Journal of Steroid Biochemistry & Molecular Biology 119 (2010) 171–179

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Metabolism of substrates incorporated into phospholipid vesicles by mouse 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1)

Edith K.Y. Tang, Kimberley J.Q. Voo, Minh N. Nguyen, Robert C. Tuckey*

School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

ARTICLE INFO

Article history:
Received 31 December 2009
Received in revised form 23 February 2010
Accepted 23 February 2010

Keywords:
Vitamin D
CYP27B1
Cytochrome P450
Phospholipid vesicles
25-Hydroxyvitamin D3

ABSTRACT

CYP27B1 catalyzes the 1α-hydroxylation of 25-hydroxyvitamin D3 to 1α,25-dihydroxyvitamin D3, the hormonally active form of vitamin D3. To further characterize mouse CYP27B1, it was expressed in *Escherichia coli*, purified and its activity measured on substrates incorporated into phospholipid vesicles, which served as a model of the inner mitochondrial membrane. 25-Hydroxyvitamin D3 and 25-hydroxyvitamin D2 in vesicles underwent 1α-hydroxylation with similar kinetics, the catalytic rate constants (k_{cat}) were 41 and 48 mol/min/mol P450, respectively, while K_m values were 5.9 and 4.6 mmol/mol phospholipid, respectively. CYP27B1 showed inhibition when substrate concentrations in the membrane were greater than 4 times K_m, more pronounced with 25-hydroxyvitamin D3 than 25-hydroxyvitamin D2. Higher catalytic efficiency was seen in vesicles prepared from dioleoyl phosphatidylcholine and cardiolipin than for dimyristoyl phosphatidylcholine vesicles. CYP27B1 also catalyzed 1α-hydroxylation of vesicle-associated 24*R*,25-dihydroxyvitamin D3 and 20-hydroxyvitamin D3, and 25-hydroxylation of 1α-hydroxyvitamin D3 and 1α-hydroxyvitamin D2, but with much lower efficiency than for 25(OH)D3. This study shows that CYP27B1 can hydroxylate 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 associated with phospholipid membranes with the highest activity yet reported for the enzyme. The expressed enzyme has low activity at higher concentrations of 25-hydroxyvitamin D in membranes, revealing that substrate inhibition may contribute to the regulation of the activity of this enzyme.

1. Introduction

25-Hydroxyvitamin D 1α-hydroxylase (CYP27B1) hydroxylates 25-hydroxyvitamin D3 (25(OH)D3) in the 1α-position producing 1α,25-dihydroxyvitamin D3 (1,25(OH)$_2$D3), the hormonally active form of vitamin D [1,2]. 1,25(OH)$_2$D3 stimulates calcium absorption, and to a lesser extent phosphorous absorption, from the small intestine [1]. 1,25(OH)$_2$D3 also has anticarcinogenic properties, affecting proliferation, differentiation and apoptosis in cells of different lineages [1,3,4]. The major location of CYP27B1 is in mitochondria of the proximal renal tubule, but it is also found in a number of extra-renal sites including skin, brain, colon, prostate and breast [3,5,6]. This gives rise to autocrine and paracrine roles of 1,25(OH)$_2$D3, particularly in relation to anti-proliferative and pro-differentiation actions.

Despite its importance, CYP27B1 has been poorly studied to date due to its very low concentration in kidney mitochondria and its lability once extracted with detergents [7,8]. Hiwatashi et al. [7] reported purifying a small amount of bovine CYP27B1 to electrophoretic homogeneity. More recently, Uchida et al. [9] have reported the purification of mouse CYP27B1 expressed in *E. coli*.

CYP27B1 belongs to the mitochondrial (Type-1) cytochrome P450 class which receive electrons to support their hydroxylation reactions from NADPH via adrenodoxin reductase and adrenodoxin [10]. Two of the mitochondrial P450s, CYP27A1 and CYP11A1, appear to be anchored to the mitochondrial membrane primarily by a region involving the F-G loop [11–14]. These P450s can hydroxylate both vitamin D and cholesterol [12,15–18], and substrates appear to reach the active site from the membrane phase [11,13,14,19]. Since CYP27B1 belongs to the same family as CYP27A1, is associated with the inner mitochondrial membrane and uses relatively hydrophobic hydroxyvitamin D metabolites as substrates, it is reasonable to predict that it is also anchored to the membrane by its F-G loop region and accesses substrate from the membrane phase. Its substrate, 25(OH)D3, has been shown to partition efficiently (95%) into the membrane phase of liposomes made

Abbreviations: 1(OH)D2, 1α-hydroxyvitamin D2; 1(OH)D3, 1α-hydroxyvitamin D3; 20(OH)D3, 20-hydroxyvitamin D3; 25(OH)D2, 25-hydroxyvitamin D2; 25(OH)D3, 25-hydroxyvitamin D3; 1,25(OH)$_2$D2, 1α,25-dihydroxyvitamin D2; 1,25(OH)$_2$D3, 1α,25-dihydroxyvitamin D3; 24,25(OH)$_2$D3, 24*R*, 25-dihydroxyvitamin D3; cyclodextrin, 2-hydroxypropyl-β-cyclodextrin.

* Corresponding author. Tel.: +61 864883040; fax: +61 864881148.
E-mail address: rtuckey@cyllene.uwa.edu.au (R.C. Tuckey).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.02.022

from egg phosphatidylcholine [20]. In the present study, we have investigated the ability of expressed mouse CYP27B1 to metabolize substrates incorporated into phospholipid vesicles. We show that 25(OH)D3 in vesicles is efficiently metabolized by CYP27B1 at low substrate concentrations, whereas at high substrate concentrations, marked substrate inhibition is observed.

2. Materials and methods

2.1. Materials

20(OH)D3 was produced enzymatically by the action of CYP11A1 on vitamin D3 and purified by TLC and reverse-phase HPLC as described before [18]. Vitamin D3, 1,25$(OH)_2$D2, dioleoyl phosphatidylcholine and bovine heart cardiolipin were from Sigma (St. Louis, MO). 1(OH)D2, 1(OH)D3, 25(OH)D3, 25(OH)D2 and CHAPS were from Merck (Darmstadt, Germany). 1,25$(OH)_2$D3 and 24,25$(OH)_2$D3 were a gift from Dr Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ). [^{3}H]25(OH)D3 and [4-^{14}C]cholesterol were from PerkinElmer Life Science (Boston, MA). The plasmid pGro7 was from Takara Bio Inc. (Shiga, Japan). Affi-Gel 15 was from Bio-Rad (CA). Bovine adrenodoxin reductase and adrenodoxin were purified from bovine adrenal mitochondria as before [21].

2.2. Expression of mouse CYP27B1 in Escherichia coli

The cDNA for mature mouse CYP27B1 [22], encoding a 4-His-tag at the C-terminus, was chemically synthesized by GenScript Corporation (Piscataway, NJ). The site of cleavage of the mitochondrial target sequence was assumed to be between Ser-32 and Val-33, as predicted by alignment with CYP24 [23]. In our construct, Ser-32 was replaced by Met as the first amino acid of the mature sequence and Val-33 was replaced by Ala. Silent mutations were made in the first 6 codons to enhance the AT-richness of the region [24]. The cDNA was ligated into the expression vector, pTrc99A, using NcoI (5′-end) and KpnI (3′-end) restriction sites. Competent *E. coli* (JM109) cells containing pGro7 plasmid (encoding the chaperones, GroEL/ES, under the control of the araB promoter) were transformed with the pTrc99A-CYP27B1 construct. A 1:100 dilution of an overnight culture was made into 500 mL Terrific Broth (pH 7.5) containing ampicillin (50 μg/mL) and chloramphenicol (20 μg/mL), and incubated at 37 °C until the absorbance at 600 nm was 0.6. L-Arabinose (4 mg/mL), isopropyl-β-D-thiogalactopyranoside (1 mM) and δ-aminolevulinic acid (0.5 mM) were added and the cultures incubated with shaking (220 rpm) for a further 46 h at 26 °C, followed by 22 °C for 3 h.

2.3. Purification of CYP27B1

Bacterial cells from a total of 2 L of culture (above) were harvested by centrifugation (4 °C) and resuspended in 100 mL of 100 mM potassium phosphate pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 20% glycerol (v/v) and 1% CHAPS (w/v). Cells were then sonicated on ice using a Branson sonicator with four 2-min pulses with 2 min cooling intervals. Particulate material was removed by centrifugation (107 000 × *g* for 60 min) and the supernatant dialyzed against 100 volumes buffer A (50 mM sodium phosphate pH 7.4, 0.3 M NaCl, 0.1 mM PMSF and 20% glycerol (v/v)), then applied to a nickel-affinity column (1.5 cm × 3 cm, Ni-NTA His-Bind Resin) equilibrated with the same buffer. The column was washed with 100 mL buffer A containing 20 mM imidazole and the CYP27B1 eluted with an imidazole gradient (20–500 mM). The eluant was dialyzed against 100 volumes buffer B (10 mM potassium phosphate pH 7.4, 0.1 mM PMSF, 0.1 mM EDTA, 0.1 mM DTT and 20% glycerol (v/v)) and concentrated to 2 mL. This solution was incubated with 10 μM 25(OH)D3 for 30 min on ice then 1.5 mL of Affi-Gel 15 coupled with bovine adrenodoxin was added. After 3 h at 4 °C the gel was placed in a column and washed with 20 mM NaCl in buffer B. The CYP27B1 was eluted with 1 M NaCl in buffer B and the eluant applied to a Fast Flow octyl Sepharose column (0.75 cm × 3 cm) equilibrated with buffer C (buffer A plus 0.1 mM EDTA and 0.1 mM DTT). The column was washed with 20 mL buffer C plus 0.2% CHAPS and the CYP27B1 eluted with 1.0% CHAPS in buffer C. Fractions with an absorbance ratio 416 nm/280 nm ≥ 0.5 were pooled, dialyzed against 100 volumes buffer C without NaCl for 3 h and concentrated to 1–2 μM for storage at −80 °C.

2.4. Bacterial expression and purification of mouse adrenodoxin

cDNA encoding mature mouse adrenodoxin [25] was chemically synthesized by GenScript and cloned into pTrc99A using NcoI and KpnI sites in a similar fashion to that described for human adrenodoxin [26]. Bacterial cultures were also grown and the adrenodoxin purified as for human adrenodoxin [26].

2.5. Preparation of phospholipid vesicles

Dioleoyl phosphatidylcholine (1.08 μmol), cardiolipin (0.19 μmol) and hydroxyvitamin D substrates (as required), were placed in tubes and the ethanol solvent removed under nitrogen. To prepare large vesicles, 1.0 mL of vesicle buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.1 mM DTT and 0.1 mM EDTA) was added, tubes vortexed and the mixture extruded through a 1.0 μm membrane 12 times using a Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL). The resulting vesicles have a diameter of approximately 1.0 μm. Small unilamellar vesicles were prepared by adding 0.5 mL vesicle buffer to the dry lipid mixture and sonicating for 10 min in a bath-type sonicator as described before [19]. Vesicles prepared by this method have a diameter of approximately 40–50 nm [27].

2.6. Measurement of 25(OH)D3 association with phospholipid vesicles

Large vesicles were made from dioleoyl phosphatidylcholine and cardiolipin by extrusion as described above except that the initial mixture of lipid also contained [4-^{14}C]cholesterol (7 nCi), [^{3}H]25(OH)D3 (20 nCi) and unlabelled 25(OH)D3 (0.0031–0.125 nmol). Vesicles (0.9 mL) were diluted to 6.0 mL with vesicle buffer and centrifuged at 107 000 × *g* for 6 h at 20 °C. The supernatant was removed and the ^{14}C and ^{3}H radioactivity determined by scintillation counting using a dual isotope program. The vesicle pellet was resuspended in 1.0 mL vesicle buffer and the radioactivity similarly measured. The percentage of vesicles sedimented (greater than 99.5%) was determined from the proportion of [4-^{14}C]cholesterol in the pellet. The degree of association of 25(OH)D3 with the vesicles was determined from the proportion of [^{3}H]25(OH)D3 in the pellet.

2.7. Measurement of CYP27B1 activity

Phospholipid vesicles containing hydroxyvitamin D substrates (as required) were prepared as described above. Activity measurements were performed in a similar way to that described for CYP11A1 [18,28,29]. The incubation mixture comprised vesicles (510 μM phospholipid), CYP27B1 (8–200 nM), 15 μM mouse adrenodoxin, 0.4 μM bovine adrenodoxin reductase, 2 mM glucose 6-phosphate, 2 U/mL glucose 6-phosphate dehydrogenase and 50 μM NADPH, in the buffer used for preparation of vesicles. Following dialysis and enzyme dilution the remaining CHAPS concentration in the incubation was typically below 0.06 μM. Samples were preincubated for 8 min, reactions started by the addition of

NADPH and incubations carried out at 37 °C in a shaking water bath. Typical incubation volumes were 0.2–1.0 mL. Incubation times were kept short (typically 2.0 min) in experiments designed to measure the kinetic constants of CYP27B1 to ensure initial rates were measured. Reactions were stopped by the addition of 2 mL ice-cold dichloromethane and the aqueous phase extracted with dichloromethane [29]. Reverse phase HPLC of samples and measurement of CYP27B1 activity in 0.45% cyclodextrin were also carried out as before [28,29].

2.8. Analysis of kinetic data

Kinetic parameters were determined by fitting the Michaelis–Menten equation to the experimental data using Kaleidagraph 3.6. For metabolism of 25(OH)D3 and 25(OH)D2 where substrate inhibition was observed, we also fitted the following equation to the data:

$$V = k_{cat}[E]\left(1 + \left(\frac{[S]}{K_i}\right)^2 + \frac{K_m}{[S]}\right)$$

This equation is derived from a model where two molecules of substrate (S) binds to the enzyme–substrate complex (ES) simultaneously with equal K_i values to form an ES_3 complex that is catalytically inactive. This equation provides the K_m and k_{cat} as well as the inhibitor constant for substrate (K_i).

2.9. Other procedures

The concentration of cytochrome CYP27B1 was determined from the CO-reduced minus reduced difference spectrum using an extinction coefficient of 91 000 $M^{-1}cm^{-1}$ for the absorbance difference between 450 and 490 nm [30]. The concentration of hydroxyvitamin D3 was determined using an extinction coefficient of 18 000 $M^{-1}cm^{-1}$ at 263 nm [7]. SDS-polyacrylamide gel electrophoresis was carried out as before [31]. Protein was determined by the Ponceau S procedure [32]. For the measurement of the K_m and k_{cat} for adrenodoxin, the concentration of free adrenodoxin was calculated as described by Hanukoglu and Jefcoate [33].

3. Results

3.1. Expression and purification of mouse CYP27B1 and adrenodoxin

The level of expression of mouse CYP27B1 was 20 nmol/L culture. This was measured after Ni-affinity chromatography since the low level of expression and the presence of some cytochrome P420 prevented measurement of its concentration via CO-reduced minus reduced difference spectroscopy directly in the CHAPS extract of the bacterial cells. Despite using a similar procedure to Uchida et al. [9], including co-expression with chaperones GroES and GroEL, and optimizing expression time, our expression level was considerably lower. CYP27B1 expression levels were similar for the pTrc99A-CYP27B1 construct in *E. coli* strains JM109 and DH5α, the latter being the strain used by Uchida et al. [9]. Due to the low expression, Ni-affinity chromatography alone was insufficient to provide reasonably pure enzyme for activity studies, so adrenodoxin-affinity chromatography and octyl Sepharose chromatography were employed to further purify the cytochrome (Fig. 1A). The band at 56 kDa (Fig. 1A), clearly visible after Ni-affinity chromatography and subsequent steps, was close to the predicted mass for CYP27B1 (53.4 kDa) and was confirmed to be CYP27B1 by electrospray ionization mass spectrometry following trypsin digestion. The final preparation of CYP27B1 was predominantly low spin with a 417 nm/280 nm absorbance ratio of 0.6, similar to that reported by Uchida et al. [9], and was relatively free of cytochrome P420 (Fig. 2A).

CYP27B1 proved to be labile during purification despite the presence of 20% glycerol as a stabilizing agent. It was found important to avoid prolonged standing of the enzyme at 4 °C, including overnight dialysis, as this caused marked loss of the holo-enzyme (up to 50%). Losses were minimized by carrying out the purifica-

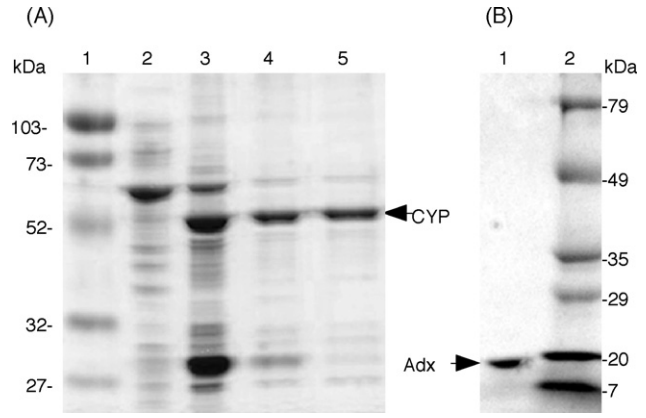

Fig. 1. Analysis of the purity of expressed mouse CYP27B1 and mouse adrenodoxin. CYP27B1 samples were run on a 12.5% SDS-polyacrylamide gel (A). Lane 1, molecular weight markers; lane 2, CHAPS extract of *E. coli* cells (4 μg); lane 3, CYP27B1 after nickel-affinity chromatography (4 μg); lane 4, CYP27B1 following nickel- and adrenodoxin-affinity chromatography (2 μg); lane 5, CYP27B1 following nickel affinity-, adrenodoxin affinity- and octyl Sepharose-chromatography (2 μg). Purified mouse adrenodoxin was run on a 4–20% gradient gel (B). Lane 1, purified mouse adrenodoxin (2 μg); lane 2, molecular weight markers. Arrows indicate the protein of interest. CYP, CYP27B1; Adx, adrenodoxin.

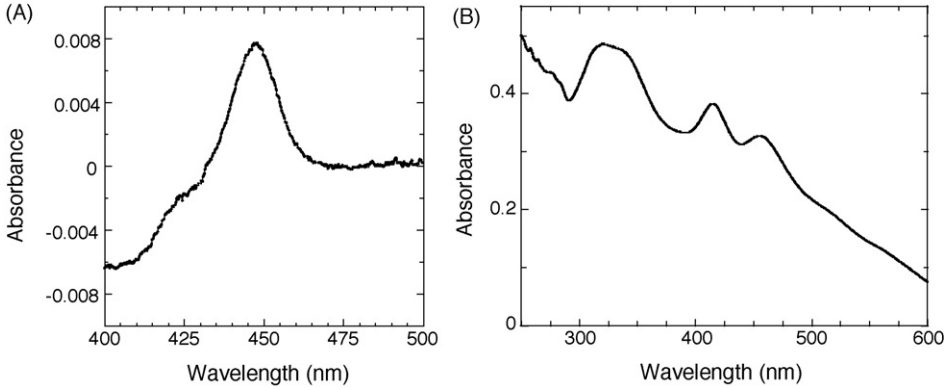

Fig. 2. Spectral analysis of purified mouse CYP27B1 and adrenodoxin. (A) CO-reduced minus reduced difference spectrum of mouse CYP27B1 following nickel affinity-, adrenodoxin affinity- and octyl Sepharose-chromatography. (B) Absolute absorbance spectrum of purified mouse adrenodoxin.

Table 1
Association of 25(OH)D3 with phospholipid vesicles.

25(OH)D3/phospholipid (mol/mol)	Vesicles sedimented (%)	25(OH)D3 sedimented (%)
0.0025	99.6	97.5
0.020	99.6	97.4
0.100	99.8	97.0

Phospholipids vesicles were prepared by extrusion of an aqueous suspension of phospholipid, [^{3}H]25(OH)D3 and [^{14}C]cholesterol through a 1.0 μm membrane and then sedimented by ultracentrifugation. The proportion of vesicles sedimented was determined from the amount of [^{14}C]cholesterol in the vesicle pellet.

tion procedures quickly at 4 °C and storing the enzyme at −80 °C between steps, as required.

In contrast to CYP27B1, mouse adrenodoxin expressed at high levels providing 3.0 μmol of pure protein per litre culture. The final preparation ran as a single band (14.5 kDa) on an SDS-polyacrylamide gel stained with Coomassie blue (Fig. 1B), close to the predicted size of 13.7 kDa. It displayed a typical adrenodoxin absorption spectrum with a 414 nm/280 nm absorbance ratio of 0.88 (Fig. 2B).

3.2. Association of 25(OH)D3 with membranes

Merz and Sternberg [20] have shown that 95.4% of 25(OH)D3 associates with egg phospholipid liposomes. We tested the degree of association of 25-[26,27-^{3}H]-hydroxyvitamin D3 ([^{3}H]25(OH)D3) with large phospholipid vesicles (liposomes) prepared from dioleoyl phosphatidylcholine and cardiolipin, which resemble the inner mitochondrial membrane. These large vesicles, produced by extrusion through a 1.0 μm membrane, were quantitatively (greater than 99%) sedimented by ultracentrifugation. At all three ratios of 25(OH)D3 to phospholipid tested, between 97.0% and 97.5% of the radiolabelled 25(OH)D3 was sedimented with the vesicles (Table 1) showing that it strongly partitions into the membrane bilayer.

3.3. Binding and metabolism of 25(OH)D3 in phospholipid vesicles by CYP27B1

The addition of purified CYP27B1 to vesicles containing 25(OH)D3 caused a change towards the high spin state indicating that CYP27B1 can access substrate from the vesicle membrane (Fig. 3). When adrenodoxin, adrenodoxin reductase and NADPH were added to the vesicles, 0.5 μM CYP27B1 was able to metabolize 95% of the 25(OH)D3 in the vesicle membrane in 2 min (Fig. 4). The product was identified as the expected 1,25(OH)$_2$D3 based on an identical retention time to authentic standard by HPLC (Fig. 4) and identical R_f values by TLC (not shown). Thus, CYP27B1 can rapidly access essentially all the 25(OH)D3 in both halves of the phospholipid bilayer. A similar result was observed for 25-hydroxyvitamin D2 (25(OH)D2) with the product being identified as 1α,25-dihydroxyvitamin D2 (1,25(OH)$_2$D2) from authentic standard (not shown).

Time courses for the metabolism of 25(OH)D3 in phospholipid vesicles by CYP27B1 were determined for large vesicles prepared by extrusion through a 1.0 μm membrane and small unilamellar vesicles prepared by sonication (Fig. 5). The time courses were linear for 2–3 min with the initial rate of product formation for small vesicles being 50% higher than for large vesicles. The loss of linearity after 2–3 min of incubation appears to be due to the instability of the CYP27B1. CO-reduced minus reduced difference spectra recorded, prior to and at the end of a 10 min incubation at 37 °C, showed a 50% reduction in P450 concentration by the end of the incubation.

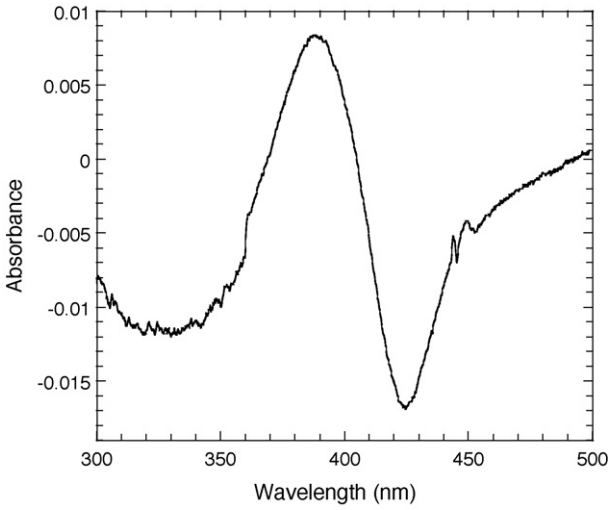

Fig. 3. Substrate-induced difference spectrum of CYP27B1. CYP27B1 was added to phospholipid vesicles alone (reference cuvette) or to phospholipid vesicles containing 25(OH)D3 (test cuvette, 25(OH)D3/phospholipid = 0.05 mol/mol) and the difference spectrum recorded. The phospholipid vesicles were prepared from prepared from dioleoyl phosphatidylcholine and cardiolipin and were used at a phospholipid concentration of 510 μM. The CYP27B1 concentration was 0.7 μM.

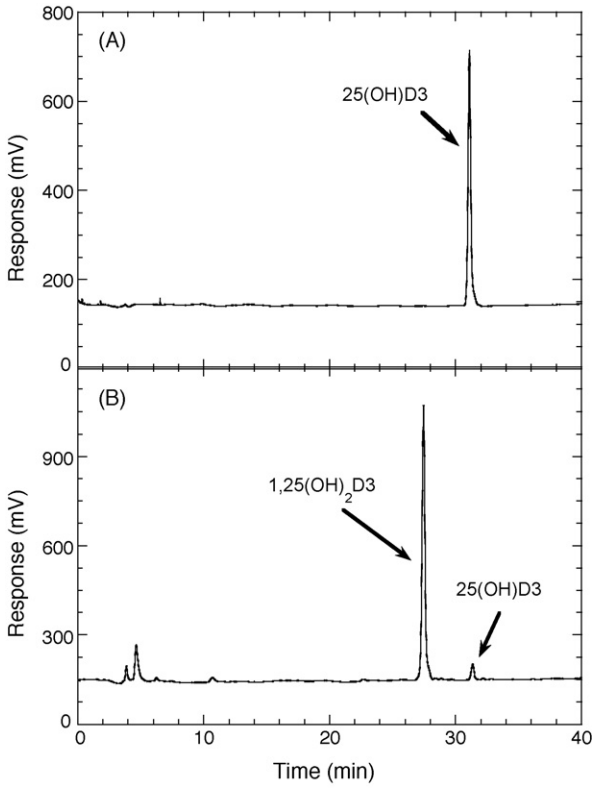

Fig. 4. Chromatograms showing metabolism of 25(OH)D3 in phospholipid vesicles by CYP27B1. Small phospholipid vesicles were prepared from dioleoyl phosphatidylcholine and cardiolipin with 25(OH)D3 present at a molar ratio of 0.05 mol/mol phospholipid, by sonication. Vesicles (510 μM phospholipid) were incubated with CYP27B1 (0.5 μM) in the presence of bovine adrenodoxin reductase (0.4 μM), mouse adrenodoxin (15 μM) and NADPH (50 μM) for 2 min at 37 °C, then the reaction stopped and secosteroids extracted with dichloromethane. Substrate and products were separated by reverse phase HPLC on a C18 column. (A) Zero time control where the reaction mixture was extracted prior to starting the reaction with NADPH; (B) test reaction.

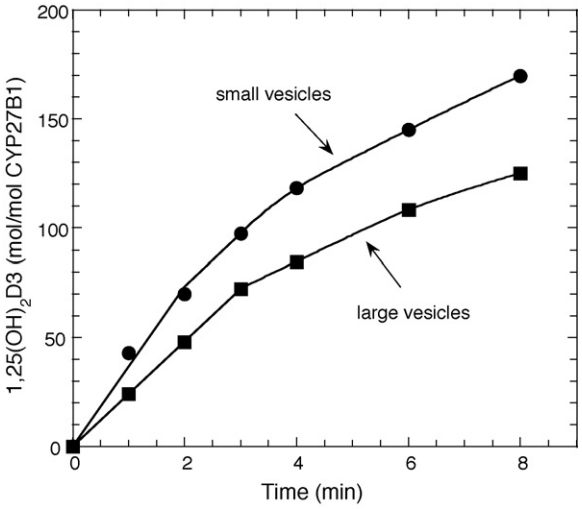

Fig. 5. Comparison of time courses for hydroxylation of 25(OH)D3 in small and large phospholipid vesicles by CYP27B1. Phospholipid vesicles were prepared from dioleoyl phosphatidylcholine and cardiolipin with 25(OH)D3 present at a molar ratio of 0.15 mol/mol phospholipid, by sonication (small vesicles) or extrusion through a 1.0 μm membrane (large vesicles). Vesicles were incubated with 12.5 nM CYP27B1 in a reconstituted system as in Fig. 4 and the amount of $1,25(OH)_2D3$ formed measured by HPLC.

3.4. Comparison between mouse and bovine adrenodoxin for supporting the activity of mouse CYP27B1

Since other researchers have used bovine adrenodoxin to measure the catalytic activity of mouse CYP27B1 [9,23,41], in contrast to our study employing expressed mouse adrenodoxin, we compared the activity of mouse CYP27B1 supported by the two different species of adrenodoxin. Mouse and bovine adrenodoxin did display similar abilities to support CYP27B1 activity. At a 25(OH)D3 concentration of 0.035 mol/mol phospholipid, mouse adrenodoxin gave K_m and k_{cat} values of 3.2 ± 0.3 μM and 34.2 ± 0.8 mol/min/mol CYP27B1, respectively, while K_m and k_{cat} values for bovine adrenodoxin were 4.6 ± 1.2 μM and 35.8 ± 2.5 mol/min/mol CYP27B1, respectively.

3.5. Kinetics of 1α-hydroxylation of different substrates by CYP27B1

When the hydroxylation of 25(OH)D3 was measured as a function of increasing 25(OH)D3 concentration the reaction rate increased, but as substrate became near-saturating strong inhibition was observed (Fig. 6A). The data gave a good fit to a hyperbolic curve up to a substrate concentration of 0.025 mol 25(OH)D3 per mol phospholipid. This permitted estimation of the K_m and k_{cat} values which are listed in Table 2. When a similar kinetic analysis was done using 25(OH)D2 as substrate, inhibition was also observed at high substrate concentrations, but was much less marked than for 25(OH)D3 (Fig. 6B). The K_m and k_{cat} values from a hyperbolic curve fitted to the data for 25(OH)D2 are listed in Table 2 and are similar to the values for 25(OH)D3. The inhibition at high substrate concentrations was seen in three replicate experiments for both 25(OH)D3 and 25(OH)D2 and with three separate preparations of CYP27B1. In contrast to phospholipid vesicles, no substrate inhibition was seen when 25(OH)D3 was dissolved in 0.45% 2-hydroxypropyl-β-cyclodextrin (cyclodextrin) (Fig. 6C). In this system a good fit to a hyperbolic curve was seen with substrate concentrations as high as $8K_m$. The k_{cat} for CYP27B1 in the cyclodextrin system was approximately 30% of that seen in vesicles. We have previously shown that the cyclodextrin concentration markedly affects both the k_{cat} and K_m for metabolism of vitamin D3 and cholesterol by CYP11A1 [29]. The cyclodextrin forms a complex with the vitamin D substrate and holds it in solution and catalytic activity is influenced by the relative binding of the substrate to the cyclodextrin and the active site of the enzyme [29].

Substrate inhibition has been observed previously for microsomal P450s with a number of substrates [34–36]. Equations based on one catalytic and one inhibitory site for substrate binding have been derived to describe this inhibition but gave a poor fit to our experimental data. A reasonable fit was given by an equation we developed which assumes one catalytic and two inhibitory sites on the P450 for substrate (see Section 2). The curve fitted to the data for 25(OH)D3 using this equation (Fig. 6A) gave a K_i value approximately $4K_m$ (Table 2). K_m and k_{cat} values were not significantly different from those provided by the hyperbolic curve fit (Table 2) due to the large standard errors accompanying the substrate inhibition model. The substrate inhibition model fitted the data for 25(OH)D2 very well (Fig. 6B) giving K_m and k_{cat} values close to those determined from the hyperbolic curve fit, and a high K_i relative to K_m (Table 2).

We also determined the kinetics of metabolism of two other hydroxyvitamin D compounds by CYP27B1. 24*R*,25-dihydroxyvitamin D3 ($24,25(OH)_2D3$) was metabolized to 1α,24*R*,25-trihydroxyvitamin D3 with a similar k_{cat} to that for 25(OH)D3, but with a K_m 4.4-fold higher and therefore a correspondingly lower k_{cat}/K_m. No substrate inhibition was observed at the maximum substrate concentration tested ($2K_m$). 20-Hydroxyvitamin D3 (20(OH)D3) is a product of CYP11A1 action on vitamin D3 [15,17,18,29] and is biologically active on

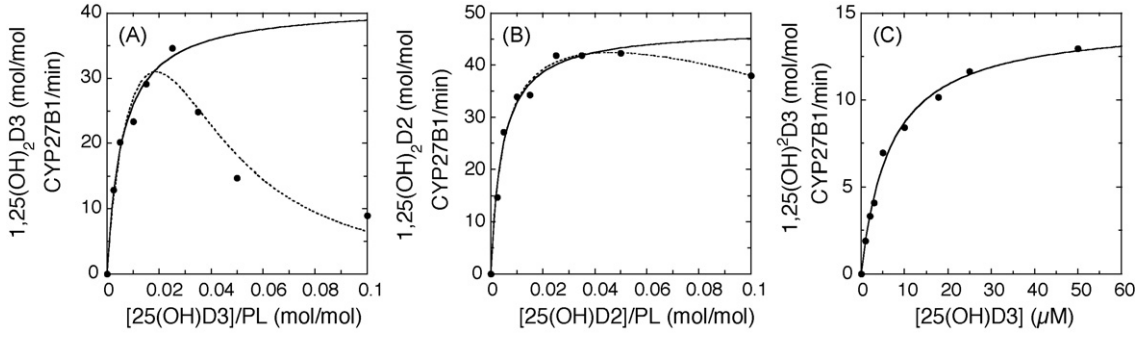

Fig. 6. The effect of substrate concentration on the rate of hydroxylation of 25(OH)D3 and 25(OH)D2 by CYP27B1 in phospholipid vesicles. Reaction rates were determined for 25(OH)D3 (A) and 25(OH)D2 (B) using substrates incorporated into small phospholipid vesicles prepared by sonication of dioleoyl phosphatidylcholine and cardiolipin, in a 2 min incubation as described in the Experimental Procedures. The activity of CYP27B1 was also measured for 25(OH)D3 in 0.45% cyclodextrin (C). Data for high substrate concentrations where inhibition was observed were excluded from the Michaelis–Menten fits (solid lines). A curve for a model describing substrate inhibition was also fitted to the data for vesicles as described in Section 2 (dashed lines).

Table 2
Kinetic parameters for metabolism of different substrates in phospholipid vesicles by CYP27B1.

Substrate	K_m (mmol/mol PL)	K_i (mmol/mol PL)	k_{cat} (min^{-1})	k_{cat}/K_m ($(mmol/mol\ PL)^{-1}\ min^{-1}$)
25(OH)D3[a]	5.9 ± 1.2		41 ± 3	6.9 ± 1.9
25(OH)D3[b]	9.7 ± 5.3	37 ± 10	55 ± 15	5.7 ± 4.6
25(OH)D2[a]	4.6 ± 0.8		48 ± 2	10.4 ± 2.2
25(OH)D2[b]	4.8 ± 0.7	204 ± 36	49 ± 2	10.2 ± 1.9
24,25$(OH)_2$D3[a]	26 ± 5		54 ± 5	2.1 ± 0.6
20(OH)D3[a]	49 ± 21		8.5 ± 1.7	0.17 ± 0.11

CYP27B1 activity was determined with substrates in small unilamellar phospholipid (PL) vesicles as described in Fig. 7. Data are mean ± SE of the curve fit for representative experiments.

[a] Kinetic parameters determined by Michaelis–Menten curve fit.

[b] Kinetic parameters determined by the kinetic model with substrate binding at two inhibitory sites.

Table 3
The effect of vesicle composition on the kinetic parameters for metabolism of 25(OH)D3.

Phospholipid	K_m (mmol/mol PL)	k_{cat} (min^{-1})	k_{cat}/K_m ($(mmol/mol\ PL)^{-1}\ min^{-1}$)
DOPC	8.2 ± 2.0	49 ± 4	5.9 ± 1.9
DOPC + cardiolipin	4.6 ± 1.4	41 ± 4	8.9 ± 3.6
DMPC	15.8 ± 2.1	11 ± 1	0.70 ± 0.16

Vesicles were prepared by sonication and kinetic parameters determined from Michaelis–Menten curve fits, excluding data above $4K_m$ where substrate inhibition was observed. DOPC, dioleoyl phosphatidylcholine. DMPC, dimyristoyl phosphatidylcholine. Cardiolipin, when present, was 15% of the total moles of phospholipid.

skin cells working as a partial receptor agonist for the vitamin D3 receptor [37,38]. It was metabolized by CYP27B1 to 1α,20-dihydroxyvitamin D3, identified from its identical retention time with authentic standard by HPLC. It displayed a K_m 8-fold higher than for 25(OH)D3 and a k_{cat} 5-fold lower giving it a 40-fold lower k_{cat}/K_m value (Table 2). No substrate inhibition was observed at the maximum substrate concentration tested ($2K_m$).

3.6. The effect of vesicle phospholipid composition on metabolism of 25(OH)D3 by CYP27B1

The value for k_{cat}/K_m for 25(OH)D3 in vesicles made from dioleoyl phosphatidylcholine was slightly lower than in vesicles comprising dioleoyl phosphatidylcholine plus 15% cardiolipin (Table 3). This was due to cardiolipin causing at 44% decrease in the K_m for 25(OH)D3 with little effect on k_{cat}. In contrast, vesicles prepared from dimyristoyl phosphatidylcholine gave a 12.7-fold lower k_{cat}/K_m for 25(OH)D3 metabolism than that seen with dioleoyl phosphatidylcholine and cardiolipin, caused by an increase in K_m and a decrease in k_{cat}. Substrate inhibition at high substrate concentrations (above $4K_m$) was observed for all three vesicle compositions analyzed.

3.7. Testing metabolism of vitamin D and 1α-hydroxyvitamin D by CYP27B1

Since both 1α-hydroxyvitamin D3 (1(OH)D3) and vitamin D3 itself have been reported to serve as substrates for CYP27B1 in a detergent reconstituted system [9], we tested the metabolism of these two substrates plus their vitamin D2 counterparts, in phospholipid vesicles. Incubations were carried out for 20 min with vesicles prepared from dioleoyl phosphatidylcholine and cardiolipin using a high concentration of CYP27B1 (0.2 μM). No metabolism of either vitamin D2 or D3 was observed (not shown). Both 1(OH)D2 and 1(OH)D3 were hydroxylated in the 25-position producing 1,25$(OH)_2$D2 and 1,25$(OH)_2$D3, respectively (Fig. 7). While a detailed kinetic analysis was not carried out, we measured the initial rate of hydroxylation of both 1(OH)D2 and 1(OH)D3 at molar ratios to phospholipid of 0.025 in a 2 min incubation. Rates were 0.73 and 0.28 mol/min/mol CYP27B1 for 1(OH)D3 and 1(OH)D2, respectively, both low compared to 1α-hydroxylation of 25(OH)D2 and 25(OH)D3.

4. Discussion

We obtained relatively low expression of mouse CYP27B1 compared to that reported by Uchida et al. [9] using a similar procedure. These workers reported a 10-fold increase in expression to levels as high as 300 nmol/L culture by co-expression with chaperones GroEL/ES but in our hands these chaperones only marginally increased CYP27B1 expression. Despite the low expression we were able to obtain a reasonably pure preparation of enzyme for catalytic studies from a combination of Ni affinity-, adrenodoxin affinity- and octyl Sepharose-chromatography. Mouse adrenodoxin was expressed in *E. coli*, purified and used for the first time as the electron donor in assays of mouse CYP27B1 activity.

Our study shows that mouse CYP27B1 efficiently utilizes substrates incorporated into the bilayer of phospholipid vesicles of a composition reflecting the inner mitochondrial membrane where the enzyme is located *in vivo* [39,40]. The k_{cat} of CYP27B1 for membrane-associated 25(OH)D3 in our study (41–55 min^{-1}) is 4–20-fold higher than previous reports of the k_{cat} for the purified enzyme measured in detergent micelles [8,9,41]. Higher CYP27B1 activity was seen with small phospholipid vesicles prepared by sonication than with large vesicles prepared by extrusion, as we have observed for CYP11A1 [42]. Vesicle size has previously been shown to affect the activity of CYP11A1 in a similar fashion and is suggested to be due to the high radius of curvature of small vesicles causing looser packing of lipids in the outer half of the bilayer [42]. Attempts to address whether purified CYP27B1 permanently or transiently interacts with the vesicle membrane were made using gel filtration and centrifugation to separate free cytochrome from vesicle-bound cytochrome, but proved unsuccessful because of the lability of the enzyme. Phospholipid composition was observed to strongly influence CYP27B1 activity with a lower k_{cat} and higher K_m for 25(OH)D3 being observed in vesicles made from phospholipids containing saturated fatty acid chains (dimyristoyl phosphatidylcholine) than those with mono-unsaturated chains (dioleoyl phosphatidylcholine). The presence of the mitochondrial phospholipid, cardiolipin, caused a decrease in the K_m for 25(OH)D3. Similarly, under comparable conditions cardiolipin has been shown to reduce the K_m for cholesterol of CYP11A1, another mitochondrial P450, by 2–3-fold, with no effect on k_{cat} [40,43].

This study is the first to compare the metabolism of 25(OH)D3 and 25(OH)D2 by purified CYP27B1. Results show that they are both metabolized with similar k_{cat} and K_m values with the major differ-

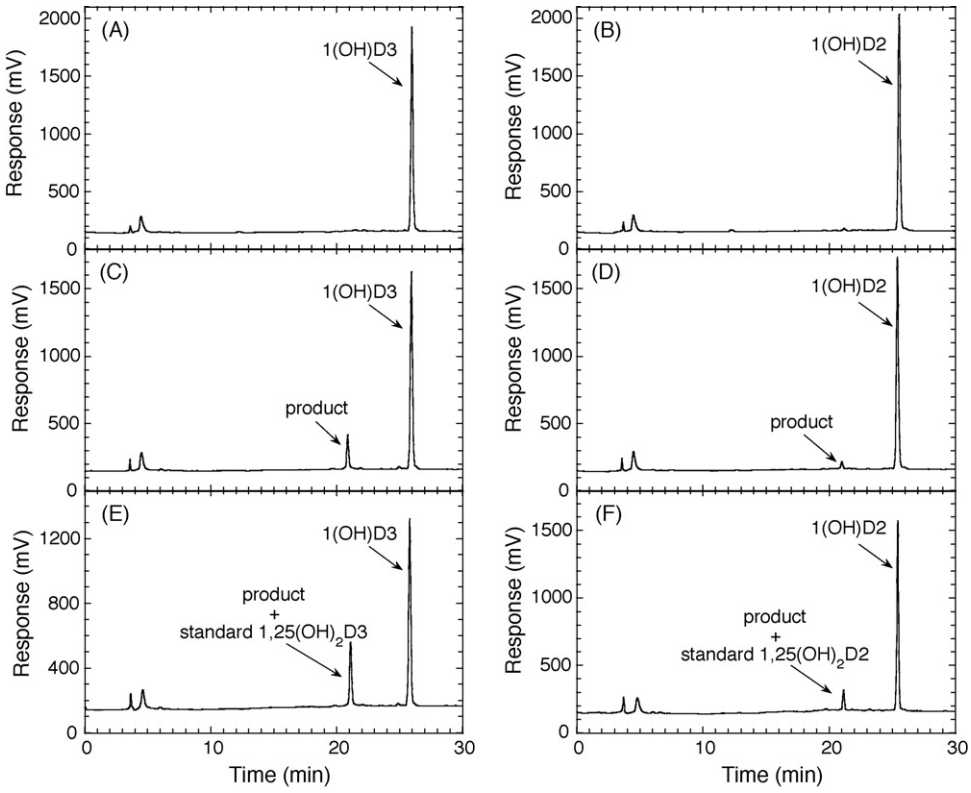

Fig. 7. Chromatograms showing metabolism of 1(OH)D3 and 1(OH)D2 by CYP27B1. Vesicles were prepared from dioleoyl phosphatidylcholine and cardiolipin with 1(OH)D3 (A, C, E) or 1(OH)D2 (B, D, F) present at ratio to phospholipid of 0.15 mol/mol. Vesicles were incubated with CYP27B1 (0.2 μM) for 20 min as in Fig. 4 and the products analyzed by HPLC. No adrenodoxin was present for control reactions (A, B). Test reactions contained adrenodoxin (C, D). The identity of products was confirmed by spiking test reaction extracts prior to HPLC with 0.3 nmol standard $1,25(OH)_2D3$ (E) or 0.09 nmol $1,25(OH)_2D2$ (F).

ence being that more pronounced substrate inhibition is observed for 25(OH)D3. Our data for the relative rates of 25(OH)D2 and 25(OH)D3 hydroxylation by CYP27B1 are consistent with early studies with kidney mitochondria from both the rat and chick, where comparable rates of metabolism of 25(OH)D2 and 25(OH)D3, presumably by CYP27B1, were reported [44]. $24,25(OH)_2D3$ is a metabolite of vitamin D3 found in the bloodstream resulting from the action of CYP24 on 25(OH)D3 and may have some direct biological activity on bone or cartilage [2]. Our study shows that $24,25(OH)_2D3$ in vesicles is metabolized with a similar k_{cat} to that for 25(OH)D3 but with a 4-fold higher K_m, and hence with a lower k_{cat}/K_m. Thus, at low substrate concentrations CYP27B1 will hydroxylate 25(OH)D2 and 25(OH)D3 in preference to $24,25(OH)_2D3$. In contrast, Sakaki et al. [23] reported that $24,25(OH)_2D3$ has a higher k_{cat}/K_m than 25(OH)D3 for expressed CYP27B1 in *E. coli* membranes. Presumably this difference is due to the different reconstituted systems used between the two studies. In the vesicle system, the presence of a hydroxyl group at either C20 or C24 markedly reduces the catalytic efficiency of 1α-hydroxylation suggesting that interaction of the side chain of the substrate with the enzyme plays an important role in positioning C1 near the heme group for optimal hydroxylation at this position. However, the presence of a hydroxyl group at C20 or C24 does not change the position of hydroxylation from the 1α-position.

20(OH)D3 is the major product of CYP11A1 action on vitamin D3 [15,17,18,29] and is biologically active on skin and other cells acting as an inhibitor of proliferation and a promoter of differentiation [37,38]. It acts as a partial receptor agonist for the vitamin D receptor and unlike $1,25(OH)_2D3$, it only weakly stimulates the expression of the CYP24 gene and does not raise serum calcium levels in rats [37,38,45]. These properties make this compound of interest for the treatment of hyperproliferative disorders including cancer. This study shows that 20(OH)D3 is a poor substrate for CYP27B1 with a k_{cat}/K_m value only 2.5% of that for 25(OH)D3. Thus, this vitamin D derivative will compete poorly with $1,25(OH)_2D3$ for metabolism by CYP27B1 and is likely to undergo 1α-hydroxylation very slowly *in vivo*. It is interesting to note that the product, $1,20(OH)_2D3$ does show some calcemic activity, although less than $1,25(OH)_2D3$ [45].

The current study on CYP27B1 was done on the mouse enzyme because of the reported difficulties in expressing and solubilizing human P450scc [46,47]. Human CYP27B1 has been expressed in *E. coli* and its activity measured in *E. coli* membranes, but it has never been successfully extracted or purified [47]. Preliminary studies in our laboratory confirm this difficulty of solubilizing the expressed human enzyme in active form for catalytic studies.

We have previously shown that there is a species difference in the ability of adrenodoxin to support the activity of human CYP11A1, with human adrenodoxin producing higher activity than bovine adrenodoxin [43]. The current investigation shows that CYP27B1 activity is not influenced by the adrenodoxin species, with mouse and bovine adrenodoxin being about equally effective in supporting 1α-hydroxylase activity of mouse CYP27B1. Our K_m for mouse adrenodoxin with mouse CYP27B1 is 4-fold higher than the value we observed for bovine adrenodoxin with bovine CYP11A1 under comparable conditions [21]. In tissues expressing a relatively high level of CYP11A1 such as the adrenal cortex, corpus luteum or placenta, adrenodoxin levels are high and are believed to be saturating, or at least near-saturating, for CYP11A1 activity [10,48–50]. The relatively high K_m of mouse CYP27B1 for adrenodoxin and the low concentrations of adrenodoxin in kidney mitochondria [51] suggest that adrenodoxin concentration may limit CYP27B1 activity *in vivo*. In support of this, Eto et al. [52] have shown that the activity

of CYP27B1 in rat kidney mitochondria is greatly increased when the mitochondria are solubilized with detergent and supplemented with exogenous adrenodoxin reductase and adrenodoxin.

Uchida et al. [9] reported that CYP27B1 in detergent micelles can hydroxylate vitamin D3 to metabolites identified as 1(OH)D3 and 1,25(OH)$_2$D3 based on HPLC retention times. They also reported that the CYP27B1 can hydroxylate 1(OH)D3 in the 25-position. Thus, they proposed that CYP27B1 alone can convert vitamin D3 to the hormonally active form, 1,25(OH)$_2$D3. We could not detect any metabolism of either vitamin D3 or D2 in vesicles by CYP27B1. Since we used a high CYP27B1 concentration, we can exclude any metabolism at a rate above our minimum detection level of approximately 0.03 mol/min/mol of CYP27B1 or 0.075% of the k_{cat} seen with 25(OH)D3. In agreement with Uchida et al. [9], we observed that CYP27B1 can slowly hydroxylate 1(OH)D3 in the 25-position and we have further shown that this reaction can also occur with 1(OH)D2. Given that we could not detect 1α-hydroxylation of vitamin D3 by CYP27B1 and that 1(OH)D3 has not been detected as a circulating form of vitamin D3 [9], the physiological relevance for this 25-hydroxylation of 1(OH)D3 is questionable.

The mechanism by which high concentrations of 25(OH)D3, and to a lesser degree 25(OH)D2, cause substrate inhibition is unclear. It is unlikely that it is a non-specific mechanism, such as perturbation of the phospholipid membrane, since the structurally similar 25(OH)D3 and 25(OH)D2 display markedly different potencies for inhibition, with K_i values of 37 and 204 mmol/mol phospholipid, respectively. Furthermore, 20(OH)D3 did not cause any inhibition at a molar ratio to phospholipid of 0.1, where strong inhibition was seen with 25(OH)D3. Substrate inhibition of microsomal drug-metabolizing P450s is well established, and in some cases is caused by two substrates occupying the active site, with one being inhibitory [34–36]. One-inhibitor site models gave a poor fit to our experimental data. The best fit was given by a two-inhibitory site model with each site being filled simultaneously with equal K_i values. Some involvement of the phospholipid bilayer in the mechanism is apparent since substrate inhibition was not seen when the 25(OH)D3 was dissolved in cyclodextrin. No substrate inhibition has been reported for 25(OH)D3 in detergent micelles [9,41,46,53], however, it is unclear whether substrate concentrations as high as the required 6K_m were tested in these studies.

The concentrations of 25(OH)D3 and 25(OH)D2 in the inner mitochondrial membrane of kidney mitochondria (and other mitochondria that contain CYP27B1) are unknown, but are likely to be low. The low K_m that CYP27B1 displays for these substrates in the membrane is compatible with the enzyme being active with low substrate concentrations. As 25(OH)D3 concentrations rise to near-saturating levels, the resulting substrate inhibition may provide a mechanism to ensure excessive amounts of the hormonally active 1,25(OH)$_2$D3 are not generated. This mechanism may be important for the short-term regulation of 1,25(OH)$_2$D3 levels, providing a more rapid response than the well characterized transcriptional regulation of the CYP27B1 and CYP24 genes [2].

Acknowledgements

This work was supported by the University of Western Australia Research Grants Scheme. We thank Prof. Paul Attwood for help in deriving kinetic equations to describe substrate inhibition.

References

[1] M.F. Holick, Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis, Am. J. Clin. Nutr. 79 (3) (2004) 362–371.
[2] D.E. Prosser, G. Jones, Enzymes involved in the activation and inactivation of vitamin D, Trends Biochem. Sci. 29 (12) (2004) 664–673.
[3] D. Bikle, Nonclassic actions of vitamin D, J. Clin. Endocrinol. Metab. 94 (1) (2009) 26–34.
[4] R. Bouillon, G. Eelen, L. Verlinden, C. Mathieu, G. Carmeliet, A. Verstuyf, Vitamin D and cancer, J. Steroid Biochem. Mol. Biol. 102 (1–5) (2006) 156–162.
[5] D.D. Bikle, M.K. Nemanic, E. Gee, P. Elias, 1,25-Dihydroxyvitamin D$_3$ production by human keratinocytes, J. Clin. Invest 78 (2) (1986) 557–566.
[6] M.F. Holick, Vitamin D: a millennium perspective, J. Cell Biochem. 88 (2) (2003) 296–307.
[7] A. Hiwatashi, Y. Nishii, Y. Ichikawa, Purification of cytochrome P-450$_{D1a}$ (25-hydroxyvitamin D$_3$-1α-hydroxylase) of bovine kidney mitochondria, Biochem. Biophys. Res. Commun. 105 (1) (1982) 320–327.
[8] Y. Nakamura, T. Eto, T. Taniguchi, K. Miyamoto, J. Nagatomo, H. Shiotsuki, H. Sueta, S. Higashi, K. Okuda, T. Setoguchi, Purification and characterization of 25-hydroxyvitamin D$_3$ 1α-hydroxylase from rat kidney mitochondria, FEBS Lett. 419 (1) (1997) 45–48.
[9] E. Uchida, N. Kagawa, T. Sakaki, N. Urushino, N. Sawada, M. Kamakura, M. Ohta, S. Kato, K. Inouye, Purification and characterization of mouse CYP27B1 overproduced by an *Escherichia coli* system coexpressing molecular chaperonins GroEL/ES, Biochem. Biophys. Res. Commun. 323 (2) (2004) 505–511.
[10] R.C. Tuckey, Progesterone synthesis by the human placenta, Placenta 26 (4) (2005) 273–281.
[11] M.J. Headlam, M.C.J. Wilce, R.C. Tuckey, The F-G loop region of cytochrome P450scc (CYP11A1) interacts with the phospholipid membrane, Biochim. Biophys. Acta Biomembr. 1617 (1–2) (2003) 96–108.
[12] I.A. Pikuleva, Cholesterol-metabolizing cytochromes P450, Drug Metab. Dispos. 34 (4) (2006) 513–520.
[13] I.A. Pikuleva, N. Mast, W. Liao, I.V. Turko, Studies of membrane topology of mitochondrial cholesterol hydroxylases CYPs 27A1 and 11A1, Lipids 43 (12) (2008) 1127–1132.
[14] K. Storbeck, P. Swart, A.C. Swart, Cytochrome P450 side-chain cleavage: insights gained from homology modeling, Mol. Cell Endocrinol. 265–266 (2007) 65–70.
[15] O. Guryev, R.A. Carvalho, S. Usanov, A. Gilep, R.W. Estabrook, A pathway for the metabolism of vitamin D3: unique hydroxylated metabolites formed during catalysis with cytochrome P450scc (CYP11A1), Proc. Natl. Acad. Sci. U.S.A. 100 (25) (2003) 14754–14759.
[16] M.N. Nguyen, A. Slominski, W. Li, Y.R. Ng, R.C. Tuckey, Metabolism of vitamin D2 to 17,20,24-trihydroxyvitamin D2 by cytochrome P450scc (CYP11A1), Drug Metab. Dispos. 37 (4) (2009) 761–767.
[17] A. Slominski, I. Semak, J. Zjawiony, J. Wortsman, W. Li, A. Szczesniewski, R.-C. Tuckey, The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism, FEBS J. 272 (16) (2005) 4080–4090.
[18] R.C. Tuckey, W. Li, J.K. Zjawiony, M.A. Zmijewski, M.N. Nguyen, T. Sweatman, D. Miller, A. Slominski, Pathways and products for the metabolism of vitamin D3 by cytochrome P450scc (CYP11A1), FEBS J. 275 (10) (2008) 2585–2596.
[19] J.D. Lambeth, S.E. Kitchen, A.A. Farooqui, R. Tuckey, H. Kamin, Cytochrome P-450scc-substrate interactions. Studies of binding and catalytic activity using hydroxycholesterols, J. Biol. Chem. 257 (4) (1982) 1876–1884.
[20] K. Merz, B. Sternberg, Incorporation of vitamin D$_3$-derivatives into liposomes of different lipid types, J. Drug Target. 2 (5) (1994) 411–417.
[21] R.C. Tuckey, P.M. Stevenson, Properties of ferredoxin reductase and ferredoxin from the bovine corpus luteum, Int. J. Biochem. 16 (5) (1984) 489–495.
[22] K. Takeyama, S. Kitanaka, T. Sato, M. Kobori, J. Yanagisawa, S. Kato, 25-Hydroxyvitamin D$_3$ 1α-hydroxylase and vitamin D synthesis, Science 277 (5333) (1997) 1827–1830.
[23] T. Sakaki, N. Sawada, K.I. Takeyama, S. Kato, K. Inouye, Enzymatic properties of mouse 25-hydroxyvitamin D3 1α-hydroxylase expressed in *Escherichia coli*, Eur. J. Biochem. 259 (3) (1999) 731–738.
[24] A. Wada, M.R. Waterman, Identification by site-directed mutagenesis of two lysine residues in cholesterol side chain cleavage cytochrome P450 that are essential for adrenodoxin binding, J. Biol. Chem. 267 (32) (1992) 22877–22882.
[25] M. Stromstedt, M.R. Waterman, A full-length cDNA encoding mouse adrenodoxin, Biochim. Biophys. Acta 1261 (1) (1995) 126–128.
[26] S.T. Woods, J. Sadleir, T. Downs, T. Triantopoulos, M.J. Headlam, R.C. Tuckey, Expression of catalytically active human cytochrome P450scc in *Escherichia coli* and mutagenesis of isoleucine-462, Arch. Biochem. Biophys. 353 (1) (1998) 109–115.
[27] J.D. Lambeth, H. Kamin, D.W. Seybert, Phosphatidylcholine vesicle reconstituted P-450scc. Role of the membrane in control of activity and spin state of the cytochrome, J. Biol. Chem. 255 (17) (1980) 8282–8288.
[28] R.C. Tuckey, Z. Janjetovic, W. Li, M.N. Nguyen, M.A. Zmijewski, J.K. Zjawiony, A. Slominski, Metabolism of 1α-hydroxyvitamin D3 by cytochrome P450scc to biologically active 1α,20-dihydroxyvitamin D3, J. Steroid Biochem. Mol. Biol. 112 (4–5) (2008) 213–219.
[29] R.C. Tuckey, M.N. Nguyen, A. Slominski, Kinetics of vitamin D3 metabolism by cytochrome P450scc (CYP11A1) in phospholipid vesicles and cyclodextrin, Int. J. Biochem. Cell Biol. 40 (11) (2008) 2619–2626.
[30] T. Omura, R. Sato, The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, J. Biol. Chem. 239 (7) (1964) 2370–2378.
[31] R.C. Tuckey, J.F. Holland, Comparison of pregnenolone synthesis by cytochrome P-450scc in mitochondria from porcine corpora lutea and granulosa cells of follicles, J. Biol. Chem. 264 (10) (1989) 5704–5709.
[32] M.A. Pesce, C.S. Strande, A new micromethod for determination of protein in cerebrospinal fluid and urine, Clin. Chem. 19 (11) (1973) 1265–1267.
[33] I. Hanukoglu, C.R. Jefcoate, Mitochondrial cytochrome P-450$_{scc}$. Mechanism of electron transport by adrenodoxin, J. Biol. Chem. 255 (7) (1980) 2057–3061.
[34] K.R. Korzekwa, N. Krishnamachary, M. Shou, A. Ogai, R.A. Parise, A.E. Rettie, F.J. Gonzalez, T.S. Tracy, Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simulta-

neously bind to cytochrome P450 active sites, Biochemistry 37 (12) (1998) 4137–4147.
[35] Y. Lin, P. Lu, C. Tang, Q. Mei, G. Sandig, A.D. Rodrigues, T.H. Rushmore, M. Shou, Substrate inhibition kinetics for cytochrome P450-catalysed reactions, Drug Metab. Dispos. 29 (4) (2001) 368–374.
[36] S. Zhou, Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4, Curr. Drug Metab. 9 (4) (2008) 310–322.
[37] Z. Janjetovic, M.A. Zmijewski, R.C. Tuckey, D.A. DeLeon, M.N. Nguyen, L.M. Pfeffer, A.T. Slominski, 20-Hydroxycholecalciferol, product of vitamin D3 hydroxylation by P450scc, decreases NF-κB activity by increasing IκBα levels in human keratinocytes, PLoS ONE 4 (6) (2009) e5988, doi:10.1371/journal.pone.0005988.
[38] B. Zbytek, Z. Janjetovic, R.C. Tuckey, M.A. Zmijewski, T.W. Sweatman, E. Jones, M.N. Nguyen, A.T. Slominski, 20-hydroxyvitamin D3, a product of vitamin D3 hydroxylation by cytochrome P450scc, stimulates keratinocytes differentiation, J. Invest. Dermatol. 128 (2008) 2271–2280.
[39] N.S. Cunningham, B.S. Lee, H.L. Henry, The renal mitochondrial metabolism of 25-hydroxyvitamin D-3: a possible role for phospholipids, Biochim. Biophys. Acta 881 (1) (1986) 480–488, 64.
[40] R.C. Tuckey, P.M. Stevenson, Purification and analysis of phospholipids in the inner mitochondrial membrane fraction of the bovine corpus luteum, and properties of cytochrome P-450scc incorporated into vesicles prepared from these phospholipids, Eur. J. Biochem. 148 (2) (1985) 379–384.
[41] N. Urushino, K. Yamamoto, N. Kagawa, S. Ikushiro, M. Kamakura, S. Yamada, S. Kato, K. Inouye, T. Sakaki, Interaction between mitochondrial CYP27B1 and adrenodoxin: role of arginine 458 of mouse CYP27B1, Biochemistry 45 (14) (2006) 4405–4412.
[42] R.C. Tuckey, H. Kamin, Kinetics of the incorporation of adrenal cytochrome P-450scc into phosphatidylcholine vesicles, J. Biol. Chem. 257 (6) (1982) 2887–2893.
[43] P. Kisselev, R.C. Tuckey, S. Woods, T. Triantopolous, D. Schwarz, Enzymatic properties of vesicle-reconstituted human cytochrome P450scc (CYP11A1). Differences in functioning of the mitochondrial electron transfer chain using human and bovine adrenodoxin and activation by cardiolipin, Eur. J. Biochem. 260 (3) (1999) 768–773.
[44] G. Jones, H. Schnoes, H.F. DeLuca, Isolation and identification of 1,25-dihydroxyvitamin D_2, Biochemistry 14 (6) (1975) 1250–1256.
[45] A.T. Slominski, Z. Janjetovic, B. Fuller, M.A. Zmijewski, R.C. Tuckey, M.N. Nguyen, T. Sweatman, W. Li, J. Zjawiony, D. Miller, T.C. Chen, G. Lozanski, M.F. Holick, Products of vitamin D3 or 7-dehydrocholesterol metabolism by cytochrome P450scc show anti-leukemia effects, having low or absent calcemic activity, PloS ONE (2010), in press.
[46] K. Yamamoto, E. Uchida, N. Urushino, T. Sakaki, N. Kagawa, N. Sawada, M. Kamakura, S. Kato, K. Inouye, S. Yamada, Identification of the amino acid residue of CYP27B1 responsible for binding of 25-hydroxyvitamin D_3 whose mutation causes vitamin D-dependent rickets type 1, J. Biol. Chem. 280 (34) (2005) 30511–30516.
[47] N. Sawada, T. Sakaki, S. Kitanaka, K. Takeyama, S. Kato, K. Inouye, Enzymatic properties of human 25-hydroxyvitamin D_3 1α-hydroxylase. Coexpression with adrenodoxin and NADPH-adrenodoxin resuctase in *Escherichia coli*, Eur. J. Biochem. 265 (1999) 950–956.
[48] I. Hanukoglu, Z. Hanukoglu, Stoichiometry of mitochondrial cytochromes P-450, adrenodoxin and adrenodoxin reductase in adrenal cortex and corpus luteum. Implications for membrane organization and gene regulation, Eur. J. Biochem. 157 (1) (1986) 27–31.
[49] R.C. Tuckey, Z. Kostadinovic, P.M. Stevenson, Ferredoxin and cytochrome P-450scc concentrations in granulosa cells of porcine ovaries during follicular cell growth and luteinisation, J. Steroid Biochem. 31 (2) (1988) 201–205.
[50] R.C. Tuckey, S.T. Woods, M. Tajbakhsh, Electron transfer to cytochrome P-450scc limits cholesterol side-chain-cleavage activity in the human placenta, Eur. J. Biochem. 244 (3) (1997) 835–839.
[51] N. Maruya, A. Hiwatashi, Y. Ichikawa, T. Yamano, Purification and characterization of renal ferredoxin form bovine renal mitochondria, J. Biochem. 93 (5) (1983) 1239–1247.
[52] T.A. Eto, Y. Nakamura, T. Taniguchi, K. Miyamoto, J. Nagatomo, Y. Maeda, S. Higashi, K. Okuda, T. Setoguchi, Assay of 25-hydroxyvitamin D_3 1α-hydroxylase in rat kidney mitochondria, Anal. Biochem. 258 (1) (1998) 53–58.
[53] N. Urushino, S. Nakabayashi, M.A. Arai, A. Kittaka, T.C. Chen, K. Yamamoto, K. Hayashi, S. Kato, M. Ohta, M. Kamakura, S. Ikushiro, T. Sakaki, Kinetic studies of 25-hydroxy-19-nor-vitamin D_3 and 1a,25-dihydroxy-19-nor-vitamin D_3 hydroxylation by CYP27B1 and CYP24A1, Drug Metab. Dispos. 35 (9) (2007) 1482–1488.