Purification from pig kidney of a microsomal cytochrome P_{450} catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3

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Abstract **A cytochrome P_{450} catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3 was purified from pig kidney microsomes. The enzyme preparation showed one protein band on gel electrophoresis with apparent M_r of 52,500 and a specific cytochrome P_{450} content of 10.7 nmol/mg of protein. The 25-hydroxyvitamin D_3 1α-hydroxylase copurified with the vitamin D_3 25-hydroxylase during purification. A cytochrome P_{450} catalyzing 1α-hydroxylation was purified also from liver microsomes. The apparently homogeneous enzyme showed the same catalytic properties and apparent M_r as the kidney enzyme. The results of the present communication demonstrate the presence in kidney of a previously unknown microsomal 1α-hydroxylase in addition to the assumed specific mitochondrial 1α-hydroxylase. The possibility that microsomal 1α-hydroxylation in pig kidney and liver is catalyzed by the previously described porcine microsomal vitamin D 25-hydroxylase(s) is discussed.**

Key words: Cytochrome P_{450}; 25-Hydroxyvitamin D_3 1α-hydroxylase; Renal 1α-hydroxylation; Hepatic 1α-hydroxylation; Microsomal 1α-hydroxylation

1. Introduction

The activation of vitamin D_3 to its hormonal form, 1α,25-dihydroxyvitamin D_3, involves an initial 25-hydroxylation in the liver. The subsequent 1α-hydroxylation of 25-hydroxyvitamin D_3 is thought to be catalyzed mainly by a mitochondrial cytochrome P_{450} in kidney [1–5]. A lot of effort trying to purify and characterize the mitochondrial 1α-hydroxylase in kidney has been made — so far without success [4,6–9]. There are no reports concerning a possible 1α-hydroxylation of 25-hydroxyvitamin D_3 in the microsomal fraction of the kidney. Extrarenal microsomal 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 has been reported [5,10,11]. Hollis [11] found 1α-hydroxylase activity both in the microsomal and the mitochondrial fractions of pig liver homogenate. None of these 1α-hydroxylases was further purified or characterized [11]. We have recently shown that CYP27, a mitochondrial sterol 27-hydroxylase, is responsible for the main 1α-hydroxylation of 25-hydroxyvitamin D_3 in the mitochondrial fraction of the liver [12]. CYP27 is present also in kidney [13]. Another example of vitamin D hydroxylating cytochrome P_{450} that is present in both liver and kidney is the porcine microsomal vitamin D 25-hydroxylase(s) [14,15]. The primary aim of this report was to study the presence of possible 25-hydroxyvitamin D_3 1α-hydroxylase in the microsomal fraction of kidney. A kidney microsomal cytochrome P_{450} catalyzing 1α-hydroxylation was purified to apparent homogeneity. For reasons of comparison also the liver microsomal cytochrome P_{450} catalyzing 1α-hydroxylation was purified and studied.

2. Materials and methods

2.1. Chemicals and other materials

25-Hydroxy[23,24(n)-^{3}H]vitamin D_3 (105.5 Ci/mmol) and 1α,25-dihydroxy-[23,24(n)-^{3}H]vitamin D_3 (91.4 Ci/mmol) were obtained from Amersham Int. (Amersham, Bucks., UK). Unlabeled vitamin D_3 and 25-hydroxyvitamin D_3 were obtained from Sigma Chemical Co. and Solvay Duphar BV, respectively. Ketoconazole and 1,2-dianilinoethane were obtained from Division of Janssen Pharmaceutica NV and Sigma Chemical Co., respectively. Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad. Hydroxylapatite 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). Q-Sepharose fast flow and S-Sepharose fast flow were obtained from Pharmacia. Emulgen 913 was obtained from Kao Chemicals, Tokyo, Japan. The remaining chemicals were reagent grade. Livers and kidneys from castrated, otherwise untreated, 6-month-old male pigs were from the local slaughter house.

2.2. Purification of enzymes

Microsomes and cytochrome P_{450} from pig kidney and liver were prepared, cholate solubilized, fractionated with poly(ethylene)glycol 6000, applied to octylamine-Sepharose, hydroxylapatite and anion exchange chromatography as described [15] except that the hydroxylapatite was eluted with 300 mM phosphate in the kidney preparations. Enzyme activity was followed in all purification steps, including the side fractions. The fractions with the major part of the 1α-hydroxylase activity and the highest specific activity were pooled and further purified. 1α-Hydroxylase activity was found also in the side fractions. The ratio between the three activities followed, i.e. the 1α- and 26(27)-hydroxylase activities towards 25-hydroxyvitamin D_3 and the 25-hydroxylase avtivity towards vitamin D_3, was around the same in all fractions, including the side fractions not chosen for further purification.

NADPH-cytochrome P_{450} reductase was prepared from pig liver microsomes as described by Yasukochi and Masters [16].

Ferredoxin and ferredoxin reductase from bovine adrenal mitochondria were prepared as described previously [17].

2.3. Incubation procedure and analysis of enzymatically formed products

Incubations were performed and analyzed as described previously [15]. The incubation time was 60 min, the concentration of substrate was 62.5 μM and of cytochrome P_{450} 0.25 μM. The enzymatically produced 1α,25-dihydroxyvitamin D_3 was identified by combined gas chromatography-mass spectrometry.

2.4. Other methods

Electrophoresis, silver staining, protein and cytochrome P_{450} determinations were performed as described previously [15,18,19].

3. Results

3.1. Purification of kidney microsomal cytochrome P_{450} catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3

Initial experiments were performed with partially purified

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Table 1
Purification from pig kidneys of microsomal cytochrome P_{450} catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3

	Cytochrome P_{450} (nmol·mg of protein^{-1})	25-Hydroxyvitamin D_3		Vitamin D_3 25-Hydroxylation (pmol·min^{-1}·mg of protein^{-1})
		1α-Hydroxylation (pmol·min^{-1}·mg of protein^{-1})	26(27)-Hydroxylation (pmol·min^{-1}·mg of protein^{-1})	
Microsomes	N.D.*	≤0.2	≤0.2	≤0.2
Octylamine-Sepharose	0.4	0.6	3.5	1.4
Hydroxylapatite (300 mM phosphate eluate)	2.2	4.1	43.4	33.8
Q-Sepharose (nonbound fraction)	3.4	14.6	75.1	168.0
S-Sepharose (0 mM sodium acetate eluate)	10.7	53.5	283.6	661.3

Details of the purification and incubation procedures are given in section 2.
*N.D., not determined.

cytochrome P_{450} prepared by chromatography of solubilized microsomal protein on octylamine-Sepharose and hydroxylapatite. Incubation of partially purified cytochrome P_{450} from pig kidney microsomes resulted in formation of 1α,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3. In addition, incubations of 25-hydroxyvitamin D_3 resulted in the formation of another product with retention times on both the straight and reversed HPLC steps identical with 25,26-dihydroxyvitamin D_3. The stereochemistry at C-25 could not be determined with the methods used. The partially purified cytochrome P_{450} also catalyzed 25-hydroxylation of vitamin D_3. On the basis of these results, purification was carried out and the activities in all purification steps, including the side fractions were analyzed. The 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 copurified with the 26(27)-hydroxylase activity towards 25-hydroxyvitamin D_3 as well as the 25-hydroxylase activity towards D_3 (Table 1). The purified enzyme fraction showed one protein band on SDS-PAGE, corresponding to an apparent M_r of 52,500 (Fig. 1A). It had a specific cytochrome P_{450} content of 10.7 nmol·mg of protein^{-1}. The 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 was 5.0 pmol·nmol cytochrome P_{450}^{-1}·min^{-1}, the 26(27)-hydroxylase activity towards 25-hydroxyvitamin D_3 26.5 pmol·nmol cytochrome P_{450}^{-1}·min^{-1} and the 25-hydroxlase activity towards D_3 61.8 pmol·nmol cytochrome P_{450}^{-1}·min^{-1}. To exclude the possibility that 1α,25-dihydroxyvitamin D_3 was formed by nonenzymatical, free radical reactions [5], a series of experiments was performed. The 1α-hydroxylase activity in the purified fraction was unaffected by 1,2-dianilinoethane (10 μM) and was inhibited to about 80% by ketoconazole (300 μM). The activity was dependent on microsomal NADPH-cytochrome P_{450} reductase. The microsomal NADPH-cytochrome P_{450} reductase could not be replaced by the mitochondrial electron-transport system involving ferredoxin and ferredoxin reductase. The identity of 1α,25-dihydroxyvitamin D_3 produced was confirmed by combined gas chromatography-mass spectrometry. The amount of purified 1α-hydroxylating cytochrome P_{450} recovered from 1 kg of kidney tissue was 8 nmol.

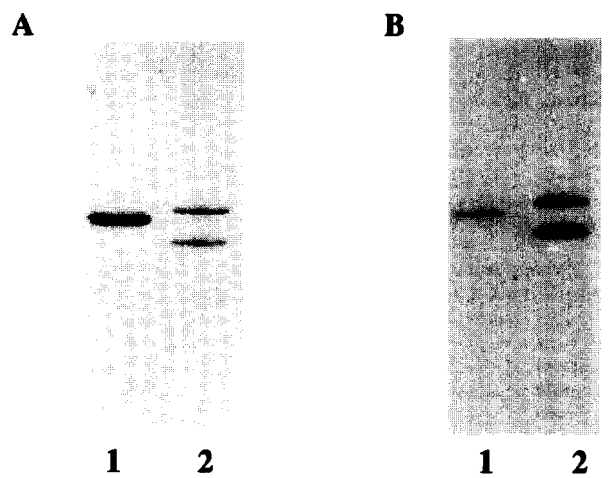

Fig. 1. SDS-PAGE of purified microsomal cytochromes P_{450} from pig kidney (A) and liver (B). (A) Lane 1 = purified 1α-hydroxylase from kidney (3 μg); lane 2 = a mixture of cytochrome P_{450} IA2 (55 kDa) and cytochrome P_{450} IIB4 (48 kDa) from rabbit liver microsomes used as M_r standards (1 μg of each). (B) Lane 1 = purified 1α-hydroxylase from liver (0.5 μg); lane 2 = standards as in (A). The electrophoresis and staining were performed as described in [15].

3.2. *Purification of liver microsomal cytochrome P_{450} catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3*

Purification of liver microsomal 1α-hydroxylase resulted in a similar purification profile and product formation as in kidney microsomes (Table 2). Thus, the 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 copurified with the 26(27)-hydroxylase activity towards 25-hydroxyvitamin D_3 as well as the 25-hydroxylase activity towards vitamin D_3. The activities were analyzed in all purification steps, including the side fractions. The final enzyme fraction showed one protein band on SDS-PAGE, corresponding to an apparent M_r of 52,500 (Fig. 1B). The specific cytochrome P_{450} content was 13.0 nmol·mg of protein^{-1}. The 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 was 7.9 pmol·nmol cytochrome P_{450}^{-1}·min^{-1}, the 26(27)-hydroxylase activity towards 25-hydroxyvitamin D_3 36.0 pmol·nmol cytochrome P_{450}^{-1}·min^{-1} and the 25-hydroxylase activity towards vitamin D_3 284.3 pmol·nmol cytochrome P_{450}^{-1}·min^{-1}. The amount of purified 1α-hydroxylating cytochrome P_{450} recovered from 1 kg of liver tissue was 218 nmol. In a separate SDS-PAGE experiment (not shown), the liver and kidney enzymes were found to have the same apparent M_r as the previously purified vitamin D 25-hydroxylase from liver microsomes [15].

4. Discussion

The results of the present communication show the presence in pig kidney of a microsomal cytochrome P_{450} active in the 1α-hydroxylation of 25-hydroxyvitamin D_3. In contrast to

Table 2
Purification from pig liver of microsomal cytochrome P_{450} catalyzing 1α-hydroxylation of 25-hydroxyvitamin D_3

	Cytochrome P_{450} (nmol·mg of protein^{-1})	25-Hydroxyvitamin D_3		Vitamin D_3 25-Hydroxylation (pmol·min^{-1}·mg of protein^{-1})
		1α-Hydroxylation (pmol·min^{-1}·mg of protein^{-1})	26(27)-Hydroxylation (pmol·min^{-1}·mg of protein^{-1})	
Microsomes	0.6	≤0.2	≤0.2	1.2
Octylamine-Sepharose	2.3	2.3	11.5	50.8
Hydroxylapatite (120 mM phosphate eluate)	3.6	9.0	38.9	328.0
Q-Sepharose (nonbound fraction)	5.4	23.8	84.2	596.2
S-Sepharose (150 mM sodium acetate eluate)	13.0	102.7	468.0	3695.9

Details of the purification and incubation procedures are given in section 2.

mitochondrial cytochrome P_{450} catalyzing 1α-hydroxylation, the apparently homogeneous microsomal 1α-hydroxylase required microsomal NADPH-cytochrome P_{450} reductase for activity and was inactive in presence of mitochondrial electron transport system involving ferredoxin and ferredoxin reductase. The physiological importance of the kidney microsomal 1α-hydroxylase in the bioactivation of vitamin D_3 to its biologically active hormone form, 1α,25-dihydroxyvitamin D_3, cannot be fully assessed at present. The kidney is the major site of 1α,25-dihydroxyvitamin D_3 production [5], although extrarenal 1α-hydroxylation exists [5,11]. It is generally considered that mitochondrial 1α-hydroxylation is responsible for the renal 1α,25-dihydroxyvitamin D_3 production. The kidney microsomal 1α-hydroxylase studied in the present communication shows 1α-hydroxylase activity higher or comparable to that of previously reported mitochondrial 1α-hydroxylase preparations [4,6–8]. The purified kidney microsomal 1α-hydroxylase should be sufficiently active catalytically to play a role in 1α,25-dihydroxyvitamin D_3 production in vivo. The daily production of 1α,25-dihydroxyvitamin D_3 in man has been estimated to be about 0.14–0.68 μg [20] and it might be assumed that the production in pig is of about the same order. On the basis of catalytic activity and yield of purified microsomal 1α-hydroxylase it can be calculated that this 1α-hydroxylase could catalyze the formation of at least 5 μg/day in kidney.

The liver microsomal 1α-hydroxylase preparation showed the same properties as the kidney enzyme and catalyzed also 25-hydroxylation of vitamin D_3. The apparently homogeneous protein was purified according to the same procedures as described for microsomal vitamin D 25-hydroxylase [15] and showed the same apparent M_r as this enzyme. These findings together with the finding that the ratio between 1α- and 25-hydroxylation was constant during purification of both the kidney and liver enzymes indicate that microsomal 1α-hydroxylation is catalyzed by the previously described microsomal vitamin D 25-hydroxylase(s) in pig kidney and liver [14,15]. The ability of purified and recombinantly expressed mitochondrial CYP27 to catalyze both 25-hydroxylation and 1α-hydroxylation in vitamin D bioactivation was recently reported [12]. It may be concluded that in addition to the assumed specific cytochrome P_{450} in kidney mitochondria catalyzing 1α-hydroxylation which has been partially purified [4,6–8], there are other 1α-hydroxylating cytochromes P_{450} that have now been purified to apparent homogeneity or recombinantly expressed. Their relative physiological importance in the bioactivation of vitamin D_3 remains to be established.

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