochrome $P-450_{26}$ (0.1–0.2 nmol) was incubated in a total volume of 1 ml with the indicated amounts of Sepharose-bound MAb 26C5 (□ and ○) or irrelevant monoclonal antibody (■ and ●) and assayed for hydroxylase activity as described in the Experimental section. The 100 % of control value for hydroxylase activity in tubes containing no antibody was, for 26-hydroxylation of 25-dihydroxyvitamin D_3 54 pmol/min per nmol of cytochrome $P-450$ in the kidney and, for 26-hydroxylation of cholesterol, 152 pmol/min per nmol of cytochrome $P-450$. In the liver, the 100 % of control value was for 25-hydroxyvitamin D_3 166 pmol/min per nmol of cytochrome $P-450$ and, for cholesterol 849 pmol/min per nmol of cytochrome $P-450$.

purification of an electrophoretically homogeneous cytochrome $P-450$ from kidney mitochondria of female rats treated with vitamin D_3 . Although the preparation was apparently homogeneous and catalysed a very efficient 24-hydroxylation of 25-hydroxyvitamin D_3 , the cytochrome $P-450$ content was not higher than that in the present partially purified preparation from pig kidney mitochondria. Concerning the cytochrome $P-450$ from liver mitochondria, it should be mentioned that a cytochrome $P-450$ from rabbit liver mitochondria active in 26-hydroxylation of 5β -cholestane- $3\alpha,7\alpha,12\alpha$ -triol was found to be susceptible to proteolytic degradation during the purification [9]. Low haem contents in apparently homogeneous cytochrome $P-450$ preparations have been explained by haem losses during the purification procedure [10,24]. It is likely that haem losses and proteolytic degradation might also occur in the preparation of cytochromes $P-450_{26}$ from pig kidney and liver.

The catalytic properties of the cytochrome $P-450_{26}$ preparation and the original mitochondria from kidney are summarized in Table 1. The hydroxylations studied were the 26-hydroxylation of 25-hydroxyvitamin D_3 , cholesterol and 5β -cholestane- $3\alpha,7\alpha$ -diol. The 26-hydroxylase activity, expressed as pmol of product formed/min per mg of protein, towards 25-hydroxyvitamin D_3 was 950 times higher and towards cholesterol and 5β -cholestane- $3\alpha,7\alpha$ -diol 100 times higher with the purified cytochrome $P-450$

than with the mitochondria. In Table 2 the catalytic properties of the cytochrome $P-450_{26}$ and the original mitochondria from liver are summarized. The 26-hydroxylase activity was 1000 times higher for 25-hydroxyvitamin D_3 and cholesterol and 800 times higher for 5β -cholestane- $3\alpha,7\alpha$ -diol in the purified cytochrome $P-450$ than in the mitochondria. All incubations were performed with saturating substrate concentrations.

Various amounts of 25-hydroxyvitamin D_3 were incubated with purified cytochromes $P-450_{26}$ from pig kidney and liver. The K_m for 26-hydroxylation of 25-hydroxyvitamin D_3 calculated from Lineweaver–Burk plots was about $2\ \mu\text{M}$ with the kidney preparation (Fig. 2a) and about $5\ \mu\text{M}$ with the liver preparation (Fig. 2b). Fig. 2 also shows experiments in which the various amounts of 25-hydroxyvitamin D_3 were incubated in the presence of a fixed amount of cholesterol. The results from these experiments show that cholesterol competitively inhibits the 26-hydroxylation of 25-hydroxyvitamin D_3 by both the kidney (Fig. 2a) and liver (Fig. 2b) preparation. This finding indicates that the two substrates compete for the same active site.

Several experiments showed that incubation of liver mitochondria with low concentrations of 25-hydroxyvitamin D_3 , $60\ \mu\text{M}$ or less, resulted in no detectable 26-hydroxylase activity. This inhibiting effect at lower 25-hydroxyvitamin D_3 concentrations is noteworthy and suggests the possible presence

Fig. 4. SDS/PAGE and immunoblotting of cytochrome *P*-450₂₆ in pig kidney and liver mitochondria

Samples containing the indicated amounts of proteins were subjected to SDS/PAGE, electrophoretic transfer and immunoblotting with MAb 26C5 as described in the Experimental section. Migration was from top to bottom. Lane 1, cytochrome *P*-450₂₆ from liver mitochondria (1 µg); lane 2, cytochrome *P*-450₂₆ from kidney mitochondria (5 µg); lane 3, liver mitochondria (100 µg); lane 4, kidney mitochondria (100 µg).

Table 3. *N*-Terminal amino acid sequences of mitochondrial 26-hydroxylases from pig liver, rabbit liver, rat liver and pig kidney as well as of microsomal 25-hydroxylase from pig kidney

	1	2	3	4	5	6	7	8	9
Pig liver cytochrome <i>P</i> -450 ₂₆	A	L	K	G	Q	G	P	F	G
Rabbit liver cytochrome <i>P</i> -450 ₂₆	A	L	P	A	D	E	A	A	E
Rat liver cytochrome <i>P</i> -450 ₂₆	A	I	P	A	A	L	R	D	H
Pig kidney cytochrome <i>P</i> -450 ₂₆	G	L	T	X	G	L	X	L	G
Pig kidney cytochrome <i>P</i> -450 ₂₅	G	L	L	T	G	L	L	L	G

of an inhibitor of 26-hydroxylation in intact mitochondria. An inhibitor has been previously shown for microsomal 25-hydroxylation of vitamin D₃ in rat liver [25]. An experiment with increasing substrate concentrations showed that it was possible to overcome the inhibiting effect at a 25-hydroxyvitamin D₃ concentration of 100 µM. Two possible explanations may be offered. Since cholesterol has been shown to inhibit competitively the 26-hydroxylation of 25-hydroxyvitamin D₃ (cf. Fig. 2), the very low 26-hydroxylase activity could be due to endogenous cholesterol in mitochondria competing with 25-hydroxyvitamin D₃. The inhibition may also be due to the presence of an inhibiting protein such as a 25-hydroxyvitamin D₃-binding protein.

A monoclonal antibody against cytochrome *P*-450₂₆ in liver mitochondria, designated MAb 26C5, was prepared by immunization of mice with purified cytochrome *P*-450₂₆. MAb 26C5 was found to belong to the IgG1 subclass. To demonstrate the reactivity of this antibody, it was coupled to Sepharose and incubated with purified cytochrome *P*-450₂₆ from both liver and kidney. After incubation, the antibody-Sepharose was removed and the supernatant was assayed in a reconstituted system for 26-hydroxylase activity towards 25-hydroxyvitamin D₃ and cholesterol. As a control, Sepharose was coupled to an

irrelevant monoclonal antibody, directed against a rat liver microsomal protein of unknown function. Increasing amounts of MAb 26C5 but not the irrelevant antibody were able to bind more than 80 % of the 26-hydroxylase activity from both organs towards both substrates (Figs. 3a–3d). SDS/PAGE and immunoblotting analyses with MAb 26C5 of cytochrome *P*-450₂₆ in liver and kidney mitochondria (Fig. 4) showed that the antibody recognized purified cytochrome *P*-450₂₆ from liver mitochondria (lane 1) and kidney mitochondria (lane 2) as well as a protein of the same *M_r* in original liver mitochondria (lane 3). No protein was detected in original kidney mitochondria (lane 4), probably owing to the small amount of cytochrome *P*-450 in kidney mitochondria [20–22]. Experiments with MAb 26C5 indicated that similar species of mitochondrial cytochrome *P*-450 are involved in 26-hydroxylation of 25-hydroxyvitamin D₃ and C₂₇ steroids in pig liver and kidney. These results, together with the finding that cholesterol is a competitive inhibitor of 26-hydroxylation of 25-hydroxyvitamin D₃ (Figs. 2a and 2b), strongly indicate the presence of a single enzyme in each tissue responsible for the 26-hydroxylation of both substrates.

N-Terminal amino acid sequences of MAb 26C5-Sepharose-bound cytochrome *P*-450₂₆ are summarized in Table 3. The sequences are compared with the *N*-terminal sequences of rabbit [9] and rat [26,27] liver mitochondrial 26-hydroxylases as well as with the pig kidney microsomal 25-hydroxylase, catalysing 25-hydroxylation of vitamin D₃ and C₂₇ steroids [13]. The amino acid composition of cytochromes *P*-450₂₆ from liver and kidney was similar to that of other purified mammalian cytochromes *P*-450 [28]. The *N*-terminal amino acid sequence of cytochrome *P*-450₂₆ in pig kidney mitochondria resembled that reported for the pig kidney microsomal 25-hydroxylase, active in 25-hydroxylation of vitamin D₃ and C₂₇ steroids [13], whereas the sequence of cytochrome *P*-450₂₆ in liver mitochondria differed from those of rabbit [9] and rat [26,27] liver mitochondrial 26-hydroxylases as well as from those of other isolated mammalian cytochromes *P*-450 [28].

The present paper describes for the first time the presence of 26-hydroxylase activity towards 25-hydroxyvitamin D₃ in liver. The role in vitamin D₃ metabolism of 25,26-dihydroxyvitamin D₃ formation in the liver relative to that in the kidney cannot be assessed at present.

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