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Microsomal 25-Hydroxylation of Vitamin D_2 and Vitamin D_3 in Pig Liver

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A microsomal cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_2 was purified from both male and female pigs to apparent homogeneity and a specific cytochrome *P*-450 content of 13 and 15.4 nmol × mg of protein^{-1}, respectively. The enzyme also catalysed 25-hydroxylation of vitamin D_3. The ratio between the 25-hydroxylase activities towards vitamin D_2 and D_3 was essentially the same in the different purification steps as well as in the apparently homogeneous enzyme preparation. The two enzyme activities showed the same pH optimum and decreased in parallel upon partial denaturation of the enzyme. Cholecalciferol competitively inhibited 25-hydroxylation of vitamin D_2 and *vice versa*. The non-steroidal cytochrome *P*-450 inhibitor ketoconazole inhibited both enzyme activities and the K_i values were the same. The cytochrome *P*-450 showed the same apparent M_r, substrate specificity and N-terminal amino acid sequence as the previously purified vitamin D_3 25-hydroxylase from pig liver microsomes. A monoclonal antibody raised against the vitamin D_3 25-hydroxylase also recognized the vitamin D_2 25-hydroxylase. The antibody immunoprecipitated the 25-hydroxylase activity towards both vitamin D_2 and D_3 in the purified enzyme. Taken together, the results show that the 25-hydroxylation of vitamin D_2 and D_3 is catalysed by the same microsomal cytochrome *P*-450 in pig liver microsomes. The properties of this 25-hydroxylase are discussed in relation to present knowledge concerning previously well-characterized vitamin D_3 25-hydroxylases that are not able to catalyse 25-hydroxylation of vitamin D_2.

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INTRODUCTION

Vitamin D_2 which differs structurally from vitamin D_3 in the side chain is formed by ultraviolet irradiation of the plant sterol ergosterol. Vitamin D_2 has been frequently used to treat and prevent vitamin D deficiency and is used in parenteral vitamin formulations. A 25-hydroxylation is the first step in the bioactivation of both vitamin D_3 and D_2 into their active hormonal forms [1, 2]. The 25-hydroxylation of vitamin D_3 is catalysed by cytochrome *P*-450 enzymes in both the microsomal and mitochondrial fractions of liver [3–6]. The relative importance of microsomal and mitochondrial 25-hydroxylation in the bioactivation process is not known at present. A male-specific microsomal enzyme from rat liver has been extensively studied [3, 4, 7]. It is apparently identical to rat liver microsomal testosterone 16α-hydroxylase CYP 2C11 [8]. A mitochondrial vitamin D_3 25-hydroxylase which is present in rabbit [5], rat [6] and man [9] appears to be identical to sterol 27-hydroxylase, CYP 27 [10–13]. Much less but also conflicting information is available about the enzyme(s) catalysing 25-hydroxylation of vitamin D_2 [3, 9, 14–17]. Surprisingly, none of the vitamin D_3 25-hydroxylases studied previously appears to be active in 25-hydroxylation of vitamin D_2. Thus, Andersson *et al.* [3] reported that the apparently homogeneous, male-specific, microsomal 25-hydroxylase from rat liver did not show 25-hydroxylase activity towards vitamin D_2. In a recent report, Guo *et al.* [9] reported that COS-cells transfected with cDNA encoding human CYP 27 expressed 25-hydroxylase activity towards vitamin D_3 but not towards vitamin D_2. In fact, no cytochrome *P*-450 active in 25-hydroxylation of vitamin D_2 has been purified to homogeneity or characterized so far. Consequently, it is not known whether vitamin D_2 and D_3 are 25-hydroxylated by the same or separate species of cytochrome *P*-450 in liver.

Recently a microsomal vitamin D_3 25-hydroxylase was purified from pig liver [18]. The aim of the present

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Table 1. Hydroxylase activities at different steps in the purification of a microsomal cytochrome P-450 vitamin D_2 25-hydroxylase from liver of untreated castrated pigs

	Cytochrome *P*-450 (nmol × mg of protein^{-1})	Vitamin D_2 25-hydroxylation (pmol × min^{-1} × mg of protein^{-1})	Vitamin D_2 25-hydroxylation (pmol × min^{-1} × nmol of *P*-450^{-1})	Vitamin D_3 25-hydroxylation (pmol × min^{-1} × mg of protein^{-1})	Vitamin D_3 25-hydroxylation (pmol × min^{-1} × nmol of *P*-450^{-1})
Microsomes	0.5	0.6	1.2	0.8	1.6
Polyethylene glycol (8–15%) precipitate	0.3	0.4	1.3	1.3	4.3
Octylamine–Sepharose	1.4	5.2	3.7	9.4	6.7
Hydroxylapatite (120 mM-phosphate eluate)	1.9	62.4	32.8	101.9	53.6
Q-Sepharose (non-bound fraction)	4.4	233.0	53.0	611.1	138.9
S-Sepharose (25 mM-sodium acetate eluate)	13.0	1270.0	97.7	2407.0	185.1

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

study was to purify and characterize a cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_2. The same procedures were applied as used for isolation of the vitamin D_3 25-hydroxylase [18]. Strong evidence is provided that the same microsomal cytochrome *P*-450 catalyses the 25-hydroxylation of both vitamin D_2 and D_3 in pig liver.

EXPERIMENTAL

Materials

25-Hydroxy[23,24(*n*)-^{3}H]vitamin D_3 (105.5 Ci/mmol), 1α,25-dihydroxy-[23,24(*n*)^{3}H]vitamin D_3 (91.4 Ci/mmol), and [4-^{14}C]cholesterol (61 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., England). 5β-[7β-^{3}H]Cholestane-3α,7α-diol (400 mCi/mmol) was prepared as described previously [19, 20]. Unlabelled vitamin D_2 and D_3 were obtained from Sigma Chemical Co. (St Louis, MO). Unlabelled 1α-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_2 were generous gifts from Lövens (Copenhagen, Denmark) and Roche Products Ltd (Stockholm, Sweden), respectively. Unlabelled 25-hydroxyvitamin D_3 was obtained from Solvay Duphar B.V. (the Netherlands). Unlabelled 1α,25-dihydroxyvitamin D_3 was obtained from Roche. Unlabelled 25-hydroxyvitamin D_2 and ketoconazole were generous gifts from Dr Inger Holmberg and Janssen Pharmaceutica, respectively. Hydroxylapatite (Bio-Rad, Richmond, CA) 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, Uppsala, Sweden). 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 male (castrated) or female pigs were used as a source of enzyme. Cytochrome

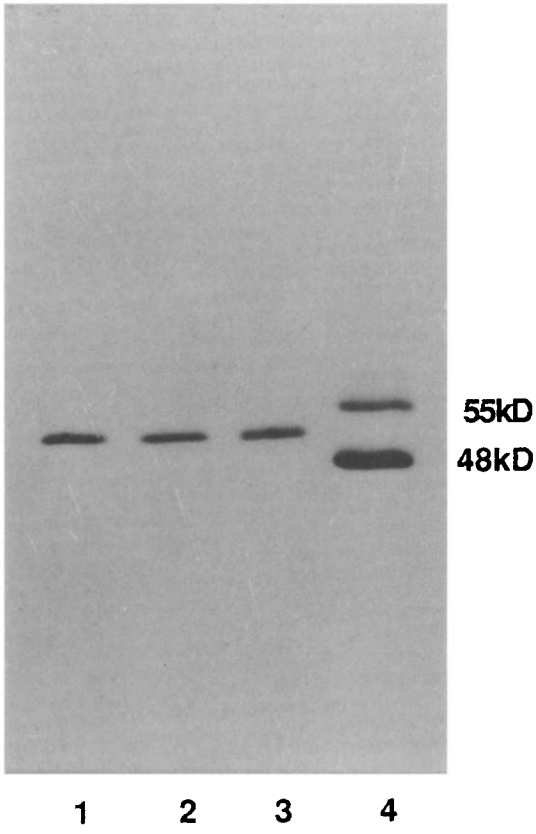

Fig. 1. SDS–PAGE of purified microsomal 25-hydroxylating cytochromes *P*-450 from pig liver. The protein samples were pretreated with SDS and mercaptoethanol at 100°C for 2 min and submitted to polyacrylamide-gel electrophoresis in the presence of SDS. Migration was from top to bottom. Gel electrophoresis was performed according to Laemmli [32] with 15% acrylamide and 0.09% bisacrylamide slab gels (15 × 10 × 0.1 cm) containing 0.1% (w/v) SDS. The gels were polymerized by addition of 0.1% (v/v) tetramethylenediamine 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.* [33]. Lane 1, purified vitamin D_2 25-hydroxylase from female pig (1 μg); lane 2, purified vitamin D_2 25-hydroxylase from castrated pig (1 μg); lane 3, purified vitamin D_3 25-hydroxylase [*cf* 18]; lane 4, M_r standards were a mixture of cytochromes *P*-450 IA2 and IIB4 from rabbit liver microsomal fraction, prepared in this laboratory as described by Haugen and Coon [34].

Table 2. N-terminal amino acid sequences of microsomal and mitochondrial cytochromes P-450 active in the 25-hydroxylation of vitamin D_3 from pig, rat, rabbit and human liver

Source	Residue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Pig liver microsomal 25-hydroxylase		G	L	L	T	G	D	L	L	G	I	L	A	L	A	M	V	I	F	L	L
Rat liver microsomal CYP 2C11		M	D	P	V	L	V	L	V	L	T	L	S	S	L	L	L	L	S	L	W
Rat liver mitochondrial CYP 27		M	A	V	L	S	R	M	R	L	R	W	A	L	L	D	T	R	V	M	G
Rabbit liver mitochondrial CYP 27		A	L	P	A	D	E	A	A	Q	A	P	G	A	G	P	G	D	R	R	R
Human liver mitochondrial CYP 27		A	L	P	S	D	K	A	T	G	A	P	G	A	G	P	G	V	R	R	R

P-450, active in 25-hydroxylation of vitamin D_2, was purified as described previously for the isolation of a vitamin D_3 25-hydroxylase from pig liver microsomes [18]. NADPH–cytochrome P-450 reductase was prepared from liver microsomes of phenobarbital-treated rats as described by Yasukochi and Masters [21]. Protein and cytochrome P-450 were determined as described by Lowry *et al.* [22] and Omura and Sato [23], respectively.

Incubation procedures

Incubations were performed as described previously [18] to incubations with vitamin D_2 and 1α-hydroxyvitamin D_2 no labelled products were added. It was assumed that the recovery for vitamin D_2 metabolites was the same as for corresponding vitamin D_3 metabolites. The retention time for 25-hydroxyvitamin D_2 was 13.5 min and for 1α,25-dihydroxyvitamin D_2

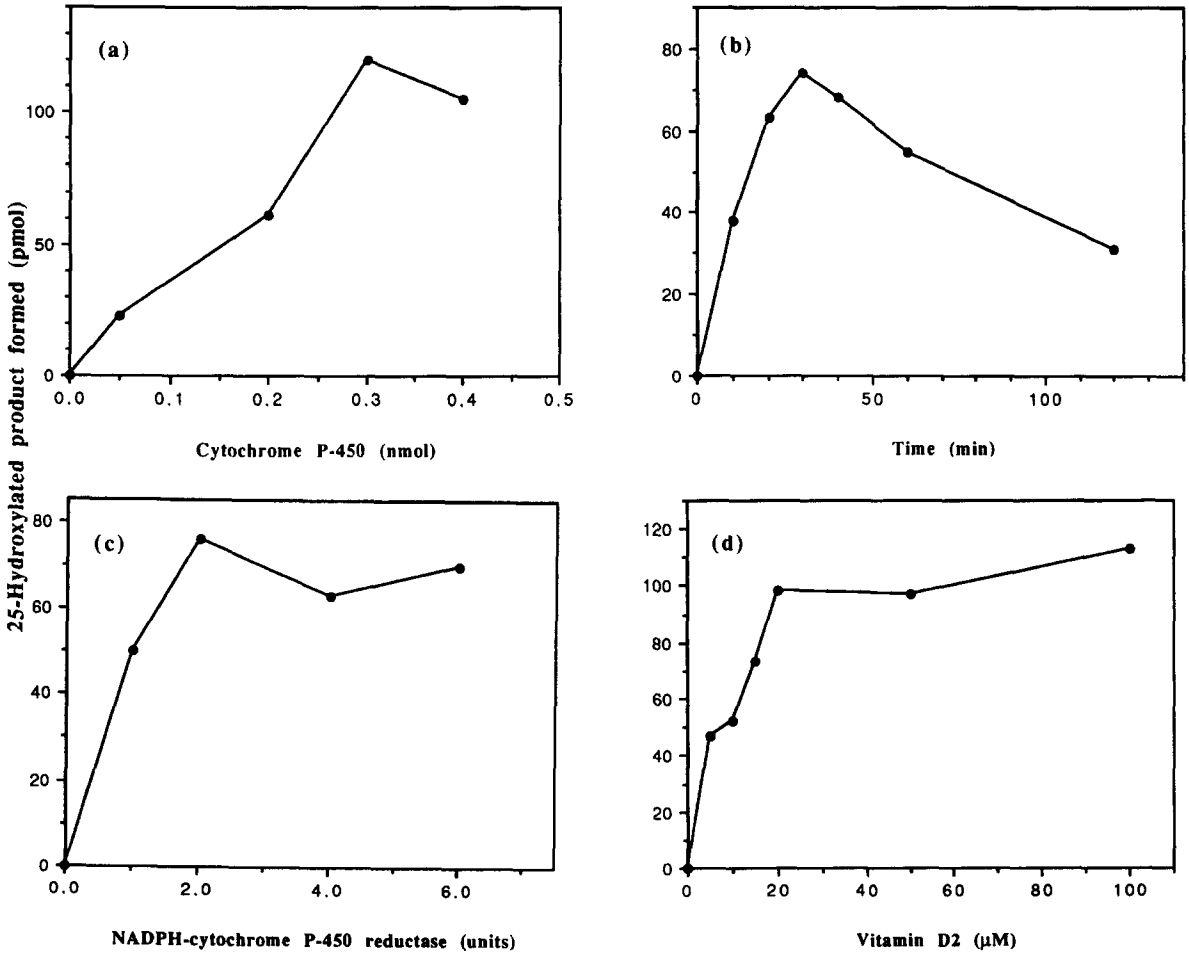

Fig. 2. Effects of cytochrome P-450 concentration (a), time (b), NADPH–cytochrome P-450 reductase concentration (c) and vitamin D_2 concentration (d) on the rate of 25-hydroxylation of vitamin D_2. Incubations were performed as described in the Experimental section except when the concentration of a component was varied. The results from three experiments are given as the means.

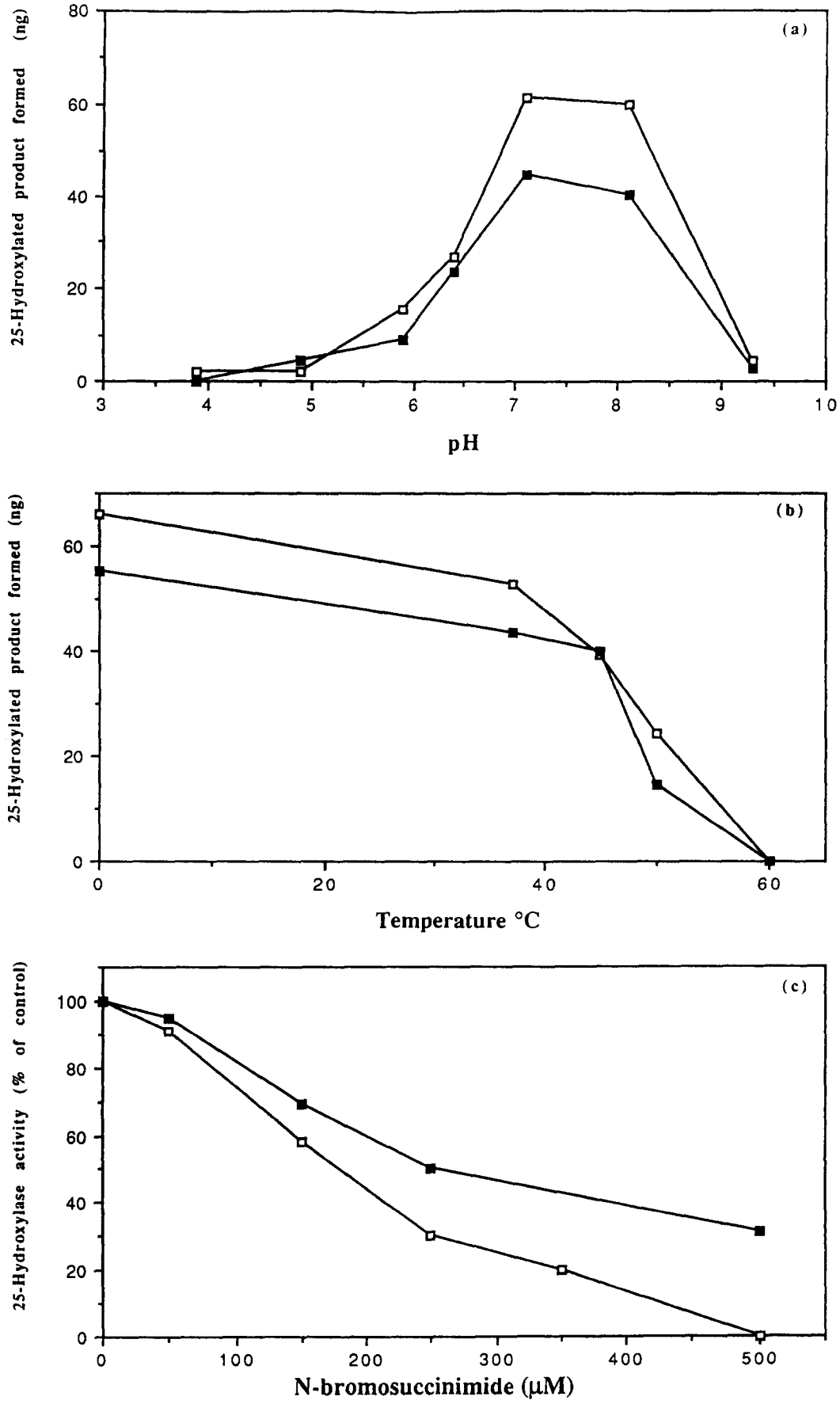

Fig. 3. Effects of pH, temperature and *N*-bromosuccinimide on the 25-hydroxylase activity towards vitamin D_2 and D_3. Incubations were performed as described in the Experimental section except that the substrate concentration was 25 μM and the incubation time was 30 min. Incubations with vitamin D_2 is shown as (■) and incubations with vitamin D_3 as (□). In (a) the incubations were performed at the indicated pH. In (b) the enzyme was preincubated for 3 min at the indicated temperature, chilled on ice and then the other components were added for incubation. In (c) the enzyme was preincubated with the indicated amount of *N*-bromosuccinimide for 1 h at 0°C. The *N*-bromosuccinimide was dissolved in 50 mM-potassium phosphate, pH 7.4, 20% glycerol and 0.1 mM EDTA.

Table 3. 25-Hydroxylase activities in microsomes and for the purified vitamin D_2 25-hydroxylase from pig liver

Reaction measured	Microsomal activity (pmol × min^{-1} × nmol of cytochrome P-450^{-1})	Cytochrome P-450 activity (pmol × min^{-1} × nmol of cytochrome P-450^{-1})
Vitamin D_2 25-hydroxylation	1.2 ± 0.2	98 ± 5
Vitamin D_3 25-hydroxylation	1.5 ± 0.4	188 ± 36
1α-Hydroxyvitamin D_2 25-hydroxylation	ND	756 ± 122
1α-Hydroxyvitamin D_3 25-hydroxylation	3.4 ± 0.5	841 ± 65
5β-Cholestane-3α,7α-diol 25-hydroxylation	≤20	1900 ± 487
Cholesterol 25-hydroxylation	<1	<1

Incubations were performed as described in the Experimental section. The results from five experiments are given as the means ± SD.
ND, not determined.

11.0 min in the straight-phase HPLC system. In the reverse-phase HPLC system the retention times were 6.4 and 7.8 min, respectively. The mobile phases in straight- and reverse-phase HPLC were the same as used for corresponding vitamin D_3 metabolites. The identity of the enzymatically formed 25-hydroxyvitamin D_2 was confirmed by combined gas chromatography-mass spectrometry [15, 24].

Incubations with antibody-coupled Sepharose

A monoclonal antibody, mAb 25H6, raised against the pig liver microsomal vitamin D_3 25-hydroxylase [18] and an irrelevant monoclonal antibody raised against a mitochondrial 27-hydroxylase were coupled to CNBr–Sepharose. Cytochrome P-450 (0.2 nmol) was incubated for 1 h with the indicated amounts of Sepharose-bound monoclonal antibody in 0.4 ml of 50 mM-Tris/acetate buffer, pH 7.4, containing 20% glycerol, 0.1 mM-EDTA and 0.7% (w/w) CHAPS. Sepharose without antibody was used as a control. The Sepharose was washed twice with the same buffer without CHAPS and pooled. The reaction mixture, 1 ml, 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 N-terminal sequence determination. N-terminal sequence analysis was performed with an Applied Biosystems 470A instrument. Phenylthiohydantoin derivatives were identified by reverse-phase HPLC [25].

RESULTS

Purification of microsomal vitamin D_2 25-hydroxylating cytochrome P-450

Cytochrome P-450, active in the 25-hydroxylation of vitamin D_2, was isolated from liver microsomes of castrated pigs, by solubilization with sodium cholate, precipitation with polyethylene glycol 6000 and chro-

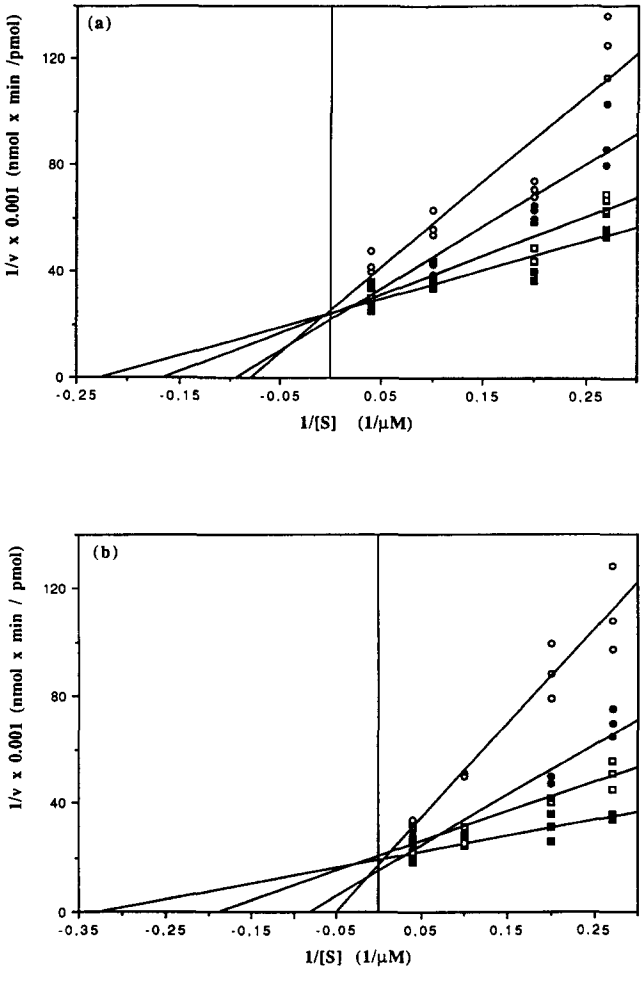

Fig. 4. Effects of various amounts of substrate and inhibitory effect of vitamin D_3 on the rate of 25-hydroxylation of vitamin D_2 and *vice versa* by the vitamin D_2 25-hydroxylase. Incubations were performed as described in the Experimental section, except that the concentrations of the substrates vitamin D_2 (a) and vitamin D_3 (b) were varied. In inhibition experiments, a constant concentration of vitamin D_3; 0 μM (■); 5 μM (□); 10 μM (●); 15 μM (○) [Fig. 5(a)] and vitamin D_2; 0 μM (■); 2.5 μM (□); 5 μM (●); 15 μM (○) [Fig. 5(b)], respectively, was added as inhibitor. The continuous line is the corresponding linear regression fitting while the data points are from the three experimental values with $r \geq 0.77$ (0.77; 0.88; 0.88; 0.83) for (a) and $r \geq 0.80$ (0.80; 0.86; 0.89; 0.94) for (b).

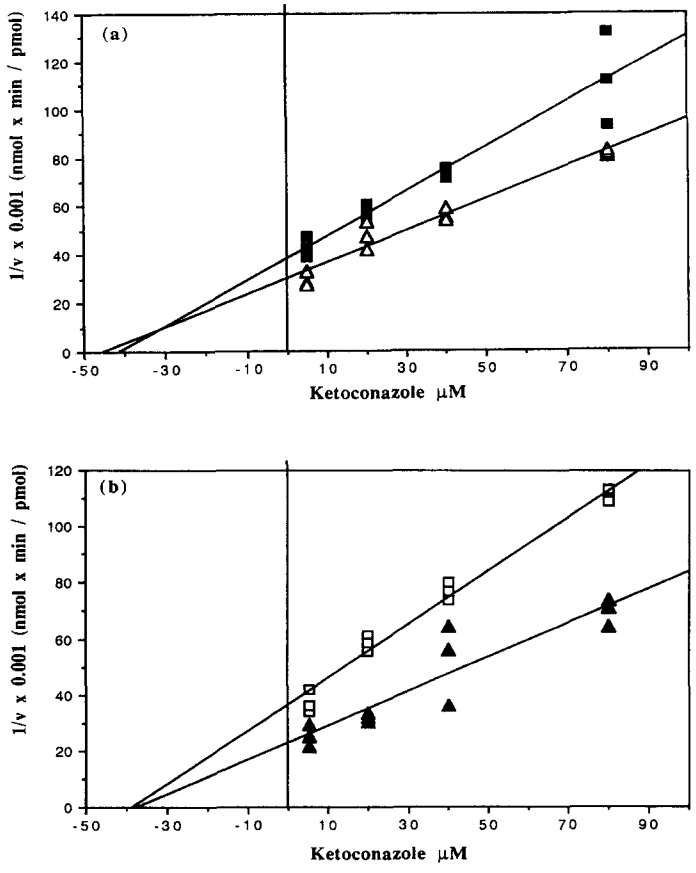

Fig. 5. Inhibitory effect of ketoconazole on 25-hydroxylation of vitamin D_2 and D_3. The incubations were performed as described in the Experimental section except that the incubation time was 20 min. Ketoconazole was dissolved in dimethylsulphoxide. In (a) the concentration of vitamin D_2 was 12.5 μM (■) and 25.0 (△) and in (b) the concentration of vitamin D_3 was 12.5 μM (□) and 25.0 (▲). The continuous line is the corresponding linear regression fitting while the data points are from three experimental values, with $r \geq 0.90$ (0.90; 0.95) for (a) and $r \geq 0.85$ (0.98; 0.85) for (b) The intersection of the two lines define the K_i [31].

matography on octylamine–Sepharose, hydroxylapatite, Q-Sepharose and S-Sepharose. The 25-hydroxylase activity against vitamin D_2 and D_3 was analysed in the different purification steps (Table 1). The highest activities towards both substrates were found in the same fractions during purification. The highest vitamin D_2 25-hydroxylase activity and the highest cytochrome *P*-450 content were in the 25 mM sodium acetate fraction from the S-Sepharose column. This fraction was used as the final vitamin D_2 25-hydroxylase preparation and it had a specific content of 13.0 nmol of cytochrome *P*-450 × mg of protein^{-1}. The specific 25-hydroxylase activity expressed as pmol of product formed × min^{-1} × mg of protein^{-1} was 1270 for 25-hydroxyvitamin D_2 and 2407 for 25-hydroxyvitamin D_3 (Table 1). The ratio between 25-hydroxylase activities towards vitamin D_2 and D_3 was about 1:2 in all purified fractions including the side fractions. The enzyme preparation showed a single protein band with an apparent M_r of 50,500 upon gel electrophoresis (Fig. 1). The enzymatically formed product was identified as 25-hydroxyvitamin D_2 by combined gas chromatography-mass spectrometry.

Structural characterization

The purified microsomal vitamin D_2 25-hydroxylating cytochrome *P*-450 (1 nmol) was analysed with respect to N-terminal amino acid sequence. The sequence of the first 25 amino acids (97% repetitive yield) was as follows: Gly-Leu-Leu-Thr-Gly-Asp-Leu-Leu-Gly-Ile-Leu-Ala-Leu-Ala-Met-Val-Ile-Phe-Leu-Leu-Leu-Val-Asp-Leu-Met. The first 16 amino acids are identical with those reported for the previously purified microsomal vitamin D_3 25-hydroxylase from pig liver in which case the sequence of only 16 N-terminal amino acids was determined [18]. The sequence differs from those of the male-specific rat liver microsomal and the mitochondrial vitamin D_3 25-hydroxylases reported previously [7, 13, 26, 27; cf Table 2].

Catalytic properties

Figure 2 shows that the conversion of vitamin D_2 into 25-hydroxyvitamin D_2 was about linear with the amount of cytochrome *P*-450 up to 0.3 nmol and with time up to 30 min. Longer incubation times resulted in

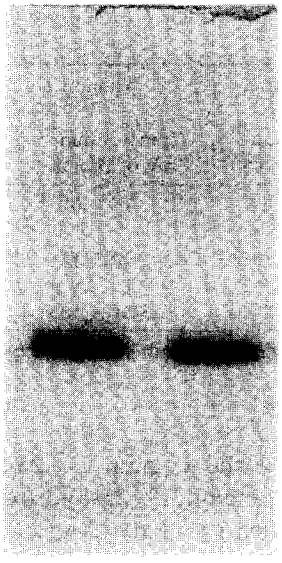

Fig. 6. SDS–PAGE and immunoblotting of purified microsomal 25-hydroxylating cytochromes *P*-450 from pig liver. The SDS–PAGE was performed as described in legend to Fig. 1. Electrophoretic transfer and immunoblotting with mAb 25H6 raised against vitamin D_3 25-hydroxylase [18] was performed as described previously [7]. Lane 1, vitamin D_2 25-hydroxylase (1 μg); lane 2, vitamin D_3 25-hydroxylase [*cf* 18] (1 μg).

decreased product formation. The system was saturated with 2 units of NADPH–cytochrome *P*-450 reductase and with 40 μM of vitamin D_2. The apparent K_m for the 25-hydroxylation of vitamin D_2 in the reconstituted system was 4.5 μM (Fig. 5). Table 3 summarizes the specificity of the purified vitamin D_2 25-hydroxylase with vitamin D_2, vitamin D_3, 1α-hydroxyvitamin D_2, 1α-hydroxyvitamin D_3, 5β-cholestane-3α,7α-diol and cholesterol as substrates. The enzyme catalysed 25-hydroxylation of all substrates except cholesterol. No 27-hydroxylase activity towards 5β-cholestane-3α,7α-diol could be detected.

Effects of pH and partial denaturation of the enzyme by temperature and N-bromosuccinimide on the 25-hydroxylation of vitamin D_2 and D_3

A series of experiments was performed to exclude the possibility that the preparation might be heterogeneous and contain two closely related 25-hydroxylases. Figure 3(a) shows how the 25-hydroxylase activities towards both substrates were affected by different pH. The 25-hydroxylase activities towards vitamin D_2 and D_3 had pH optima between 7.0 and 8.2. Figure 3(b) shows that the enzyme activities decreased in parallel when the enzyme preparation had been treated at 37, 45, 50 and 60°C for 3 min prior to incubation.

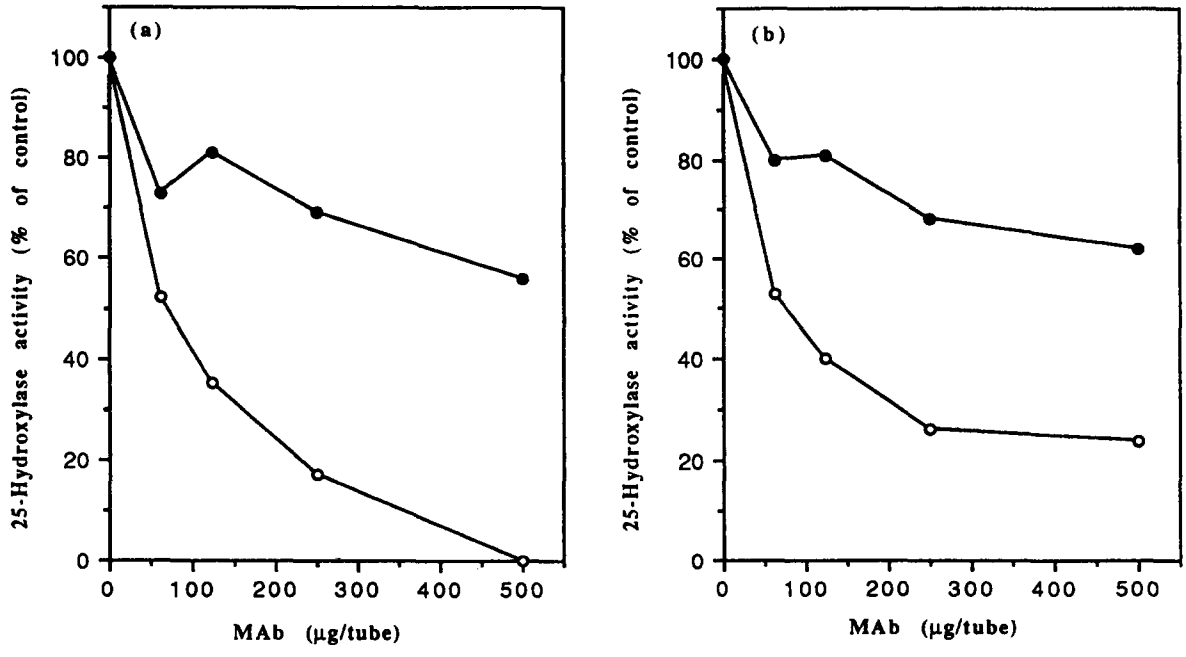

Fig. 7. Effect of mAb 25H6 (against the pig liver microsomal vitamin D_3 25-hydroxylase) on the 25-hydroxylation of vitamin D_2 (a) and D_3 (b). Cytochrome *P*-450 (0.2 nmol) was incubated with the indicated amounts of Sepharose-bound mAb 25H6 (○) or mAb 26·C·5 (●) (against a mitochondrial 26-hydroxylase 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 for vitamin D_2 was 66 pmol × min^{-1} × nmol of cytochrome *P*-450^{-1} and for vitamin D_3 112 pmol × min^{-1} × nmol of cytochrome *P*-450^{-1}. The results from three experiments are given as the means.

Table 4. Hydroxylase activities at different steps in the purification of a microsomal cytochrome P-450 vitamin D_2 25-hydroxylase from liver of female pigs

	Cytochrome *P*-450 (nmol × mg of protein^{-1})	Vitamin D_2 25-hydroxylation (pmol × min^{-1} × mg of protein^{-1})	Vitamin D_2 25-hydroxylation (pmol × min^{-1} × nmol of *P*-450^{-1})	Vitamin D_3 25-hydroxylation (pmol × min^{-1} × mg of protein^{-1})	Vitamin D_3 25-hydroxylation (pmol × min^{-1} × nmol of *P*-450^{-1})
Microsomes	0.5	0.8 ± 0.4	1.5 ± 0.8	0.9 ± 0.1	1.8 ± 0.3
Hydroxylapatite (120 mM-phosphate eluate)	2.5	130 ± 38	52 ± 15	188 ± 12	75 ± 6
S-Sepharose (25 mM-sodium acetate eluate)	15.4	2787 ± 62	181 ± 4	3450 ± 585	224 ± 38

Details of the purification and incubation procedures are given in the Experimental section. The result from five experiments are given as the means ± SD.

Figure 3(c) shows that the 25-hydroxylase activities towards vitamin D_2 and D_3 decreased upon treatment for 1 h with different concentrations of *N*-bromosuccinimide.

Competition between vitamin D_2 and D_3 as substrates for vitamin D_2 25-hydroxylase

25-Hydroxylation of vitamin D_2 by the purified cytochrome *P*-450 was inhibited by addition of increasing amounts of vitamin D_3. Conversely, the 25-hydroxylation of vitamin D_3 was inhibited by addition of vitamin D_2. Figure 4 shows double-reciprocal plots from experiments in which varying amounts of vitamin D_2 were incubated in the presence of different concentrations of vitamin D_3 (a) and of varying amounts of vitamin D_3 in the presence of different concentrations of vitamin D_2 (b). The results from these experiments show that vitamin D_3 competitively inhibits the 25-hydroxylation of vitamin D_2 and vitamin D_2 competitively inhibits the 25-hydroxylation of vitamin D_3. These results support the contention that 25-hydroxylation of vitamin D_2 and D_3 is catalysed by a common active site.

Inhibition of the 25-hydroxylation of vitamin D_2 and D_3 by ketoconazole

Ketoconazole, a non-steroidal inhibitor of several cytochromes *P*-450, was found to be a common inhibitor for 25-hydroxylation of both vitamin D_2 and D_3. Experiments were carried out with two concentrations of the respective substrates in the presence of various concentrations of ketoconazole. To obtain apparent K_i values the data were analysed using Dixon plots [28]. Figure 5 shows that ketoconazole inhibited 25-hydroxylase activities towards vitamin D_2 and D_3 with about the same apparent K_i value, 32 and 40 μM, respectively. The results confirm that a common enzyme catalyses 25-hydroxylation of the two substrates.

Comparison of electrophoretic and immunological properties with a previously reported vitamin D_3 25-hydroxylase

Figure 1 shows that the vitamin D_2 25-hydroxylase had the same apparent M_r (= 50,500) as vitamin D_3 25-hydroxylase. The immunoblotting experiments in Fig. 6 show that the monoclonal antibody (25H6) directed against the vitamin D_3 25-hydroxylase [18] recognized the vitamin D_2 25-hydroxylase. To further establish the identity of the two 25-hydroxylases the monoclonal antibody was coupled to Sepharose and incubated with the purified vitamin D_2 25-hydroxylase. After incubation, the antibody–Sepharose was removed and the supernatant was assayed for 25-hydroxylase activity towards vitamin D_2 and D_3, respectively, in a reconstituted system. As a control, Sepharose was coupled to a monoclonal antibody, directed against a mitochondrial 27-hydroxylase from pig liver. As shown in Fig. 7, increasing amounts of mAb 25H6 but not the irrelevant antibody was able to bind and decrease the 25-hydroxylase activity towards both vitamin D_2 and D_3.

25-Hydroxylase activity in liver microsomes and purified vitamin D_2 25-hydroxylase from female pig

Table 4 shows the 25-hydroxylase activity towards vitamin D_2 and D_3 in the microsomes, a partially purified cytochrome *P*-450 and an apparently homogeneous preparation of vitamin D_2 25-hydroxylase from livers of female pigs. The 25-hydroxylase activities and specific cytochrome *P*-450 content were similar as those in the same fractions from castrated male pigs. Figure 1 shows that the female enzyme has the same apparent M_r as the male enzyme. It has earlier been shown that a monoclonal antibody raised against the vitamin D_3 25-hydroxylase [*cf* 18] recognized protein both in male and female pig that are present in similar amounts with the same apparent M_r.

DISCUSSION

Previous work on 25-hydroxylation in this and other laboratories has not resulted in the purification or identification of a cytochrome *P*-450 responsible for 25-hydroxylation of vitamin D_2 [3, 9, 16]. The apparently homogeneous microsomal vitamin D_2 25-hydroxylase described in the present communication also catalysed 25-hydroxylation of vitamin D_3. Taken together, the results strongly indicate that a common enzyme catalyses 25-hydroxylation of the two vitamin D compounds in pig liver microsomes and that this

cytochrome *P*-450 is, in fact, identical with the recently purified vitamin D_3 25-hydroxylase from pig liver microsomes [18]. In view of the reports that two previously well-characterized hepatic vitamin D_3 25-hydroxylases, i.e. CYP 27 and CYP 2C11, do not show vitamin D_2 25-hydroxylase activity [3, 9] it is of interest to compare their properties with those of the microsomal 25-hydroxylase from pig liver. The turnover for 25-hydroxylation of vitamin D_3 is of the same order of magnitude for the three enzymes. The mitochondrial vitamin D 25-hydroxylase was originally purified from rabbit liver in this laboratory [5, 29] and rat liver in Okuda's laboratory [6, 30]. This enzyme, which is present also in human liver, catalyses 27-hydroxylation of C_{27}-steroids in bile acid biosynthesis with a turnover that is more than 20 times higher than that for vitamin D_3 [5, 9–12]. Okuda and coworkers [10–12] have presented overwhelming evidence that the mitochondrial vitamin D_3 25-hydroxylase is identical with the sterol 27-hydroxylase CYP 27, an obligatory cytochrome *P*-450 enzyme in bile acid biosynthesis [13, 31]. CYP 2C11 in rat liver microsomes is male specific and shows testosterone 16α-hydroxylase activity [4, 7, 8]. This cytochrome *P*-450 has not so far been detected in other species. CYP 27 and CYP 2C11 belong to separate cytochrome *P*-450 gene families [8]. The 25-hydroxylase from pig liver microsomes in the present communication showed properties which were fundamentally different from those of both CYP 27 and CYP 2C11. Thus, the N-terminal amino acid sequence was markedly different from that of the other two enzymes. Unlike the mitochondrial CYP 27 it is not active in 27-hydroxylation of bile acid intermediates. The pig liver enzyme showed no testosterone 16α-hydroxylase activity, was not sex specific and could be isolated in the same yield and with the same activity from both male and female pigs. It thus appears that the pig enzyme is sufficiently different from CYP 27 and CYP 2C11 to represent a novel 25-hydroxylase which is active towards both vitamin D_2 and D_3.

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