Purification of a cytochrome *P*-450 from pig kidney microsomes catalysing the 25-hydroxylation of vitamin D₃

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Cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D₃ was purified from pig kidney microsomes. The enzyme fraction contained 7 nmol of cytochrome *P*-450/mg of protein and showed only one protein band with an apparent *M_r* of 50 500 upon SDS/polyacrylamide-gel electrophoresis. The purified cytochrome *P*-450 catalysed 25-hydroxylation of vitamin D₃ up to 1000 times more efficiently, and 25-hydroxylation of 1 α -hydroxyvitamin D₃ up to 4000 times more efficiently, than the microsomes. The cytochrome *P*-450 required microsomal NADPH–cytochrome *P*-450 reductase for catalytic activity. Mitochondrial ferredoxin and ferredoxin reductase could not replace microsomal NADPH–cytochrome *P*-450 reductase. The enzyme preparation showed no detectable 25-hydroxylase activity towards vitamin D₂ or 1 α -hydroxylase activity towards 25-hydroxyvitamin D₃. CO inhibited the 25-hydroxylation by more than 85%. Mannitol, hydroquinone, catalase and superoxide dismutase did not affect the 25-hydroxylation. The possible role of the kidney microsomal cytochrome *P*-450 in the metabolism of vitamin D₃ is discussed.

INTRODUCTION

The 25-hydroxylation of vitamin D₃ is the first step in the bioactivation of vitamin D₃ to 1 α ,25-dihydroxyvitamin D₃, a compound possessing hormone-like properties [1]. The reaction is catalysed by cytochrome *P*-450-dependent enzyme systems in both the microsomal [2,3] and the mitochondrial [4,5] fractions of the liver.

There are reports indicating that the liver is not the only site of formation of 25-hydroxyvitamin D₃. Thus, Tucker *et al.* [6] have reported that homogenates of chick kidney and intestine, in addition to liver, show 25-hydroxylase activity. Olson *et al.* [7] showed that hepatectomized rats convert vitamin D₃ to 25-hydroxyvitamin D₃ but concluded that liver was the main source of 25-hydroxyvitamin D₃ under physiological conditions. The enzyme systems in chick kidney and intestine, catalysing 25-hydroxylation of vitamin D₃, have not been characterized. The isolation of extrahepatic 25-hydroxylase systems from mammals has not been reported.

The present communication reports the purification of an electrophoretically homogeneous cytochrome *P*-450 fraction from pig kidney microsomes, which catalyses 25-hydroxylation of vitamin D₃.

EXPERIMENTAL

Materials

25-Hydroxy[23,24(n)-³H]vitamin D₃ (84 Ci/mmol) and 1 α ,25-dihydroxy[23,24(n)-³H]vitamin D₃ (101 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, U.K.). Unlabelled 25-hydroxyvitamin D₃ was obtained from Duphar (Weesp, The Netherlands). 1 α -Hydroxyvitamin D₃ was a generous gift from Löwens (Malmö, Sweden). Vitamin D₂, vitamin D₃, sodium cholate (3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid), Lubrol PX, catalase, superoxide dismutase and

hydroquinone were obtained from Sigma, mannitol from Difco Laboratories and cofactors and Fractogel TSK DEAE-650 (s) from Merck. Octylamine-Sepharose 4B was prepared as described previously [8]. CM-Sepharose CL-6B was from Pharmacia and hydroxyapatite (Bio-Gel HTP) from Bio-Rad. Bio-Gel HTP was mixed with an equal amount of Whatman CF-1 cellulose powder before chromatography. Molecular mass was determined with SDS low molecular mass standards for polyacrylamide-gel electrophoresis (Bio-Rad). The remaining chemicals were of reagent grade. Pig kidneys from the local slaughter house were used.

Methods

Kidney cortex microsomes were prepared from 1 kg of pig kidney as described earlier for liver microsomes by Andersson *et al.* [9]. Cytochrome *P*-450 was purified essentially as described by Wikvall [10] for purification of liver mitochondrial cytochrome *P*-450, with the following modifications. Lubrol PX was used instead of Emulgen 913. Cytochrome *P*-450 was eluted from the hydroxyapatite column with 300 mM-potassium phosphate buffer instead of 150 mM-potassium phosphate, and Fractogel TSK DEAE-650 (s) was used instead of DE-52 cellulose. Cytochrome *P*-450, active in 25-hydroxylation of vitamin D₃, was eluted from the Fractogel TSK DEAE 650 (s) column with the equilibrating buffer and was applied to a CM-Sepharose CL-6B column (1 cm $\times$ 25 cm) in 10 mM-potassium phosphate buffer containing 20% glycerol/0.1% (w/v) sodium cholate/0.05% (w/v) Lubrol PX. The 25-hydroxylating cytochrome *P*-450 was eluted by a 0–0.3 M-NaCl gradient in 400 ml of the equilibrating buffer. Detergents were removed and the final cytochrome *P*-450 fraction was dialysed as described by Wikvall [10].

NADPH–cytochrome *P*-450 reductase was prepared from rat liver microsomes as described by Yasukochi & Masters [11] and ferredoxin and ferredoxin reductase

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were prepared from bovine adrenal mitochondria as described by Wikvall [10]. Cytochrome *P*-450 was determined as described by Omura & Sato [12] and protein was determined as described by Lowry *et al.* [13]. Polyacrylamide-gel electrophoresis was performed with 15% polyacrylamide (15% polyacrylamide/0.09% bis-acrylamide) slab gels containing 0.1% (w/v) SDS according to Laemmli [14]. Electrophoresis was carried out at 30 mA/slab gel and the gels were silver-stained as described by Wray *et al.* [15].

Incubation procedures and analysis of incubation mixtures

Incubations were carried out for 7 min at 37 °C. Vitamin D_2 , vitamin D_3 and 1α -hydroxyvitamin D_3 (40 μ g in 40 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. 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. Incubations were terminated by addition of 5 ml of trichloroethane/methanol (2:1, v/v).

Quantification of the incubation products was performed by h.p.l.c. using an LKB 2150 h.p.l.c. pump, LKB 2151 variable Wavelength Monitor, LKB 2210 1-channel recorder and a LiChrosorb Si column (150 mm $\times$ 3 mm i.d., 5 μ m, Merck) for the straight phase analysis. A Merck Hitachi L-6000 liquid chromatograph, Merck Hitachi 655 A Wavelength U.V. Monitor, Merck Hitachi D-2000 Chromato-Integrator and a LiChrosorb RP-18 column (150 mm $\times$ 3 mm i.d., 5 μ m, Merck) were used for reversed-phase analysis. 3 H-labelled 25-hydroxy- or $1\alpha,25$ -dihydroxyvitamin D_3 (10000 d.p.m.) was added as internal standard to the incubations immediately after termination to correct for recovery during the extraction and chromatographic procedures. The incubations were extracted twice with

trichloroethane/methanol (2:1, v/v) and the organic phases were dried down with N_2 , dissolved in 100 μ l of mobile phase and submitted to straight phase h.p.l.c. analysis. The mobile phase was hexane/isopropanol, (24:1, v/v) for 25-hydroxyvitamin D_3 and hexane/isopropanol (9:1, v/v) for $1\alpha,25$ -dihydroxyvitamin D_3 analysis. The solvent flow was 0.7 ml/min. The retention time for 25-hydroxyvitamin D_3 was 8 min and, for $1\alpha,25$ -dihydroxyvitamin D_3 , 12 min. The fractions corresponding to authentic 25-hydroxyvitamin D_3 and $1\alpha,25$ -dihydroxyvitamin D_3 were collected and taken to dryness with N_2 . The residue was dissolved in 100 μ l of the mobile phase, methanol/water (23:2, v/v) for 25-hydroxyvitamin D_3 , and methanol/water (17:3, v/v) for $1\alpha,25$ -dihydroxyvitamin D_3 , and submitted to reversed-phase h.p.l.c. analysis. The solvent flow was 0.5 ml/min and the retention time for 25-hydroxyvitamin D_3 was 6 min and, for $1\alpha,25$ -dihydroxyvitamin D_3 , 7 min. The amount of the two vitamin D_3 metabolites was quantified by comparing the height of the peak found at 265 nm with those of known standards. Correction was made for recovery of the added 3 H-labelled metabolites. Total recovery was about 60%, which is considered to be good [cf. ref. [16]].

Further identification of the products was carried out by combined g.c.-m.s. of the trimethylsilyl derivative of 25-hydroxyvitamin D_3 and of $1\alpha,25$ -dihydroxyvitamin D_3 [17,18].

RESULTS

Cytochrome *P*-450, active in 25-hydroxylation of vitamin D_3 , was isolated from pig kidney microsomes by solubilization with sodium cholate and chromatography on octylamine-Sepharose, hydroxyapatite, Fractogel TSK DEAE 650 (s) and CM-Sepharose CL 6B. The final enzyme fraction had a specific content of 7 nmol of cytochrome *P*-450/mg of protein. The cytochrome *P*-450 content in the microsomes could not be accurately

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

Incubations were performed at 37 °C for 7 min except for incubations with intact microsomes which were carried out for 20 min. The incubation mixtures contained 0.1 nmol of cytochrome *P*-450, 2 units of microsomal NADPH-cytochrome *P*-450 reductase, 1 μ mol of NADPH and 40 μ g of vitamin D_3 , vitamin D_2 or 1α -hydroxyvitamin D_3 , in a total volume of 1 ml of Tris/acetate buffer, pH 7.4. Incubations with intact microsomes were performed as described in the Experimental section. The results from five experiments are given as the means $\pm$ S.D. N.D., not determined.

Reaction measured	Hydroxylase activity		
	Microsomes (pmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	Cytochrome <i>P</i> -450	
		(pmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)	(pmol $\cdot$ min ⁻¹ $\cdot$ nmol of cytochrome <i>P</i> -450 ⁻¹)
Vitamin D_3 25-hydroxylation	0.5 $\pm$ 0.1	454 $\pm$ 83	68 $\pm$ 12
1α -Hydroxyvitamin D_3 25-hydroxylation	1.0 $\pm$ 0.2	4229 $\pm$ 591	631 $\pm$ 88
Vitamin D_2 25-hydroxylation	< 0.2	< 10	< 10
25-Hydroxyvitamin D_3 1α -hydroxylation	N.D.	< 10	< 10

Fig. 1. Polyacrylamide-gel electrophoresis of purified microsomal cytochrome *P*-450 from pig kidney

The protein samples were pretreated with SDS and mercaptoethanol at 100 °C for 2 min and subjected to polyacrylamide-gel electrophoresis in the presence of SDS. Migration was from top to bottom. Gel electrophoresis was performed according to Laemmli [14] with modifications as described in the Experimental section. The stacking gels contained 3% (w/v) acrylamide, and the separating gels, 15% (w/v) acrylamide and 0.09% (w/v) bis-acrylamide, had a length of 10 cm, and were 0.1 cm thick. A mixture of cytochrome *P*-450 LM₄ and cytochrome *P*-450 LM₂ from rabbit liver microsomes, 0.5 µg of each in lane 1, microsomal cytochrome *P*-450, 1 µg in lane 2 and kidney microsomes, 40 µg in lane 3.

measured due to presence of other haem proteins and cytochrome oxidase. A content of 0.1–0.2 nmol/mg of protein has been reported [19]. The total protein in the final cytochrome *P*-450 preparation represented about 0.3% of the protein present in the microsomes.

Table 1 shows the rate of 25-hydroxylation of vitamin D₃, 1 α -hydroxyvitamin D₃ and vitamin D₂ in the microsomes as well as in the purified cytochrome *P*-450 fraction. The 25-hydroxylase activity, expressed as pmol of product formed·min⁻¹·mg of protein⁻¹, was about 1000 times higher in the purified cytochrome *P*-450 than in microsomes for vitamin D₃ and about 4000 times higher for 1 α -hydroxyvitamin D₃. No 25-hydroxylase activity

towards vitamin D₂ could be detected in microsomes or in the purified cytochrome *P*-450 fraction. The enzyme was also inactive in 1 α -hydroxylation of 25-hydroxyvitamin D₃.

The enzymic product formed after incubations with vitamin D₃ and the purified cytochrome *P*-450 was identified as 25-hydroxyvitamin D₃ by straight-phase followed by reversed-phase h.p.l.c. The identity of 25-hydroxyvitamin D₃ was further confirmed by combined g.c.-m.s. of h.p.l.c.-purified material obtained from separate incubations to which no ³H-labelled 25-hydroxyvitamin D₃ had been added. Prominent peaks were observed at *m/z* 544 [*M*⁺ (the molecular ion)], 529 (*M*⁺–15), 439 (*M*⁺–15, –90), 413 (*M*⁺–131) and 131 (cleavage between C-24 and C-25). The same peaks with the same relative intensity were observed in the mass spectrum of the derivative of authentic 25-hydroxyvitamin D₃. The enzymic product formed after incubations with 1 α -hydroxyvitamin D₃ was identified as 1 α ,25-dihydroxyvitamin D₃ in the same way.

Fig. 1 shows the result of SDS/polyacrylamide-gel electrophoresis of the 25-hydroxylating cytochrome *P*-450. The preparation showed only one protein band corresponding to an apparent *M_r* of 50500. The reconstitution conditions for the 25-hydroxylation of vitamin D₃ were analysed in a series of experiments. The 25-hydroxylase activity showed an absolute requirement for the cytochrome *P*-450 and microsomal NADPH-cytochrome *P*-450 reductase, as well as for NADPH. The rate of 25-hydroxylation was linear with time for up to 7 min. In the presence of 0.1 nmol of the cytochrome *P*-450, the system was saturated with 2 units of NADPH-cytochrome *P*-450 reductase and with 40 µg of vitamin D₃. Microsomal NADPH-cytochrome *P*-450 reductase could not be replaced by mitochondrial ferredoxin and ferredoxin reductase. These results exclude the possibility that the cytochrome *P*-450 is of mitochondrial origin. Addition of the phospholipid dilauroylglycero-3-phosphocholine was not required for 25-hydroxylase activity.

It has been reported that hydroxyl radicals, superoxide radicals or hydrogen peroxide are involved in some reactions catalysed by microsomal cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase [20–23]. In order to examine the possibility that such reactive oxygen species participate in the 25-hydroxylation catalysed by the present cytochrome *P*-450, a series of experiments were performed. The hydroxyl radical scavengers, mannitol and hydroquinone, had no effect on the 25-hydroxylation. Catalase, which decomposes hydrogen peroxide, and superoxide dismutase were also without inhibitory effect. These results exclude the involvement of hydroxyl radicals, superoxide radicals and hydrogen peroxide in the 25-hydroxylation of vitamin D₃. CO inhibited the reaction by more than 85%.

DISCUSSION

Cytochrome *P*-450-dependent 25-hydroxylation of vitamin D₃ in liver is well established [2–5]. The present study demonstrates that pig kidney microsomes contain a cytochrome *P*-450 with the ability to catalyse 25-hydroxylation of vitamin D₃. The enzyme did not require phospholipid for activity. A similar lack of lipid dependency has been previously reported for prepara-

tions of cytochrome *P*-450 from rat liver microsomes, catalysing 25-hydroxylation of vitamin D_3 [24,25]. This is in contrast with many other reconstituted systems involving microsomal cytochrome *P*-450, which are more or less lipid dependent [26]. The rate of 25-hydroxylation with the purified enzyme preparation was comparable with that reported for partially purified cytochrome *P*-450 from rat liver mitochondria [27], and was about one-fifth of that reported for a cytochrome *P*-450 purified from liver microsomes of male rats [24]. In similarity with cytochrome *P*-450 preparations isolated from liver microsomes, the cytochrome *P*-450 preparation from kidney microsomes catalysed 25-hydroxylation of 1α -hydroxyvitamin D_3 more effectively than that of vitamin D_3 [24,25], and showed no detectable 25-hydroxylase activity towards vitamin D_2 [24]. In this connection it might be mentioned that the 25-hydroxylation of vitamin D_2 has been reported to occur in rat and human liver mitochondria [28,29]. The rate of 25-hydroxylation of vitamin D_2 was considerably lower than that of vitamin D_3 . Liver microsomes or partially purified liver microsomal cytochrome *P*-450 did not show detectable 25-hydroxylase activity towards vitamin D_2 [28,29].

The physiological importance of kidney microsomal cytochrome *P*-450 relative to the cytochromes *P*-450 in liver in the metabolism of vitamin D_3 is difficult to assess. The liver is regarded to be the most significant, if not the sole, source of production of 25-hydroxyvitamin D_3 in mammals under physiological conditions [30]. The present as well as some previous findings indicate a role also of extrahepatic tissues in 25-hydroxyvitamin D_3 production. It has been reported that patients with primary biliary cirrhosis, irrespective of its severity, have the same capacity as control subjects of similar vitamin D nutritional status to convert orally or intravenously administered vitamin D_3 to 25-hydroxyvitamin D_3 [31]. Measurements of the concentration of vitamin D_3 in various tissues have shown that vitamin D_3 is present in kidney in almost the same concentration as that found in liver [32]. It is not unlikely that vitamin D_3 present in kidney serves as substrate for the 25-hydroxylase in kidney microsomes. Since kidney mitochondria contain the 1α -hydroxylase that converts 25-hydroxyvitamin D_3 into $1\alpha,25$ -dihydroxyvitamin D_3 , the kidney would then be capable of complete conversion of vitamin D_3 to its hormonal form.

The skilful technical assistance of Mrs Britt-Marie Johansson, Mrs Angela Lannerbro and Mrs Kerstin Rönnqvist is gratefully acknowledged. We are grateful to Professor Ingemar Björkhem for placing mass spectrometric equipment at our disposal and to Mr Olle Falk for performing g.c.-m.s. analyses of 25-hydroxyvitamin D_3 and $1\alpha,25$ -dihydroxyvitamin D_3 . We are also grateful to Dr Inger Holmberg for generous gifts of authentic $1\alpha,25$ -dihydroxyvitamin D_3 and 25-hydroxyvitamin D_2 . This work was supported by the Swedish Medical Research Council (project 03X-218).

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