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A possible role for CYP27 as a major renal mitochondrial 25-hydroxyvitamin D_3 1α-hydroxylase

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Abstract A mitochondrial cytochrome P450 fraction catalyzing 1α- and 27-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3 was purified from pig kidney. The ratio between the 1α- and 27-hydroxylase activities was the same in all purification steps including a side fraction. Attempts to separate the 1α- and 27-hydroxylase activities were unsuccessful. A monoclonal antibody directed against purified pig liver CYP27 recognized a protein of the same apparent M_r and immunoprecipitated both the 1α- and 27-hydroxylase activities towards 25-hydroxyvitamin D_3 in the purified kidney enzyme fraction as well as in a solubilized, crude cytochrome P450 extract considered to represent the major part of the 25-hydroxyvitamin D_3 hydroxylases in kidney mitochondria. Taken together, the results from the purification and the experiments with CYP27 antibody, substrate inhibition, and recombinant expressed human liver CYP27 strongly indicate that CYP27 is able to catalyze 1α-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3 in kidney. In conclusion, the results provide evidence for a role for CYP27 as a major renal mitochondrial 25-hydroxyvitamin D_3 1α-hydroxylase.

Key words: Cytochrome P450; Renal mitochondrial 1α-Hydroxylation; 24-Hydroxylation

1. Introduction

From a regulatory point of view, the 1α-hydroxylation of 25-hydroxyvitamin D_3 is the most important reaction in the overall metabolism of vitamin D_3. The 1α-hydroxylation takes place primarily in the kidney and the enzyme is known to be a cytochrome P450 located in the inner mitochondrial membrane [1–3]. Several efforts have been made to try to characterize an assumed specific mitochondrial 1α-hydroxylase in kidney – so far without success [3–7]. There are two additional mitochondrial cytochromes P450, CYP24 and CYP27, that are active towards 25-hydroxyvitamin D_3 [8,9]. CYP24 catalyzes the 24-hydroxylation of 25-hydroxyvitamin D_3 in kidney and intestine but is inactive in the 1α-hydroxylation. The activated vitamin D_3 product, 1α,25-dihydroxyvitamin D_3, is known to regulate the 24- and 1α-hydroxylase activities reciprocally. Thus, CYP24 is induced whereas the mitochondrial 1α-hydroxylase activity in kidney is suppressed by this compound [8,10–13]. CYP27 catalyzes 27-hydroxylation of C_{27}-steroids and 25-hydroxyvitamin D_3 and 25-hydroxylation of vitamin D_3 in liver [10,14,15]. mRNA for CYP27 is distributed in several tissues including kidney [15]. Recently, Axén et al. [14,16] reported the surprising finding that purified liver mitochondrial CYP27 and recombinant expressed human CYP27 were able to catalyze the 1α-hydroxylation of 25-hydroxyvitamin D_3. The expression of CYP27 mRNA in rat kidney was found to be suppressed by 1α,25-dihydroxyvitamin D_3, indicating a coordinate regulation of CYP27 mRNA levels and mitochondrial 1α-hydroxylase activity by 1α,25-dihydroxyvitamin D_3 in kidney [16]. The results suggest a possible role of CYP27 as a renal 1α-hydroxylase and raise further questions on the catalytic properties of CYP27 towards 25-hydroxyvitamin D_3 in kidney.

The present investigation reports evidence that renal CYP27 is able to catalyze the 1α-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3 and that most of the 1α-hydroxylase activity in kidney appears to be associated with CYP27.

2. Materials and methods

2.1. Purification of cytochrome P450 catalyzing 1α-hydroxylation

Kidney cortex mitochondria were prepared from 20–25 kg of kidney from castrated pigs [6,17]. Phosphate buffers, used as the potassium salt, contained 1 mM dithiothreitol and 0.25 mM PMSF. Mitochondria were suspended in 10 mM phosphate buffer, pH 7.4, to a protein concentration of 25 mg/ml, homogenized and allowed to stand at 4°C overnight. The mitochondrial suspension was sonicated for 3 min at intervals of 15 s and centrifuged at $100\,000\times g$ for 30 min. The precipitate was dissolved and homogenized in 0.1 M phosphate, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. All buffers in the purification procedures contained 20% (v/v) glycerol and 0.1 mM EDTA unless otherwise stated. The suspension was diluted to a protein concentration of 20 mg/ml, sodium cholate was added to 0.8% (w/v), the suspension was stirred gently for 1 h at 4°C, and then centrifuged at $100\,000\times g$ for 30 min. The $100\,000\times g$ supernatant was subjected to chromatography on DEAE-Sepharose CL6B (8×15 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 0.5% sodium cholate. The non-bound material was applied to an aminohexyl-Sepharose column (5×30 cm), equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 0.4% sodium cholate. The cytochrome P450 fraction eluted with 0.1 M phosphate buffer, pH 7.4, containing 0.2% (w/v) polyoxyethylene 10 lauryl ether (POELE) was diluted 4-fold with 20% glycerol containing 0.1 mM EDTA and subjected to chromatography on hydroxyapatite (column, 4×20 cm), equilibrated in 10 mM phosphate buffer, pH 7.4, containing 0.2% POELE. The hydroxyapatite column was washed with 25 mM phosphate buffer, pH 7.4, containing 0.2% POELE. The cytochrome P450 fraction eluted with 150 mM phosphate buffer, pH 7.4, containing 0.2% POELE, was concentrated, dialyzed against 20 mM Tris-acetate buffer, pH 8.0, containing 0.4% POELE and then subjected to Q-Sepharose chromatography. The column (1.6×30 cm) was equilibrated in the same buffer as used for dialysis and was eluted in a linear gradient of sodium acetate (0–0.5 M) in the equilibrating buffer. The cytochrome P450 eluted with 85 mM sodium acetate was dialyzed against 10 mM phosphate buffer, pH 7.0, containing 0.1% sodium cholate, and subjected to chromatography on Q-Sepharose (column, 1.6×20 cm) equilibrated and washed in the same buffer as used for dialysis. The column was eluted with sodium acetate gradients (0–0.1, 0.1, 0.1–0.3, 0.3 M) in the equilibrating buffer. Detergents were removed and the final cytochrome P450 fraction was dialyzed as described [17].

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2.2. Preparation of a crude kidney mitochondrial cytochrome P450 extract active in 1α-, 24-, and 27-hydroxylations

Mitochondrial cytochrome P450 from 1 kg of pig kidney was cholate solubilized as described above and only partially purified by octylamine-Sepharose and hydroxyapatite chromatography [18].

2.3. Purification of human liver CYP27 expressed in E. coli

Glycerol stocks of pTrc 27H-transformed JM 105 cells served as an inoculum for all expression experiments [14]. The membranes isolated from 1 l of culture were diluted to a protein concentration of 20 mg/ml with 100 mM phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.25 mM PMSF. The membrane proteins were solubilized by addition of 0.8% sodium cholate and centrifuged at 100 000 × *g* for 60 min [14]. The supernatant was dialyzed against 50 mM phosphate buffer, pH 7.4, containing 20% glycerol, and used as a source of human liver CYP27.

2.4. Incubation procedures

25-Hydroxyvitamin D_3, 25 μg in 25 μl acetone, was incubated at 37°C for 20–60 min with 0.5 nmol of cytochrome P450 or 0.5 mg of solubilized membrane protein from *E. coli*, 4 nmol of ferredoxin, 0.4 nmol of ferredoxin reductase, and 1 μmol of NADPH in a total volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4. Incubations were terminated, extracted and analyzed by SP- and RP-HPLC as described previously [9].

2.5. Antibody production and incubations with antibody-coupled Sepharose

A monoclonal antibody, MAb 26C5, raised against pig liver mitochondrial CYP27 [9] and an irrelevant monoclonal antibody, MAb 25H6 [19], raised against pig liver microsomal vitamin D_3 25-hydroxylase were those produced and used in previous reports from this laboratory. Polyclonal antibodies raised against an unknown pig kidney mitochondrial protein with a molecular weight of approx. 55 kDa, were raised in New Zealand White rabbits. The antigen was prepared by SDS-PAGE and following transfer to nitrocellulose sheets the bands were visualized by india ink staining. The band corresponding to the 55 kDa protein was cut out, dissolved in DMSO, emulsified in an equal volume of Freund's complete or incomplete adjuvant and used for multiple immunization. The antibodies were purified from rabbit serum on protein A-Sepharose [20]. The antibodies were separately coupled to CNBr-Sepharose. Cytochrome P450 (0.5 nmol) was incubated for 1 h with the indicated amounts of Sepharose-bound antibody 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. Inactivated Sepharose without antibody was used as a control. The Sepharose was washed once with the same buffer without CHAPS and pooled. The reaction mixture, 1 ml, was incubated for 1 h at 37°C, terminated, extracted and analyzed as described [9].

2.6. Other methods

Adrenodoxin and adrenodoxin reductase from bovine adrenal mitochondria were prepared as described [17]. Protein and cytochrome P450 determinations, SDS-PAGE and silver staining were performed as described [21–24]. Immunoblotting was performed as described [20], except that the blot was incubated with the monoclonal antibody (10 μg/ml) or antiserum (diluted 300-fold with phosphate-buffered saline). An alkaline phosphatase detection system (Bio-Rad) was used to visualize the immune complexes.

3. Results and discussion

3.1. Purification of 1α-hydroxylating cytochrome P450 from pig kidney mitochondria

A cytochrome P450 fraction, active in 1α-hydroxylation of 25-hydroxyvitamin D_3, was isolated from kidney mitochondria of castrated pigs by solubilization with sodium cholate and chromatography on DEAE-Sepharose, aminohexyl-Sepharose, hydroxyapatite, and Q-Sepharose in two steps. The enzyme fraction also catalyzed 27-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3. The specific cytochrome P450 content varied between 2.4 and 3.8 nmol cytochrome P450 mg^{-1} protein in different preparations. On silver-stained SDS-PAGE (Fig. 1A) all preparations showed one major protein at 53 kDa and a minor one at 55 kDa in the molecular weight region for cytochrome P450. Owing to the very low content of cytochrome P450 and relative abundance of other kinds of hemoproteins in kidney mitochondria, it was important to purify the 1α-hydroxylase protein by measuring the catalytic activity throughout each step in the purification procedure. Attempts to separate the 1α- and 27-hydroxylase activities by various chromatographic methods were unsuccessful. No 1α- or 27-hydroxylase activity was detected in any of the side fractions with the exception of the last chromatographic step. The ratio between the 27- and 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 was about 4:1 in all purification steps and in all preparations (Table 1). The side fraction from the last chromatographic step catalyzed both 1α- and 27-hydroxylation of 25-hydroxyvitamin D_3. The ratio between 27- and 1α-hydroxylase activity was also 4:1 in this fraction although the rate of hydroxylation and the cytochrome P450 content were several-fold lower than in the main fraction.

3.2. A monoclonal antibody against CYP27 inhibits renal 1α-hydroxylase activity

SDS-PAGE and immunoblotting analyses of the purified cytochrome P450 fraction were performed with a monoclonal antibody (MAb 26C5) raised against pig liver CYP27 [9] and with a polyclonal antibody (HP3) against a 55 kDa pig kidney protein present in the final cytochrome P450 fraction of all preparations. Fig. 1B shows that the antibodies recognized protein with M_r of 53 kDa and 55 kDa, respectively. The two antibodies were separately coupled to Sepharose and incubated with the purified cytochrome P450 fraction. After

Table 1
Catalytic activities of purified pig kidney mitochondrial cytochrome P450 catalyzing 1α- and 27-hydroxylation of 25-hydroxyvitamin D_3

	P450 content (nmol mg^{-1} protein)	25-Hydroxyvitamin D_3				Ratio
		27-hydroxylation		1α-hydroxylation		27:1α
		(pmol min^{-1} mg^{-1} protein)	(pmol min^{-1} $nmol^{-1}$ P450)	(pmol min^{-1} mg^{-1} protein)	(pmol min^{-1} $nmol^{-1}$ P450)	
Prep. I	2.6	143	33	33	8	4.3
Prep. II	2.4	256	58	73	17	3.5
Prep. III	3.7	516	141	118	32	4.4
Prep. IV	3.0	133	49	29	11	4.6

Incubations were terminated, extracted and analyzed as described in Section 2. The results from four preparations are shown.

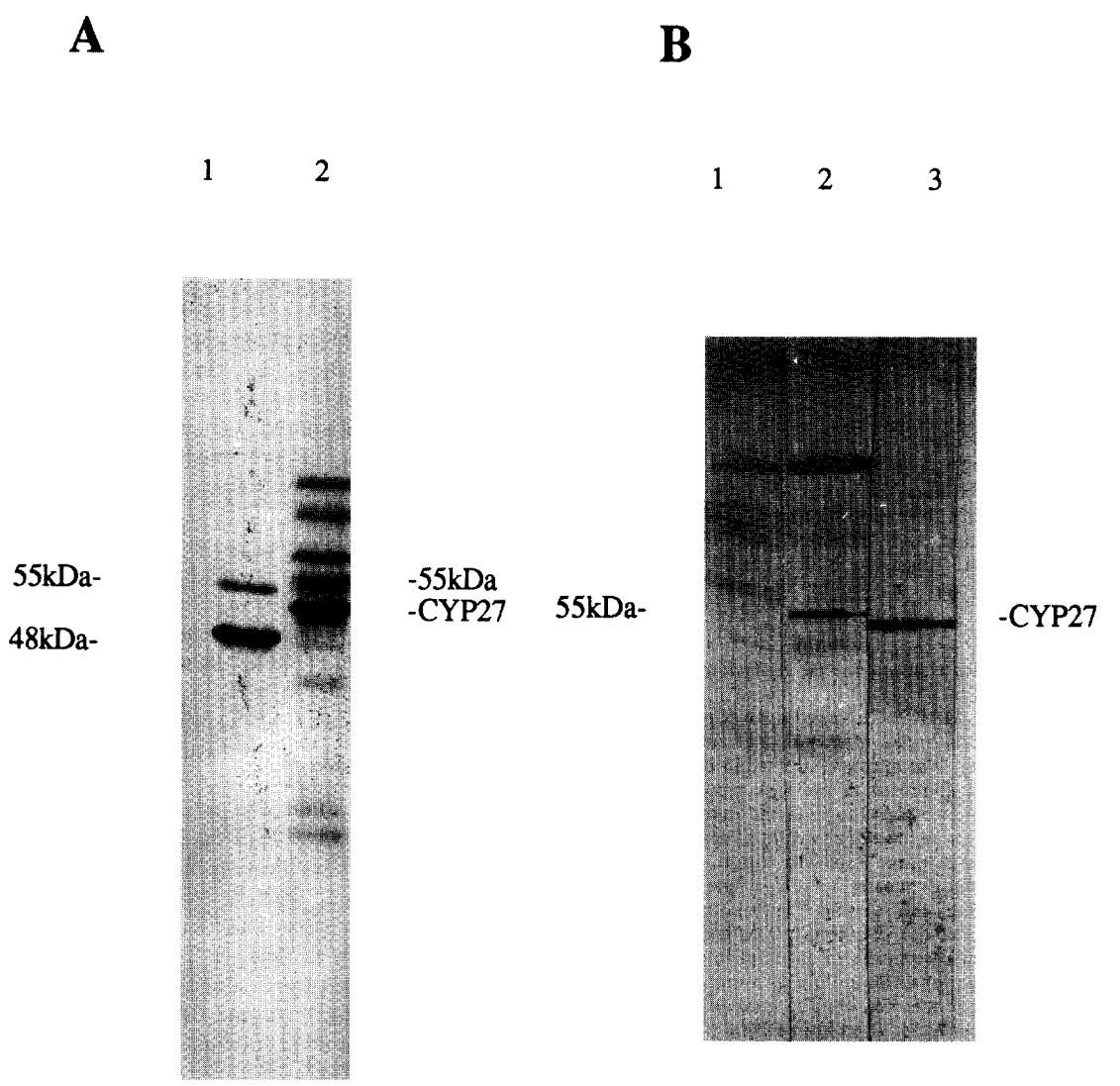

Fig. 1. SDS-PAGE of partially purified CYP27 from pig kidney mitochondria (A) and Western blotting using antibodies against pig liver CYP27 and the 55 kDa protein (B). (A) Lanes: 1, a mixture of CYP1A2 (55 kDa) and 2B4 (48 kDa) from rabbit liver microsomes used as M_r standards (1 μg of each); 2, purified kidney mitochondrial cytochrome P450 (10 μg). (B) Partially purified CYP27 from pig kidney mitochondria (5 μg/lane) was subjected to SDS-PAGE, electrophoretic transfer and immunoblotting with different antibodies. Lanes: 1, preimmune serum; 2, anti-55 kDa (HP3); 3, anti-CYP27 (MAb 26C5).

incubation, the antibody-Sepharose was removed and the supernatant assayed in a reconstituted system for 1α- and 27-hydroxylase activity towards 25-hydroxyvitamin D_3. As a control, Sepharose was coupled to an irrelevant monoclonal antibody directed against pig liver microsomal vitamin D_3 25-hydroxylase (MAb 25H6) [19]. Increasing amounts of MAb 26C5 but not HP3 or the irrelevant antibody were able to bind more than 90% of both the 1α- and the 27-hydroxylase activities towards 25-hydroxyvitamin D_3.

A separate set of experiments was carried out in an attempt to examine how much of the total 1α-hydroxylase in kidney mitochondria is represented by the 1α-hydroxylase in the purified cytochrome P450 preparation. For this purpose, a solubilized, crude kidney mitochondrial cytochrome P450 extract considered to contain most of the mitochondrial 1α-, 24- and 27-hydroxylase activities was prepared. The cytochrome P450 had to be partially purified in order to achieve a meaningful and accurate assay of the hydroxylase activities towards 25-hydroxyvitamin D_3. The monoclonal antibody raised against CYP27 (MAb 26C5) was incubated with the partially purified cytochrome P450 fraction. As shown in Fig. 2A, more than 90% of the 1α-hydroxylase and 27-hydroxylase activities were immunoprecipitated by increasing amounts of the antibody. The 24-hydroxylase activity was not immunoprecipitated in the same way. The lowest amount of antibody decreased the 24-hydroxylase activity, however, the activity was not further decreased by greater amounts of the antibody. A possible explanation for this finding might be that the antibody could bind to a part of CYP24 which is aggregated together with CYP27, whereas the non-aggregated CYP24 is not bound by the antibody.

3.3. Substrate for CYP27 inhibits 1α-hydroxylation

Since the ratio between the 1α- and 27-hydroxylase activities remained essentially constant during purification and since the purification and antibody experiments indicated

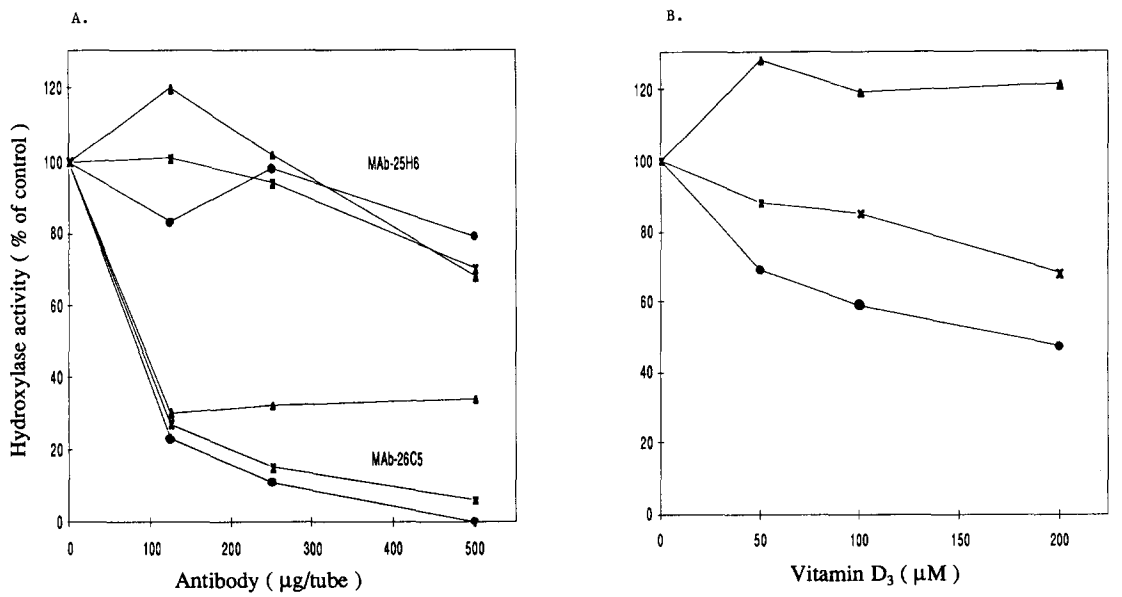

Fig. 2. Effects of antibodies (A) and CYP27 substrate (B) on 1α- (●), 24- (▲) and 27-hydroxylation (×) of 25-hydroxyvitamin D_3 by a solubilized, crude kidney mitochondrial cytochrome P450 extract. (A) Cytochrome P450 (0.5 nmol) was incubated in a total volume of 1 ml with the indicated amounts of Sepharose-bound antibody against pig liver CYP27 (MAb 26C5) or irrelevant antibody against pig liver microsomal vitamin D 25-hydroxylase (MAb 25H6) and assayed for hydroxylase activities as described in Section 2. The 100% of control value was for 1α-hydroxylation 13 pmol $nmol^{-1}$ min^{-1}, for 24-hydroxylation 5 pmol $nmol^{-1}$ min^{-1} and for 27-hydroxylation 79 pmol $nmol^{-1}$ min^{-1}. (B) 25-Hydroxyvitamin D_3 (200 μM) was incubated in a reconstituted system as described in Section 2 with the indicated concentrations of vitamin D_3.

that most of the renal mitochondrial 1α-hydroxylase activity is associated with CYP27, further comparisons with this enzyme were made. Addition of increasing amounts of vitamin D_3, a known substrate for CYP27, to the partially purified 1α-hydroxylase system decreased both the 1α- and 27-hydroxylase activities towards 25-hydroxyvitamin D_3 in a parallel fashion. Vitamin D_3 together with 25-hydroxyvitamin D_3 in equimolar concentrations decreased the 1α- and 27-hydroxylase activities by 50%, as would be expected if a single enzyme catalyzed both reactions. In contrast, the 24-hydroxylase activity was not inhibited but rather slightly increased by the addition of vitamin D_3 to the reconstituted system (Fig. 2B).

The results provide further evidence that the activity of 1α-hydroxylase but not that of 24-hydroxylase is associated with CYP27 in kidney mitochondria.

3.4. *Recombinant expressed human liver CYP27 in* E. coli *catalyzes 1α-hydroxylation but not 24-hydroxylation*

Since it has been reported that recombinant expressed human liver CYP27 catalyzes 24-hydroxylation of vitamin D_3 and vitamin D_2 [25], experiments were performed with 25-hydroxyvitamin D_3 and human liver CYP27 recombinantly expressed in pTrc27H-transformed *E. coli* [14]. The expression of human CYP27 was induced by isopropyl β-D-thiogalactopyranoside and membrane proteins were solubilized with sodium cholate. Solubilized CYP27 protein reconstituted with adrenodoxin and adrenodoxin reductase showed 1α-hydroxylase activity (10 pmol min^{-1} $nmol^{-1}$ P450) and 27-hydroxylase activity (8 pmol min^{-1} $nmol^{-1}$ P450) but not 24-hydroxylase activity (<0.1 pmol min^{-1} $nmol^{-1}$ P450) towards 25-hydroxyvitamin D_3. Control incubations without adrenodoxin and adrenodoxin reductase did not show 1α- or 27-hydroxylase activities. These results suggest that the hydroxyl group in the 25-position hinders 24-hydroxylation by CYP27 and support the contention that CYP27 is a 25-hydroxyvitamin D_3 24-hydroxylase in neither liver nor kidney.

3.5. *Biological significance*

It has recently been reported that liver mitochondrial CYP27 catalyzes 1α-hydroxylation of 25-hydroxyvitamin D_3 [14] and that 1α,25-dihydroxyvitamin D_3 suppresses both mitochondrial 1α-hydroxylase activity and the expression of CYP27 mRNA in kidney [16]. The results of the present study showing that renal CYP27 catalyzes 1α-hydroxylation but not 24-hydroxylation of 25-hydroxyvitamin D_3 provide further evidence that renal 1α-hydroxylation might be catalyzed by CYP27. In fact, the results from the purification, immunoprecipitation and substrate inhibition experiments indicate that CYP27 is responsible for most if not all of the 1α-hydroxylase activity in kidney mitochondria. However, the existence of another 1α-hydroxylase different from CYP27 cannot be completely ruled out. All the final cytochrome P450 fractions showed not only the major CYP27 protein at 53 kDa but also a minor protein at 55 kDa on silver-stained SDS-PAGE. Several laboratories have estimated the M_r of their putative 1α-hydroxylase to be about 53–59 kDa [26–28]. The possibility that the 55 kDa protein might be a 1α-hydroxylase seems to be excluded, since polyclonal antibodies (HP3) raised against the 55 kDa protein were unable to immunoprecipitate the 1α- and 27-hydroxylase activities.

The conclusion that renal CYP27 might be a major mitochondrial 1α-hydroxylase is both supported and disfavoured by a study on patients with the rare inherited disease cerebrotendinous xanthomatosis having defective sterol 27-hydroxylation of bile acid intermediates [29]. On the one hand, it was demonstrated that extensive osteoporosis and increased risk of bone fractures are components of the disease; on the other,

the serum concentrations of 1α,25-dihydroxyvitamin D_3 were not significantly lower than in healthy subjects [29].

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