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Metabolism of cholesterol, vitamin D3 and 20-hydroxyvitamin D3 incorporated into phospholipid vesicles by human CYP27A1

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Abstract

CYP27A1 is a mitochondrial cytochrome P450 which can hydroxylate vitamin D3 and cholesterol at carbons 25 and 26, respectively. The product of vitamin D3 metabolism, 25-hydroxyvitamin D3, is the precursor to the biologically active hormone, 1α,25-dihydroxyvitamin D3. CYP27A1 is attached to the inner mitochondrial membrane and substrates appear to reach the active site through the membrane phase. We have therefore examined the ability of bacterially expressed and purified CYP27A1 to metabolize substrates incorporated into phospholipid vesicles which resemble the inner mitochondrial membrane. We also examined the ability of CYP27A1 to metabolize 20-hydroxyvitamin D3 (20(OH)D3), a novel non-calcemic form of vitamin D derived from CYP11A1 action on vitamin D3 which has anti-proliferative activity on keratinocytes, leukemic and myeloid cells. CYP27A1 displayed high catalytic activity towards cholesterol with a turnover number (k_{cat}) of 9.8 min^{-1} and K_m of 0.49 mol/mol phospholipid (510 μM phospholipid). The K_m value of vitamin D3 was similar for that of cholesterol, but the k_{cat} was 4.5-fold lower. 20(OH)D3 was metabolized by CYP27A1 to two major products with a k_{cat}/K_m that was 2.5-fold higher than that for vitamin D3, suggesting that 20(OH)D3 could effectively compete with vitamin D3 for catalysis. NMR and mass spectrometric analyses revealed that the two major products were 20,25-dihydroxyvitamin D3 and 20,26-dihydroxyvitamin D3, in almost equal proportions. Thus the presence of the 20-hydroxyl group on the vitamin D3 side chain enables it to be metabolized more efficiently than vitamin D3, with carbon 26 in addition to carbon 25 becoming a major site of hydroxylation. Our study reports the highest k_{cat} for the 25-hydroxylation of vitamin D3 by any human cytochrome P450 suggesting that CYP27A1 might be an important contributor to the synthesis of 25-hydroxyvitamin D3, particularly in tissues where it is highly expressed.

Keywords

CYP27A1; vitamin D; cholesterol; cytochrome P450; phospholipid vesicles

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1. Introduction

CYP27A1 is a multifunctional enzyme involved in the initial activation of vitamin D3, producing 25-hydroxyvitamin D3 (25(OH)D3), as well as in the biosynthesis of acidic and neutral bile acids. In the acidic bile acid pathway, CYP27A1 is responsible for the rate limiting step of 26-hydroxylation of cholesterol forming 26-hydroxycholesterol. Furthermore it has the ability to subsequently hydroxylate carbon 26 several times to yield 3β-hydroxy-5-cholestenoic acid [1–3]. In the neutral bile acid pathway, CYP27A1 serves to hydroxylate bile acid intermediates, 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, to initiate side chain cleavage, forming cholic acid and chenodeoxycholic acid, respectively [4]. Although primarily expressed in the liver, CYP27A1 has also been detected in keratinocytes, dermal fibroblasts, osteoblasts, arterial endothelium, parathyroid gland, ovaries and duodenum, where it could play a role in the local synthesis of 25-hydroxyvitamin D3 [5–10].

Once formed, 25(OH)D3 is further activated by the mitochondrial 1α-hydroxylase (CYP27B1) to produce 1α,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3), the biologically active form of vitamin D3. 1,25$(OH)_2$D3 is essential for calcium and phosphorous homeostasis and thus skeletal integrity [11, 12]. In addition, 1,25$(OH)_2$D3 has tumorostatic and anti-carcinogenic properties, where it promotes differentiation in normal and transformed cells including melanoma, leukemia, prostate, breast, keratinocytes and hematopoietic cells [13, 14]. As a result 1,25$(OH)_2$D3 has the potential to treat hyperproliferative diseases such as psoriasis and cancer [14]. However supraphysiological doses of 1,25$(OH)_2$D3 are needed and this has limited its therapeutic use due to the resulting calcemic effect. As a result there is considerable interest in finding vitamin D analogs which retain the anti-proliferative property but are non-calcemic. One source of vitamin D analogs with these properties is from the metabolism of vitamin D by CYP11A1, with the major metabolite being 20-hydroxyvitamin D3 (20(OH)D3) [15–17]. This product as well as its sequential metabolites are biologically active exhibiting anti-proliferative and pro-differentiation effects on a range of cell lines including keratinocytes, leukemic and myeloid cells [18–20]. It also inhibits NF-κB activity [21] but shows no calcemic activity in rats at doses as high as 4 μg/kg [18]. Structurally similar 20(OH)D2 shows similar properties [22]. Thus 20(OH)D3 has the potential to be used as a therapeutic drug for the treatment of hyperproliferative and inflammatory disorders. The addition of a 1α-hydroxyl group to 20(OH)D3 by CYP27B1, produces 1,20-dihydroxyvitamin D3, which exhibits moderate calcemic activity when administered at comparable doses to 20(OH)D3 [18]. However, it remains to be determined if 20(OH)D3 can undergo 25-hydroxylation by CYP27A1 or other P450s, and whether these novel products have an altered biological activity.

CYP27A1 belongs to the mitochondrial Type I cytochrome P450 family, which receives its electrons from NADPH via adrenodoxin reductase and its redox partner adrenodoxin [23, 24]. CYP27A1 interacts with the matrix side of the inner mitochondrial membrane [25]. The F-G loop and the N-terminal part of the G helix have been identified as the sites of membrane attachment, similar to what has been reported for CYP24 and CYP11A1 [26–28]. As membrane bound P450s acquire their hydrophobic substrates such as vitamin D3 from the membrane phase of the phospholipid bilayer, it is important to characterize P450 activity in a membrane environment. Murtazina *et al.* [29] found that the activity of CYP27A1 was altered according to the presence of different phospholipid species, such as phosphatidylglycerol and phosphatidylethanolamine. However, these phospholipids are found predominantly in bacterial membranes and while they can influence the properties of the purified expressed enzyme, they are not representative of phospholipids of the inner mitochondrial membrane. Recently, unilamellar phospholipid vesicles have been used to characterize the kinetics of vitamin D metabolism by CYP11A1 and CYP27B1 [30–32].

This membrane system uses dioleoyl phosphatidylcholine and cardiolipin to closely mimic the composition of the inner mitochondrial membrane [33].

While CYP27A1 can metabolize a range of substrates including cholesterol, oxysterols and vitamin D, kinetic comparisons of the ability of CYP27A1 to metabolize different substrates are lacking. Even though one study did show that the activity of CYP27A1 towards cholesterol was about 4-fold higher than that for vitamin D3, the incubation conditions were not identical for both substrates and were not under initial rate conditions [34]. In the current study we address this deficiency by comparing the kinetic parameters for vitamin D3 and cholesterol metabolism in the phospholipid vesicle system. In addition, we describe the ability of CYP27A1 to hydroxylate the novel non-calcemic vitamin D3 analog, 20(OH)D3.

2. Materials and Methods

2.1. Materials

20(OH)D3 was enzymatically synthesized by the action of CYP11A1 on vitamin D3 and purified as described before [15]. Vitamin D3, 2-hydroxypropyl-β-cyclodextrin (cyclodextrin), NADPH, dioleoyl phosphatidylcholine, bovine heart cardiolipin and cholesterol were from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). The pGro7 plasmid was from Takara Bio Inc. (Shiga, Japan). The silica gel plates were from Alugram Sil G, Macherey-Nagel, Inc. (Easton, PA). The [4-^{14}C]cholesterol and emulsifier safe scintillant were from PerkinElmer Life Science (Boston, MA). 26-Hydroxycholesterol (25(*R*)-cholest-5-ene-3β,26-diol) was purchased from Research Plus Inc. (Barnegat, NJ).

2.2. Preparation of enzymes

Human adrenodoxin and adrenodoxin reductase were expressed in *Escherichia coli* with the coexpression of molecular chaperones, GroEL/ES, and purified as previously described [35, 36]. The cDNA sequence of human CYP27A1 used for expression was as reported by Cali *et al.* [37], with the addition of a C-terminal 6 His tag and the 5′ modifications as reported by Pikuleva *et al.* [2]. This construct was chemically synthesized by GenScript Corporation (Piscataway, NJ) and ligated into the expression vector, pTrc99A. *Escherichia coli* JM109 containing the pGro7 plasmid was transformed with the CYP27A1-pTrc99A construct. The cultivation and induction of bacteria, as well as the purification of the expressed CYP27A1 were carried out in a similar manner to that described for the expression of mouse CYP27B1 [30], except the detergent cholate was used instead of CHAPS. The expression level measured after nickel affinity chromatography was 126 nmol/L culture. After octyl Sepharose chromatography, the final preparation of expressed CYP27A1 was largely free from P420 and had a 414/280 absorbance ratio of 0.80.

2.3. Small scale incubations to measure CYP27A1 activity towards substrates incorporated in phospholipid vesicles

Phospholipid vesicles were prepared from dioleoyl phosphatidylcholine and bovine heart cardiolipin at a molar ratio of 85:15. Vitamin D3, cholesterol or 20(OH)D3 were added to the phospholipids as required (see Results) and the ethanol solvent removed under nitrogen. For incubations involving cholesterol, both [4-^{14}C]cholesterol (100 000 dpm) and unlabelled cholesterol were present. Buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 mM dithiothreitol and 0.1 mM EDTA was added to the dry lipid mixture and sonicated for 10 min in a bath-type sonicator [38]. Reactions were carried at a concentration of 510 μM phospholipid in the above buffer to which 15 μM human adrenodoxin, 0.5 μM human adrenodoxin reductase, 2 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase and 50 μM NADPH were added, similar to reactions described for CYP11A1 and CYP27B1 [15, 30, 31]. The purified CYP27A1 was preincubated with the

vesicles for 6 min at 37°C. Adrenodoxin was added last to initiate the reaction. For kinetic experiments, the incubations were typically 0.5 mL and were carried out over the initial linear period of the reaction (10 min for vitamin D3 and cholesterol and 30 min for 20(OH)D3). Ice-cold dichloromethane (3 mL) was added to stop the reactions and samples were then extracted as before [35] for HPLC analysis (see Section 2.5). The kinetic parameters were determined by fitting hyperbolic curves described by the Michaelis-Menten equation using Kaleidagraph 3.6, similar to what was described previously [30].

2.4. Small scale incubations to determine CYP27A1 activity towards substrates dissolved in cyclodextrin

Vitamin D3 and 20(OH)D3 stock solutions were prepared in 45% cyclodextrin by stirring in the dark for 2 days at room temperature [31, 39]. Incubations were carried out in a similar fashion to that described above for phospholipid vesicles, except that the vesicles were replaced with substrates in cyclodextrin with the final cyclodextrin concentration being 0.45% (w/v).

2.5. HPLC analysis of vitamin D3 metabolites

For the separation of vitamin D3 metabolites, HPLC was carried out using a Perkin-Elmer HPLC (Perkin-Elmer Life Sciences Inc., Walthan, MA, USA) equipped with a C18 column (GraceSmart, 15 cm × 4.6 mm, particle size 5 μm). Vitamin D3 metabolites were separated using a 75% to 100% methanol in water gradient for 10 min, followed by 100% methanol for 15 min, at a flow rate of 0.5 mL/min. The separation of 20(OH)D3 and its metabolites was carried out with a C18 column (Grace Alltima, 25 cm × 4.6 mm, particle size 5 μm) using a 44% to 58% acetonitrile in water gradient for 25 min followed by a 58% to 100% acetonitrile in water gradient for 15 min, and ending with 100% acetonitrile for 25 min, at a flow rate of 0.5 mL/min. All these vitamin D compounds were detected with the UV monitor set at 265 nm. The amounts of product formed following peak integration were calculated as before [31].

2.6. TLC analysis and liquid scintillation counting of cholesterol metabolites

The [4-^{14}C]cholesterol extracts were dissolved in 50 μL chloroform and applied to Alugram silica G gel plates (20 cm × 10 cm × 0.2 mm thick). Authentic standards of cholesterol and 26-hydroxycholesterol were also applied on either side of the plate. The plates were developed twice in hexane/acetone (7:3 v/v) with drying in between. To visualize the cholesterol standards, the section containing the standards was removed and sprayed with a solution of 2 mM $FeSO_4$ containing 5% concentrated sulphuric acid and 5% acetic acid, followed by charring to reveal their positions. This section of the plate was realigned with the remainder of the plate and the positions of the cholesterol and 26-hydroxycholesterol were marked. The plate was cut into areas of about 1.5 cm × 1 cm and each was placed in a scintillation vial. To each scintillation vial, 5 mL of Emulsifier safe scintillant was added and left to stand for 1 h before counting for 10 min or to an error of 2%.

2.7. Large scale preparation of CYP27A1-derived 20(OH)D3 metabolites for NMR analysis

Incubations of 20(OH)D3 with CYP27A1 were carried out with substrate dissolved in cyclodextrin in a similar manner to the small scale incubations, but in a scaled up version. A 20(OH)D3 stock solution in 4.5% cyclodextrin was added to the incubation mixture to give a final 20(OH)D3 concentration of 58 μM in 0.45% cyclodextrin. A 35 mL reaction mixture comprising expressed CYP27A1 (1.5 μM), adrenodoxin (15 μM), adrenodoxin reductase (0.5 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (2 U/mL) and NADPH (50 μM) was incubated at 37°C for 2 h in a shaking water bath. The reaction was stopped with 2 volumes of ice-cold dichloromethane and the vitamin D3 metabolites

extracted as before [35]. For the initial separation of 20(OH)D3 and its products, a C18 preparative column (Brownlee Aquapore, 25 cm × 10 mm, particle size 20 μm) was used with isocratic 80% methanol for 20 min followed by a 80–90% methanol in water gradient for 5 min, and ending with isocratic 90% methanol for 20 min, all at flow rate of 1.5 mL/min. The two major products (unseparated) were collected and subjected to further purification using a C18 Grace Alltima column as described above (Section 2.5).

2.8. NMR spectroscopy and mass spectrometry

NMR measurements were performed using an inverse triple-resonance 3 mm probe on a Varian Unity Inova 500 MHz spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). Samples were dissolved in CD_3OD and transferred to a 3 mm Shigemi NMR tube (Shigemi Inc., Allison Park, PA) or using a 1.7 mm cryogenic probe on a Bruker 600-MHz spectrometer (Bruker Biospin, Billerica, MA). Temperature was regulated at 22°C and was controlled with an accuracy of ± 0.1°C. Chemical shifts were referenced to residual solvent peaks for CD_3OD (3.31 ppm for proton and 49.15 ppm for carbon). Standard two-dimensional NMR experiments [(^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY, mixing time 80 millisecond), ^{1}H-^{13}C heteronuclear single quantum correlation spectroscopy (HSQC), and ^{1}H-^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC)] were acquired in order to fully elucidate the structures of the metabolites. All acquired NMR data were transferred to an offline PC computer and processed using ACD software version 12 (Advanced Chemistry Development, Toronto, ON, Canada), with zero-filling in the direct dimension and linear prediction in the indirect dimension.

Mass spectra were acquired in a Bruker Esquire-LC/MS system (Bruker Daltonics, Billerica, MA) utilizing the ionization source of electrospray ionization (ESI). Data were collected by Bruker EsquireControl and processed by ACD mass processor.

2.9. Other procedures

The concentration of expressed CYP27A1 was measured by reduced-CO minus reduced difference spectroscopy using an extinction coefficient of 91000 M^{-1} cm^{-1} for the absorbance difference between 450 and 490 nm [40]. The concentrations of vitamin D and other hydroxyvitamin D stock solutions were measured using an extinction coefficient of 18000 M^{-1} cm^{-1} for the absorbance at 263 nm [41].

3. Results

3.1. Metabolism of cholesterol and vitamin D3 incorporated in phospholipid vesicles

Phospholipid vesicles provide a means of mimicking the inner mitochondrial membrane environment of mitochondrial P450s. Both cholesterol and vitamin D3 partition exclusively into the bilayer of phospholipid vesicles prepared in aqueous buffer [31, 42]. 25(OH)D3 has also been shown to partition greater than 97% into phospholipid vesicles [30]. As expected, the major product of vitamin D3 metabolism was identified as 25(OH)D3 based upon its identical HPLC retention time to authentic 25(OH)D3, as well as identical R_f values by normal phase TLC (not shown). A minor product, representing 8% of the total product formed, was also detected with a retention time 30 s longer than 25(OH)D3. This is believed to be 26-hydroxyvitamin D3 based on work done by Sawada *et al.* [43]. Also as expected, 26-hydroxycholesterol was identified as the product of cholesterol metabolism by CYP27A1 based on its identical R_f value with an authentic standard. The time course for cholesterol hydroxylation was linear over the 20 min incubation period (Fig. 1). The time course for vitamin D3 metabolism was approximately linear for 120 min but based on high initial rates

seen in separate kinetic experiments a more appropriate fit was provided by a biphasic time course indicating a more rapid initial rate, as shown in Fig. 1.

CYP27A1 displayed similar K_m values for vitamin D3 and cholesterol in vesicles, 0.55 ± 0.11 and 0.49 ± 0.04 mol/mol phospholipid (510 μM phospholipid), respectively (Fig. 2). The k_{cat} value for cholesterol (9.84 ± 0.48 mol/min/mol CYP27A1) was 4.5-fold higher than that for vitamin D3 (2.09 ± 0.26 mol/min/mol CYP27A1). Thus the catalytic efficiency (k_{cat}/K_m) was highest for cholesterol at 20 ± 2.5 min^{-1}(mol/mol phospholipid)$^{-1}$ compared to 3.8 ± 1.2 min^{-1}(mol/mol phospholipid)$^{-1}$ for vitamin D3.

3.2. Metabolism of 20(OH)D3 by CYP27A1

As CYP27A1 is known to have reasonably broad substrate specificity, acting on cholesterol, bile acid intermediates, vitamin D3 and 1α-hydroxyvitamin D3 [3, 43–45], it was of interest to determine if it could metabolize the non-calcemic vitamin D analog, 20(OH)D3 [18]. At least six different products were observed when 20(OH)D3 was incorporated in phospholipid vesicles and incubated with CYP27A1 (Fig. 3). Similar metabolism was observed when the substrate was dissolved in cyclodextrin, as demonstrated by the time course (Fig. 4A). The two major products were produced in almost equal proportions and were labelled as Product A and Product B (these were subsequently shown by NMR to be 20,25-dihydroxyvitamin D3 and 20,26-dihydroxyvitamin D3, respectively, see Sections 3.4 and 3.5). The other major product, labelled as Product E, is likely to be a secondary product derived from subsequent metabolism of Products A and/or B, since it displayed a lag in its time course. Kinetic characterization of the metabolism of 20(OH)D3 by CYP27A1 was carried out with substrate dissolved in either cyclodextrin (Fig. 4B) or phospholipid vesicle (Fig. 4C). In cyclodextrin, the K_m for 20(OH)D3 was 33 ± 2.1 μM and the k_{cat} was 0.78 ± 0.02 min^{-1}. This compared to the K_m and k_{cat} values for vitamin D3 metabolism in cyclodextrin of 10.7 ± 3.1 μM and 1.7 ± 0.14 min^{-1}, respectively (Fig. 4B). For phospholipid vesicles, the k_{cat} for 20(OH)D3 was 0.755 ± 0.06 min^{-1}, similar to that observed in cyclodextrin, while the K_m was 0.078 ± 0.022 mol/mol phospholipid (510 μM phospholipid). Thus CYP27A1 displays a higher catalytic efficiency (k_{cat}/K_m) for 20(OH)D3 metabolism than for vitamin D3 metabolism in phospholipid vesicles but a lower efficiency in the cyclodextrin system.

3.3. Large scale synthesis of products of 20(OH)D3 metabolism

The cyclodextrin system was chosen to scale up the synthesis of 20(OH)D3 metabolites because of its ease of use and the ability of this system to hold a high concentration of substrate in solution (relative to its K_m). A 35 mL incubation of 58 μM 20(OH)D3 solubilized in 0.45% cyclodextrin was carried out using 1.5 μM CYP27A1 for 2 h. This resulted in 30% conversion of substrate to product. After HPLC purification, 145 nmol of Product A and 140 nmol of Product B were obtained for NMR structure determination.

3.4. Determination of product A as 20,25-dihydroxyvitamin D3

Analysis of product A by mass spectrometry showed that it was a dihydroxyvitamin D3 derivative. The observed molecular ion had a mass of 439.3 $[M + Na]^+$ giving a true mass of 416.3 (Fig. 5A). The site of hydroxylation of 20(OH)D3 was unambiguously assigned to be at the 25-position based on the NMR spectra for this metabolite. First, none of the four methyl groups (18, 21, 26, 27) are hydroxylated based on ^{1}H NMR (Fig. 5B). The doublet of 26/27-CH_3 in 20(OH)D3 became a singlet in the metabolite (^{1}H at 1.19 ppm, ^{13}C at 29.2 ppm, Fig. 5C, ^{1}H-^{13}C HSQC, projection), indicating the loss of scalar coupling from 25-CH. Second, ^{1}H-^{13}C HMBC showed correlation from 26/27-CH_3 (^{1}H at 1.19 ppm) to a carbon at 70.0 ppm (Fig. 5D), indicating that the hydroxylation must be at either 24-C or 25-C. As we have identified that that 26/27-CH_3 lost scalar coupling from 25-CH, the hydroxylation must

be at 25-C. Consistent with this assignment, the 26/27-CH_3 (^{1}H at 1.19 ppm) showed no correlation to any other protons based on ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY (Supplemental Fig. S-1C and S-1E), suggesting that 26/27-CH_3 was separated by a quaternary carbon (C25) and thus behaves as an independent spin system. From these analyses the structure of this metabolite was unambiguously established to be 20,25$(OH)_2$D3. The full assignments for this metabolite are summarized in Table 1 (for comparison, we also included the assignments for 20(OH)D3) and full spectra for all 1D/2D NMR are shown in the supplementary materials (Fig. S-1).

3.5. Determination of product B as 20,26-dihydroxyvitamin D3

Analysis of product B by mass spectrometry showed that it was also a dihydroxyvitamin D3 derivative. The observed molecular ion had a mass of 439.3 $[M + Na]^+$ giving a true mass of 416.3 (Fig. 6A). The site of hydroxylation of 20(OH)D3 was unambiguously assigned to be at the 26-position based on the NMR spectra for this metabolite. First, ^{1}H NMR (Fig. 6B) and ^{1}H-^{13}C HSQC revealed a new methylene group at 3.33/3.41 ppm, (^{13}C at 68.4 ppm, Fig. 6C inset). This methylene is in the same spin system as 26- or 27-CH_3 (^{1}H at 0.91 ppm) based on ^{1}H-^{1}H TOCSY (Fig. 6D), indicating that the hydroxylation occurred on the side chain. Second, one distinct feature for this metabolite is that only three methyl groups (18, 21, and one of 26/27) were observed (Fig. 6B and 6C), implying that the hydroxylation occurred on either 26 or 27-CH_3. Since 26- and 27-CH_3 are equivalent, we assigned this metabolite as 20,26$(OH)_2$D3. Consistent with this assignment, ^{1}H-^{13}C HMBC showed the expected correlation from 27-CH_3 (^{1}H at 0.91 ppm) to C26 (^{13}C at 68.4 ppm) (Fig. 6E). ^{1}H-^{1}H COSY also had the expected coupling from 26-CH_2 (^{1}H at 3.33/3.41 ppm) to 25-CH (^{1}H at 1.59 ppm, Fig. 6F). Thus, the structure of this metabolite was unambiguously determined as 20,26$(OH)_2$D3. The full assignments for this metabolite are summarized in Table 1 and full spectra for all 1D/2D NMR are shown in the supplementary materials (Fig. S-2).

4. Discussion

In this study we have shown that purified human CYP27A1 is catalytically active towards substrates that have been incorporated into phospholipid membranes. Kinetic analysis shows that vitamin D3 metabolism by CYP27A1 has a k_{cat} of 2.09 min^{-1}, which is 10-fold higher than what Sawada *et al.* [43] reported using bacterial membranes. Our study reports the highest k_{cat} for the 25-hydroxylation of vitamin D3 by any human cytochrome P450. Kinetic assays using membrane fractions containing CYP2R1 reported to a k_{cat} value that is 2-fold lower than our value for CYP27A1 [46]. In a more recent study, purified CYP2R1 displayed a k_{cat} value 4-fold lower than our value [47]. CYP2J2 has an even lower k_{cat} for 25-hydroxylation of vitamin D3 (0.087 min^{-1}) [48], with its primary substrate believed to be arachidonic acid, not vitamin D3. In contrast, rat CYP2J3 has a k_{cat} of 1.4 min^{-1} for the 25-hydroxylation of vitamin D3 which is 16-fold higher than its human homolog, CYP2J2 [48]. This suggests that there may be some species specificity as to which P450 enzyme metabolizes the majority of vitamin D3. Since mutations to human CYP2R1 cause rickets [49] this P450 has been implicated as the major enzyme in vitamin D3 metabolism. However, based on k_{cat} values CYP27A1 could be a major contributor, particularly in tissues with high relative expression of CYP27A1. Unfortunately it is not possible to compare the K_m values for 25-hydroxylation by CYP2R1 and CYP27A1 because of the different methods used to solubilize substrate. In the membrane environment used in the current study, CYP27A1 displays a similar K_m for vitamin D and its potentially competitive substrate, cholesterol.

Metabolism of cholesterol by CYP27A1 in a detergent (Tween 20) environment has been reported to have a k_{cat} that is 8-fold lower than that reported in this study [2]. The high k_{cat}

observed in this study for both vitamin D3 and cholesterol metabolism could be attributed to the membrane environment provided by the phospholipids, dioleoyl phosphatidylcholine and cardiolipin, which closely mimics the native inner mitochondrial membrane [33, 50]. This may give optimal access and orientation of substrates since the substrate access channel of mitochondrial P450s appears to sit within the hydrophobic domain of the membrane [26–28, 51]. The presence of the 20-hydroxyl group on the vitamin D3 side chain causes CYP27A1 substrate to display a lower K_m value for hydroxylation of this substrate in phospholipid vesicles compared to that for vitamin D3. The tendency for lower K_m values when hydroxyl groups are added to the vitamin D3 side chain has also been observed in the metabolism of these compounds by CYP11A1 [31] and may reflect increased hydrogen bonding.

As CYP27A1 has the ability to hydroxylate vitamin D3 at carbon 25 and cholesterol at carbon 26, it is not surprising that it is able to hydroxylate 20(OH)D3 at both positions, producing 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3 in approximately equal proportions. Presumably 20(OH)D3 sits in the active site of CYP27A1 with carbons 25 and 26 approximately equidistant from the heme iron. It is interesting to note that CYP11A1 can not metabolize 25(OH)D3 [16] so production of 20,25$(OH)_2$D3 can not proceed in the reverse order where CYP27A1 acts before 20-hydroxylation by CYP11A1. 20(OH)D3 is a non-calcemic form of vitamin D which can inhibit proliferation, stimulate differentiation as well as inhibit NF-κB activity in normal and cancer cells [18–22]. Consequently it has therapeutic potential for the treatment of hyperproliferative and inflammatory disorders [18–22]. The results of our study indicate that CYP27A1 could participate in the *in vivo* metabolism of this vitamin D analog, with the products, 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3, potentially being more active than the parent compound. 20,25$(OH)_2$D3, like 1,25$(OH)_2$D3, contains a hydroxyl group at carbon 25 which is known to participate in binding of 1,25$(OH)_2$D3 to the vitamin D receptor [52]. Interestingly it is the lack of the 1α-hydroxyl group in 20(OH)D3 that primarily conveys its non-calcemic activity as 1α-hydroxylation by CYP27B1 results in a product with moderate calcemic activity [18, 30]. The ability to scale up production of 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3 using CYP27A1 as a biological catalyst, as we have done to produce these compounds for NMR analysis, will enable us to test the biological activity of these novel compounds in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

20(OH)D3	20-hydroxyvitamin D3
25(OH)D3	25-hydroxyvitamin D3
cyclodextrin	2-hydroxypropyl-β-cyclodextrin
COSY	correlation spectroscopy
HMBC	heteronuclear multiple bond correlation spectroscopy
HSQC	heteronuclear single quantum correlation spectroscopy

TOCSY total correlation spectroscopy

References

1. Okuda KI. Liver mitochondrial P450 involved in cholesterol catabolism and vitamin D activation. J Lipid Res. 1994; 35(3):361–372. [PubMed: 8014573]
2. Pikuleva IA, Bjorkhem I, Waterman MR. Expression, purification, and enzymatic properties of recombinant human cytochrome P450c27 (CYP27). Arch Biochem Biophys. 1997; 343(1):123–130. [PubMed: 9210654]
3. Pikuleva IA, Babiker A, Waterman MR, Bjorkhem I. Activities of recombinant human cytochrome P450c27 (CYP27) which produce intermediates of alternative bile acid biosynthetic pathways. J Biol Chem. 1998; 273(29):18153–18160. [PubMed: 9660774]
4. Norlin M, Wikvall K. Enzymes in the conversion of cholesterol into bile acids. Curr Mol Med. 2007; 7(2):199–218. [PubMed: 17346171]
5. Ellfolk M, Norlin M, Gyllensten K, Wikvall K. Regulation of human vitamin D(3) 25-hydroxylases in dermal fibroblasts and prostate cancer LNCaP cells. Mol Pharmacol. 2009; 75(6):1392–1399. [PubMed: 19286836]
6. Gascon-Barre M, Demers C, Ghrab O, Theodoropoulos C, Lapointe R, Jones G, Valiquette L, Menard D. Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney. Clin Endocr. 2001; 54(1):107–115. [PubMed: 11167933]
7. Reiss AB, Martin KO, Rojer DE, Iyer S, Grossi EA, Galloway AC, Javitt NB. Sterol 27-hydroxylase: expression in human arterial endothelium. J Lipid Res. 1997; 38(6):1254–1260. [PubMed: 9215552]
8. Schuessler M, Astecker N, Herzig G, Vorisek G, Schuster I. Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D(3): CYP27 in epidermis completes the set of essential vitamin D(3)-hydroxylases. Steroids. 2001; 66(3–5):399–408. [PubMed: 11179749]
9. Bikle DD, Nemanic MK, Gee E, Elias P. 1,25-Dihydroxyvitamin D3 production by human keratinocytes. Kinetics and regulation. J Clin Invest. 1986; 78(2):557–566. [PubMed: 2426308]
10. Holick MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest. 2006; 116(8):2062–2072. [PubMed: 16886050]
11. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. Am J Physiol Renal Physiol. 2005; 289(1):F8–28. [PubMed: 15951480]
12. Holick MF. Vitamin D: the underappreciated D-lightful hormone that is important for skeletal and cellular health. Curr Opin Endocrinol Diabetes Obes. 2002; 91(1):87–98.
13. Holick MF. Vitamin D deficiency. New Engl J Med. 2007; 357(3):266–281. [PubMed: 17634462]
14. Masuda S. Promise of vitamin D analogues in the treatment of hyperproliferative conditions. Mol Cancer Ther. 2006; 5(4):797–808. [PubMed: 16648549]
15. Tuckey RC, Li W, Zjawiony JK, Zmijewski MA, Nguyen MN, Sweatman T, Miller D, Slominski A. Pathways and products for the metabolism of vitamin D3 by cytochrome P450scc. FEBS J. 2008; 275(10):2585–2596. [PubMed: 18410379]
16. Slominski A, Semak I, Zjawiony J, Wortsman J, Li W, Szczesniewski A, Tuckey RC. The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism. FEBS J. 2005; 272(16):4080–4090. [PubMed: 16098191]
17. Guryev O, Carvalho RA, Usanov S, Gilep A, Estabrook RW. 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. 2003; 100(25):14754–14759. [PubMed: 14657394]
18. Slominski AT, Janjetovic Z, Fuller BE, Zmijewski MA, Tuckey RC, Nguyen MN, Sweatman T, Li W, Zjawiony J, Miller D, Chen TC, Lozanski G, Holick MF. Products of vitamin D3 or 7-dehydrocholesterol metabolism by cytochrome P450scc show anti-leukemia effects, having low or absent calcemic activity. PLoS One. 2010; 5(3):e9907. [PubMed: 20360850]
19. Zbytek B, Janjetovic Z, Tuckey RC, Zmijewski MA, Sweatman TW, Jones E, Nguyen MN, Slominski AT. 20-hydroxyvitamin D3, a product of vitamin D3 hydroxylation by cytochrome

P450scc, stimulates keratinocyte differentiation. J Invest Dermatol. 2008; 128(9):2271–2280. [PubMed: 18368131]

20. Janjetovic Z, Tuckey RC, Nguyen MN, Thorpe EM Jr, Slominski AT. 20,23-dihydroxyvitamin D3, novel P450scc product, stimulates differentiation and inhibits proliferation and NF-kappaB activity in human keratinocytes. J Cell Physiol. 2009; 223(1):36–48. [PubMed: 20020487]
21. Janjetovic Z, Zmijewski MA, Tuckey RC, DeLeon DA, Nguyen MN, Pfeffer LM, Slominski AT. 20-Hydroxycholecalciferol, Product of Vitamin D3 Hydroxylation by P450scc, Decreases NF-kappa B Activity by Increasing I kappa B alpha Levels in Human Keratinocytes. PLoS One. 2009; 4(6):e5988. [PubMed: 19543524]
22. Slominski AT, Kim TK, Janjetovic Z, Tuckey RC, Bieniek R, Yue J, Li W, Chen J, Nguyen MN, Tang EKY, Miller D, Chen TC, Holick M. 20-Hydroxyvitamin D2 is a noncalcemic analog of vitamin D with potent antiproliferative and prodifferentiation activities in normal and malignant cells. Am J Physiol Cell Physiol. 2011; 300(3):C526–C541. [PubMed: 21160030]
23. Tuckey RC. Progesterone synthesis by the human placenta. Placenta. 2005; 26(4):273–281. [PubMed: 15823613]
24. Pikuleva IA, Cao C, Waterman MR. An additional electrostatic interaction between adrenodoxin and P450c27 (CYP27A1) results in tighter binding than between adrenodoxin and P450scc (CYP11A1). J Biol Chem. 1999; 274(4):2045–2052. [PubMed: 9890963]
25. Murtazina D, Puchkaev AV, Schein CH, Oezguen N, Braun W, Nanavati A, Pikuleva IA. Membrane-protein interactions contribute to efficient 27-hydroxylation of cholesterol by mitochondrial cytochrome P450 27A1. J Biol Chem. 2002; 277(40):37582–37589. [PubMed: 12124390]
26. Annalora AJ, Goodin DB, Hong WX, Zhang Q, Johnson EF, Stout CD. Crystal structure of CYP24A1, a mitochondrial cytochrome P450 involved in vitamin D metabolism. J Mol Biol. 2010; 396(2):441–451. [PubMed: 19961857]
27. Mast N, Annalora AJ, Lodowski DT, Palczewski K, Stout CD, Pikuleva IA. Structural basis for three-step sequential catalysis by the cholesterol side chain cleavage enzyme CYP11A1. J Biol Chem. 2011; 286(7):5607–5613. [PubMed: 21159775]
28. Strushkevich N, MacKenzie F, Cherkesova T, Grabovec I, Usanov S, Park HW. Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. Proc Natl Acad Sci U S A. 2011; 108(25):10139–10143. [PubMed: 21636783]
29. Murtazina DA, Andersson U, Hahn IS, Bjorkhem I, Ansari GAS, Pikuleva IA. Phospholipids modify substrate binding and enzyme activity of human cytochrome P450 27A1. J Lipid Res. 2004; 45(12):2345–2353. [PubMed: 15342675]
30. Tang EKY, Voo KJQ, Nguyen MN, Tuckey RC. Metabolism of substrates incorporated into phospholipid vesicles by mouse 25-hydroxyvitamin D3 1[alpha]-hydroxylase (CYP27B1). J Steroid Biochem Mol Biol. 2010; 119(3–5):171–179. [PubMed: 20193763]
31. Tuckey RC, Nguyen MN, Slominski A. Kinetics of vitamin D3 metabolism by cytochrome P450scc (CYP11A1) in phospholipid vesicles and cyclodextrin. Int J Biochem Cell Biol. 2008; 40(11):2619–2626. [PubMed: 18573681]
32. Tuckey RC, Janjetovic Z, Li W, Nguyen MN, Zmijewski MA, Zjawiony J, Slominski A. Metabolism of 1 alpha-hydroxyvitamin D3 by cytochrome P450scc to biologically active 1 alpha, 20-dihydroxyvitamin D3. J Steroid Biochem Mol Biol. 2008; 112(4–5):213–219. [PubMed: 19000766]
33. Tuckey RC, Stevenson PM. Purification and analysis of phospholipids in the inner mitochondrial membrane fraction of bovine corpus luteum, and properties of cytochrome P-450scc incorporated into vesicles prepared from these phospholipids. Eur J Biochem. 1985; 148(2):379–384. [PubMed: 3838724]
34. Gupta RP, Patrick K, Bell NH. Mutational analysis of CYP27A1: assessment of 27-hydroxylation of cholesterol and 25-hydroxylation of vitamin D. Metab-Clin Exp. 2007; 56(9):1248–1255. [PubMed: 17697869]
35. Tuckey RC, Li W, Shehabi HZ, Janjetovic Z, Nguyen MN, Kim TK, Chen J, Howell DE, Benson HA, Sweatman T, Baldisseri DM, Slominski A. Production of 22-hydroxy-metabolites of vitamin

D3 by cytochrome P450scc (CYP11A1) and analysis of their biological activities on skin cells. Drug Metab Dispos. 2011; 39(9):1577–1588. [PubMed: 21677063]

36. Woods ST, Sadleir J, Downs T, Triantopoulos T, Headlam MJ, Tuckey RC. Expression of catalytically active human cytochrome P450scc in Escherichia coli and mutagenesis of isoleucine-462. Arch Biochem Biophys. 1998; 353(1):109–115. [PubMed: 9578606]
37. Cali JJ, Russell DW. Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. J Biol Chem. 1991; 266(12):7774–7778. [PubMed: 1708392]
38. Tuckey RC, Kamin H. Kinetics of the incorporation of adrenal cytochrome P-450scc into phosphatidylcholine vesicles. J Biol Chem. 1982; 257(6):2887–2893. [PubMed: 7061453]
39. Decaprio J, Yun J, Javitt NB. Bile acid and sterol solubilization in 2-hydroxypropyl-beta-cyclodextrin. J Lipid Res. 1992; 33(3):441–443. [PubMed: 1569391]
40. Omura T, Sato R. The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. J Biol Chem. 1964; 239:2370–2378. [PubMed: 14209971]
41. Hiwatashi A, Nishii Y, Ichikawa Y. Purification of cytochrome P-450$_{D1\alpha}$ (25-hydroxyvitamin D3–1α-hydroxylase) of bovine kidney mitochondria. Biochem Biophys Res Commun. 1982; 105(1): 320–327. [PubMed: 6807301]
42. Merz K, Sternberg B. Incorporation of vitamin D3-derivatives in liposomes of different lipid types. J Drug Target. 1994; 2(5):411–417. [PubMed: 7704486]
43. Sawada N, Sakaki T, Ohta M, Inouye K. Metabolism of vitamin D3 by human CYP27A1. Biochem Biophys Res Commun. 2000; 273(3):977–984. [PubMed: 10891358]
44. Axen E, Postlind H, Sjoberg H, Wikvall K. Liver mitochondrial cytochrome-P450 CYP27 and recombinant-expressed human CYP27 catalyze 1-alpha-hydroxylation of 25-hydroxyvitamin D-3. Proc Natl Acad Sci U S A. 1994; 91(21):10014–10018. [PubMed: 7937829]
45. Pikuleva I, Javitt NB. Novel sterols synthesized via the CYP27A1 metabolic pathway. Arch Biochem Biophys. 2003; 420(1):35–39. [PubMed: 14622972]
46. Shinkyo R, Sakaki T, Kamakura M, Ohta M, Inouye K. Metabolism of vitamin D by human microsomal CYP2R1. Biochem Biophys Res Commun. 2004; 324(1):451–457. [PubMed: 15465040]
47. Strushkevich N, Usanov SA, Plotnikov AN, Jones G, Park HW. Structural Analysis of CYP2R1 in Complex with Vitamin D3. J Mol Biol. 2008; 380(1):95–106. [PubMed: 18511070]
48. Aiba I, Yamasaki T, Shinki T, Izumi S, Yamamoto K, Yamada S, Terato H, Ide H, Ohyama Y. Characterization of rat and human CYP2J enzymes as Vitamin D 25-hydroxylases. Steroids. 2006; 71(10):849–856. [PubMed: 16842832]
49. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci U S A. 2004; 101(20): 7711–7715. [PubMed: 15128933]
50. Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. Prog Lipid Res. 2000; 39(3):257–288. [PubMed: 10799718]
51. Headlam MJ, Wilce MC, Tuckey RC. The F-G loop region of cytochrome P450scc (CYP11A1) interacts with the phospholipid membrane. Biochim Biophys Acta. 2003; 1617(1–2):96–108. [PubMed: 14637024]
52. Collins ED, Bishop JE, Bula CM, Acevedo A, Okamura WH, Norman AW. Effect of 25-hydroxyl group orientation on biological activity and binding to the 1alpha,25-dihydroxy vitamin D3 receptor. J Steroid Biochem Mol Biol. 2005; 94(4):279–288. [PubMed: 15857747]

Highlights

- CYP27A1 metabolizes cholesterol and vitamin D3 in phospholipid vesicles
- Comparable K_m values observed for the metabolism of vitamin D3 and cholesterol
- CYP27A1 metabolizes CYP11A1-derived 20-hydroxyvitamin D3 to two major products
- Products identified as 20,25-dihydroxyvitamin D3 and 20,26-dihydroxyvitamin D3
- 20-Hydroxyvitamin D3 is metabolized more efficiently than vitamin D3

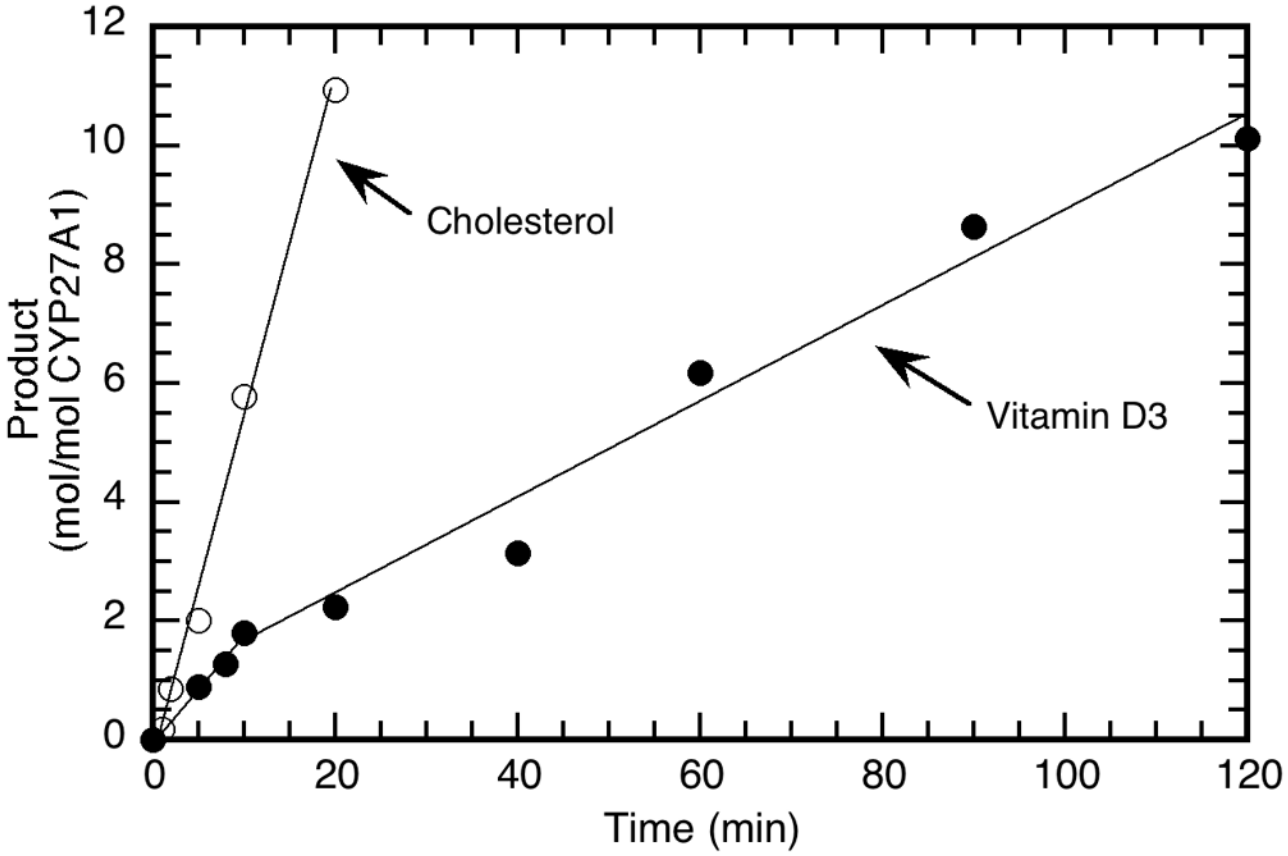


Fig. 1.

Time courses for the metabolism of cholesterol and vitamin D3 in phospholipid vesicles. Vesicles containing 0.1 mol [4-^{14}C]cholesterol/mol phospholipid (open circles) or 0.4 mol vitamin D3/mol phospholipid (closed circles) were incubated with 1 μM CYP27A1 in a reconstituted system containing 0.5 μM adrenodoxin reductase and 15 μM adrenodoxin. Products of [4-^{14}C]cholesterol metabolism were separated by TLC and quantitated by liquid scintillation counting (Section 2.6). Products of vitamin D3 metabolism were measured by reverse phase HPLC (Section 2.5).

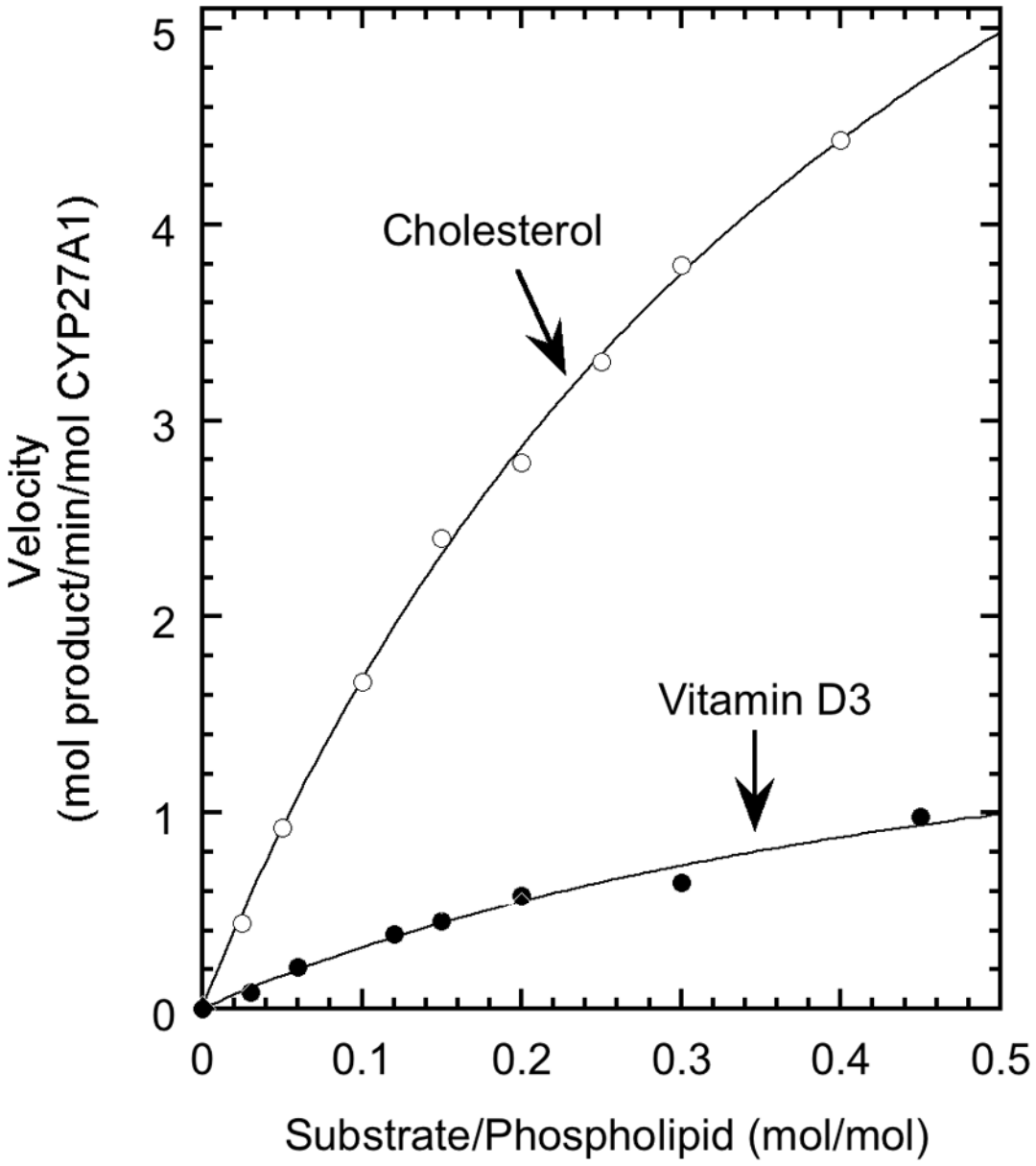


Fig. 2.

Michaelis-Menten plots for the metabolism of cholesterol and vitamin D3 in phospholipid vesicles by CYP27A1. Incubations were carried out with 0.2 μM CYP27A1 for 10 min. The hyperbolic curves were fitted using the Michaelis-Menten equation in the form: $v/[E] = (k_{cat} \times [S])/(K_m + [S])$ where v is reaction velocity, [E] is the CYP27A1 concentration, and [S] the substrate concentration . The correlation coefficients for the curve fits were 0.999 for cholesterol and 0.994 for vitamin D3.

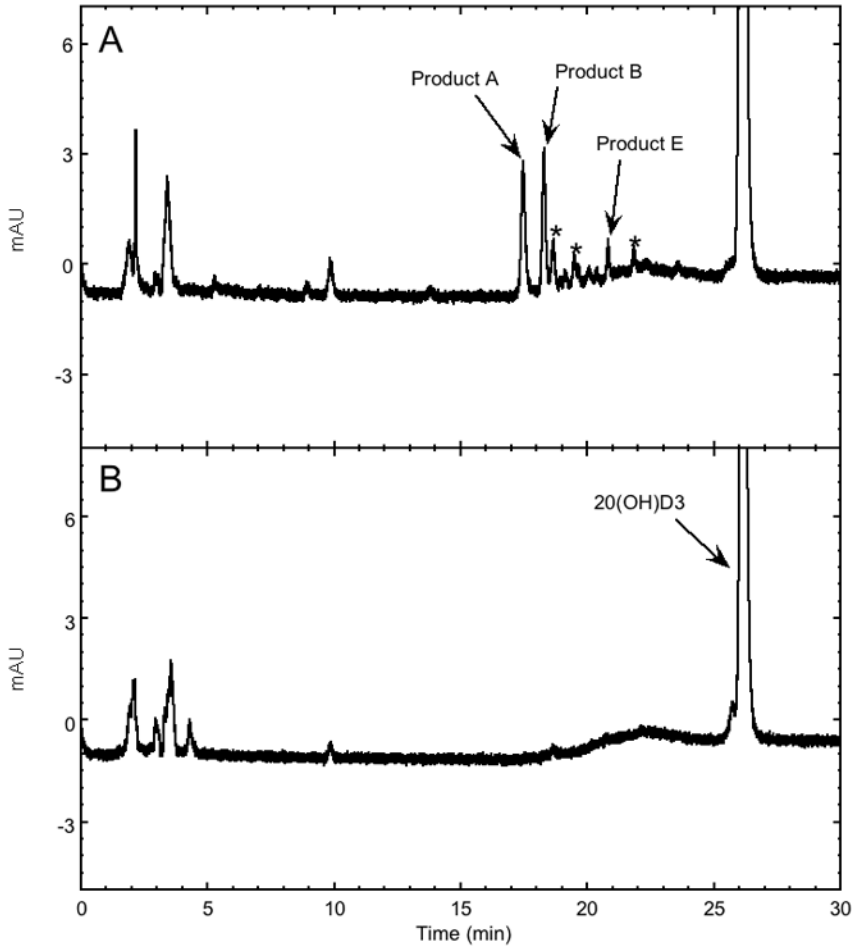


Fig. 3.

CYP27A1 metabolizes 20(OH)D3 to two major products. 20(OH)D3 was incorporated in phospholipid vesicles at a molar ratio of 0.025 mol/mol phospholipid and incubated with 0.2 μM CYP27A1 for 90 min. Samples were extracted with dichloromethane and analyzed by reverse phase HPLC as outlined in Section 2.5. (A) Test reaction showing major products A, B and E with asterisks indicating minor products; (B) control reaction with adrenodoxin omitted.

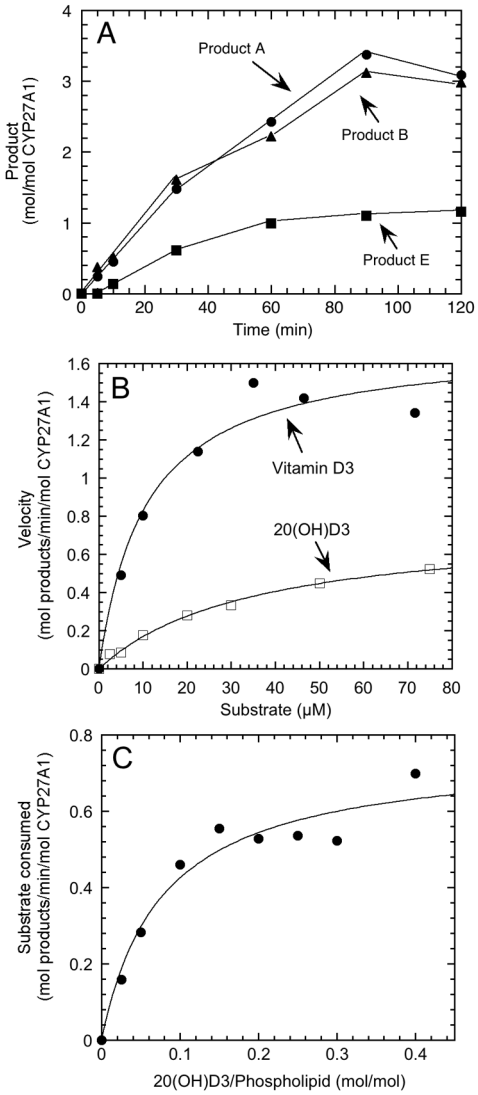


Fig. 4.

Metabolism of 20(OH)D3 in 0.45% cyclodextrin and phospholipid vesicles. (A) Time course showing the metabolism of 20(OH)D3 (56 μM) dissolved in cyclodextrin, by 1.5 μM CYP27A1 at 37°C. The products were analyzed by reverse phase HPLC (Section 2.5). (B) Michaelis-Menten plots for the metabolism of vitamin D3 and 20(OH)D3 in 0.45% cyclodextrin. Vitamin D3 and 20(OH)D3 were incubated with 0.4 μM CYP27A1 and 1 μM CYP27A1, respectively, for 10 and 30 min. The products were analyzed by reverse phase HPLC. The hyperbolic curves were fitted using the same equation as described in Fig. 2. The correlation coefficients for the curve fits were 0.983 for vitamin D3 and 0.999 for 20(OH)D3. (C) Michaelis-Menten plot for the metabolism of 20(OH)D3 in phospholipid vesicles incubated with 0.2 μM CYP27A1 for 30 min. The correlation coefficient for the curve fit was 0.978.

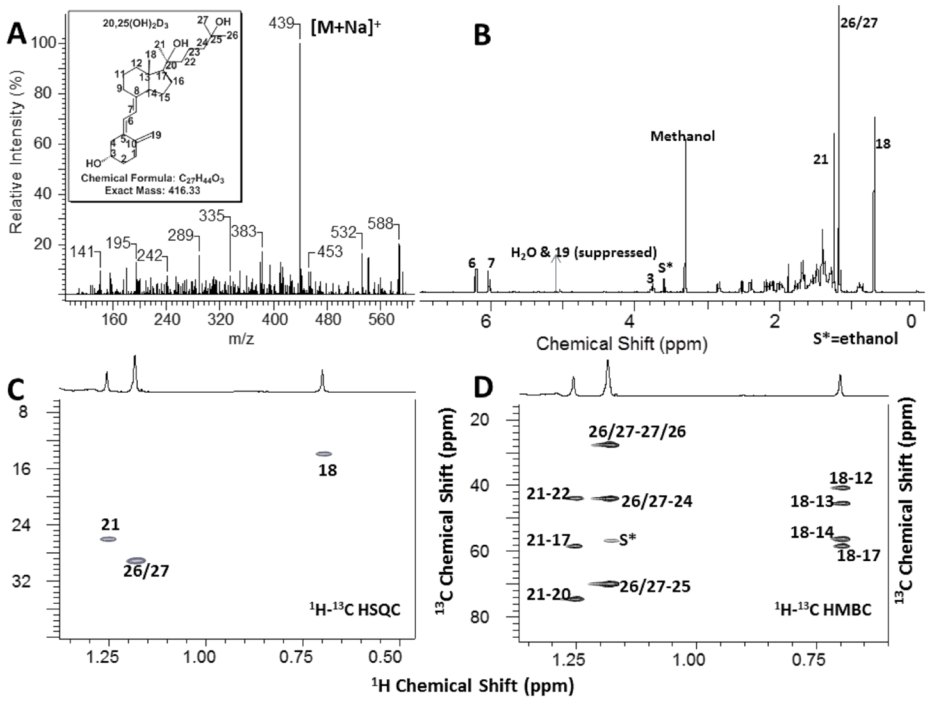


Fig. 5.

Mass and NMR spectra of 20,25(OH)$_2$D3. (A) Mass; (B) 1D Proton; (C) ^{1}H-^{13}C HSQC; (D) ^{1}H-^{13}C HMBC. Full spectra are shown in Supplemental Figures (Fig. S-1).

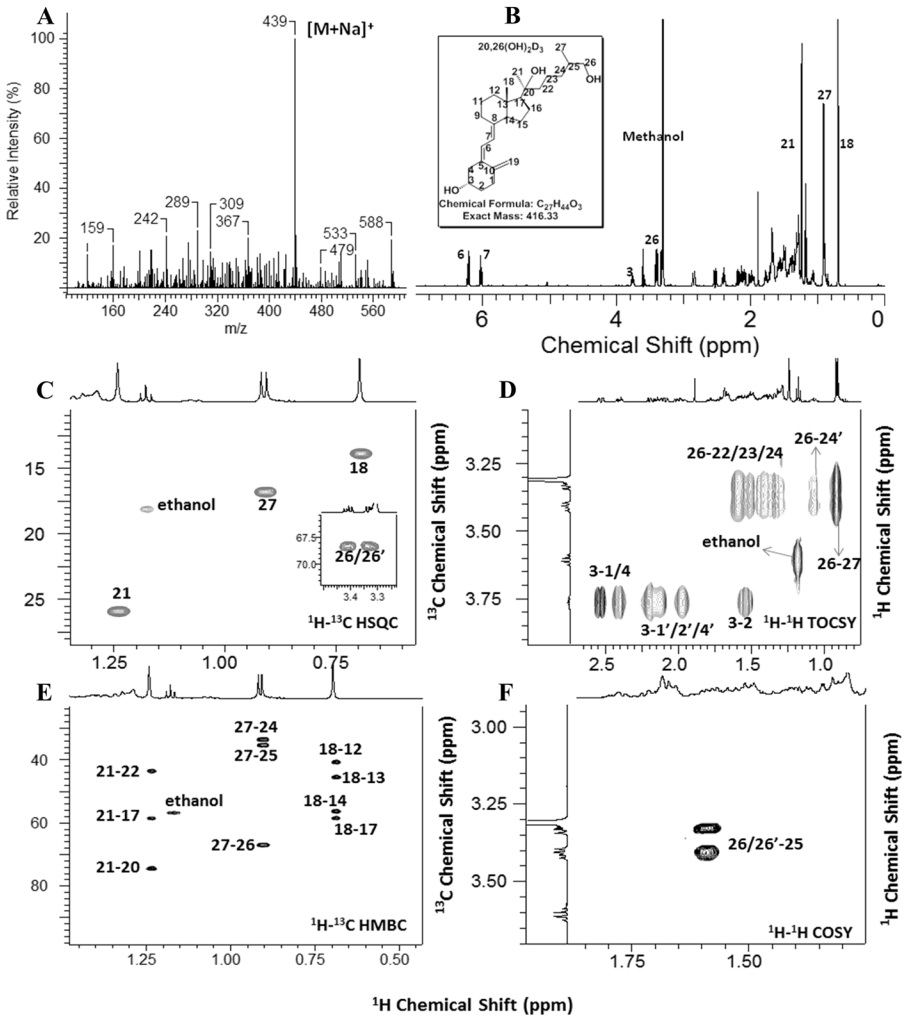


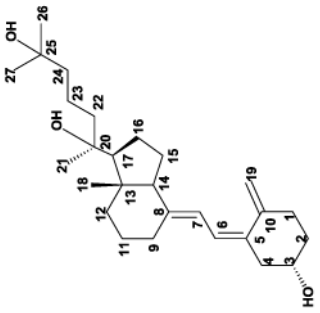
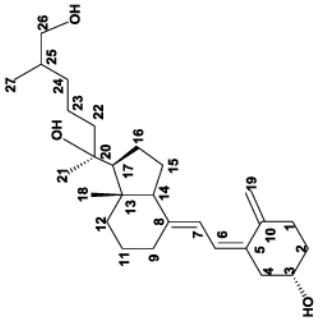
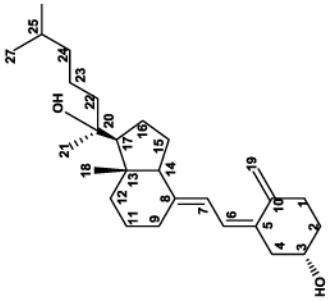
Fig. 6.

Mass and NMR spectra of 20,26(OH)$_2$D3. (A) Mass; (B) 1D Proton; (C) ^{1}H-^{13}C HSQC; (D) ^{1}H-^{1}H TOCSY; (E) ^{1}H-^{13}C HMBC; (F) ^{1}H-^{1}H COSY. Full spectra are shown in Supplemental Figures (Fig. S-2).

Table 1

NMR chemical shift assignments for 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3 analysis of their 2D NMR. (Solvent: CD_3OD)

Atom	20,25$(OH)_2$D3		20,26$(OH)_2$D3		20(OH)D3	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	2.12α, 2.41β	33.5	2.12 α, 2.42β	33.4	2.11α, 2.40β	32.2
2	1.97α, 1.54β	36.6	1.97α, 1.53β	36.3	1.97α, 1.53β	35.2
3	3.76	70.5	3.77	70.5	3.76	69.2
4	2.54α, 2.19β	47.0	2.54α, 2.20β	46.8	2.53α, 2.19β	45.6
5	NA	136.0	NA	136.0	NA	136.3
6	6.22	122.6	6.22	122.6	6.21	121.2
7	6.03	119.4	6.03	119.3	6.02	118.0
8	NA	141.1	NA	140.4	NA	141.0
9	1.69 α, 2.86β	29.7	1.69α, 2.86β	29.6	1.68α, 2.85β	28.4
10	NA	145.9	NA	145.7	NA	145.6
11	1.54α, 1.68β	22.7	1.56α, 1.67β	24.3	1.56α, 1.66β	23.1
12	1.39α, 2.10β	42.2	1.38α, 2.10β	42.1	1.37α, 2.07β	40.9
13	NA	45.4	NA	45.5	NA	45.6
14	2.02	57.8	2.01	57.7	2.00	56.4
15	1.78	22.9	1.50	22.7	1.51	21.7
16	1.68α, 1.56β	24.4	1.72α, 1.79β	22.7	1.69α, 1.78β	21.7
17	1.70	59.9	1.68	59.9	1.67	58.5
18	0.70	14.0	0.70	13.9	0.69	12.8
19	4.74, 5.04	112.64	4.75, 5.05	112.5	4.74, 5.04	111.3
20	NA	74.4	NA	74.5	NA	74.5
21	1.26	26.1	1.25	26.0	1.23	24.8
22	1.48, 1.35	45.4	1.34, 1.51	44.9	1.32, 1.46	43.9
23	1.40	19.9	1.43, 1.29	22.3	1.33	21.7
24	1.41	45.5	1.41, 1.07	34.9	1.16	39.6
25	**NA**	**70.0**	1.59	36.7	1.55	27.8
26	1.19	29.2	**3.33, 3.41**	**68.4**	0.89	21.7
27	1.19	29.2	0.91	16.8	0.89	21.7

Atom	20,25(OH)$_2$D3		20,26(OH)$_2$D3		20(OH)D3	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
						

NA – Not applicable (ternary carbons).