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# Metabolism of vitamin D by human microsomal CYP2R1☆

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**Abstract**

The activation of vitamin D requires 25-hydroxylation in the liver and 1α-hydroxylation in the kidney. However, it remains unclear which enzyme is relevant to vitamin D 25-hydroxylation. Recently, human CYP2R1 has been reported to be a potential candidate for a hepatic vitamin D 25-hydroxylase. Thus, vitamin D metabolism by CYP2R1 was compared with human mitochondrial CYP27A1, which used to be considered a physiologically important vitamin $D_3$ 25-hydroxylase. A clear difference was observed between CYP2R1 and CYP27A1 in the metabolism of vitamin $D_2$. CYP2R1 hydroxylated vitamin $D_2$ at the C-25 position while CYP27A1 hydroxylated it at positions C-24 and C-27. The $K_m$ and $k_{cat}$ values for the CYP2R1-dependent 25-hydroxylation activity toward vitamin $D_3$ were 0.45 μM and 0.97 $min^{-1}$, respectively. The $k_{cat}/K_m$ value of CYP2R1 was 26-fold higher than that of CYP27A1. These results strongly suggest that CYP2R1 plays a physiologically important role in the vitamin D 25-hydroxylation in humans.


*Keywords:* Cytochrome P450; CYP2R1; Vitamin D metabolism; Vitamin D 25-hydroxylation

It is commonly known that vitamin $D_3$ is initially converted to 25(OH)$D_3$ in the liver, and then 25(OH)$D_3$ is converted to a functionally active form, 1α,25(OH)$_2$ $D_3$, in the kidney. Hormone 1α,25(OH)$_2D_3$ plays essential roles in calcium and phosphate homeostasis, immunology, and cell differentiation [1]. The metabolic activations of vitamin $D_3$ are carried out by specific cytochromes P450, the hepatic vitamin $D_3$ 25-hydroxylase, and the renal 25(OH)$D_3$ 1α-hydroxylase (mitochondrial CYP27B1). Mitochondrial CYP27B1 is a well known key enzyme in determining the level of 1α,25(OH)$_2D_3$ because pseudovitamin D-deficient rickets is caused by the disruption of CYP27B1 gene [2]. On the other hand, a physiologically important hepatic vitamin D 25-hydroxylase has not been identified, although there are many reports of enzymes catalyzing vitamin D 25-hydroxylation.

To our knowledge, six cytochromes P450 have been reported as the hepatic vitamin D 25-hydroxylase: CYP2C11 [3], CYP2D25 [4], CYP2J3 [5], CYP3A4 [6], CYP27A1 [7,8], and CYP2R1 [9,10]. CYP2C11 is a rat male- specific P450 and shows high catalytic activity toward testosterone. CYP2D25 is considered a physiologically important vitamin D 25-hydroxylase in pigs. However, human ortholog of porcine CYP2D25, human CYP2D6, shows no activity toward vitamins $D_3$, and $D_2$ [4]. Recently, Yamasaki et al. [5] suggested that CYP2J3 is the principal P450 responsible for vitamin $D_3$ 25-hydroxylation in rat livers. Other CYP2J subfamily members including human CYP2J2 are known to be involved in the metabolism of arachidonic acid, but there are no reports on the metabolism of vitamin D by human

☆ *Abbreviations:* 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 25(OH)$D_2$, 25-hydroxyvitamin $D_2$; 1α,25(OH)$_2D_3$, 1α,25-dihydroxyvitamin $D_3$; 1α,25(OH)$_2D_2$, 1α,25-dihydroxyvitamin $D_2$; CYP, cytochrome P450; ADX, adrenodoxin; ADR, NADPH-adrenodoxin reductase.

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CYP2J2. CYP3A4, a major human hepatic microsomal P450 that is known to metabolize about 50% of therapeutic drugs, shows 25-hydroxylation activity toward vitamin $D_2$ but not toward vitamin $D_3$ [6]. Considering the importance of vitamin $D_3$ activation pathway, CYP3A4 does not seem to play an essential role in that pathway.

Mitochondrial CYP27A1 used to be a physiologically important vitamin $D_3$ 25-hydroxylase. However, its contribution to vitamin D activation has been questioned. Rosen et al. [11] reported that mice with disrupted CYP27A1 genes showed markedly reduced bile acid biosynthesis, but the plasma level of 25-hydroxyvitamin $D_3$ increased more than 2-fold. Patients with cerebrotendinous xanthomatosis (CTX) having a mutated CYP27A1 gene showed normal serum levels of 25-hydroxyvitamin $D_3$ [12]. These reports suggest that another enzyme compensates the loss of the vitamin $D_3$ 25-hydroxylation activity of CYP27A1.

Cheng et al. [9] cloned the gene coding human microsomal CYP2R1 which catalyzed vitamin D 25-hydroxylation. They succeeded in the functional expression of human CYP2R1 in HEK 293 cells and detected 25-hydroxylation activity toward vitamin $D_3$, $1\alpha(OH)D_3$, and vitamin $D_2$. Recently, they also reported that the CYP2R1 mutant L99P eliminated vitamin D 25-hydroxylase activity and that the mutation of CYP2R1 enzyme causes selective 25-hydroxyvitamin $D_3$ deficiency [10]. These data suggest that CYP2R1 is a physiologically important vitamin D 25-hydroxylase. However, such enzymatic characteristics as substrate specificity and the kinetic parameters of CYP2R1 were not determined.

In this study, we revealed the enzymatic properties of human CYP2R1 on the metabolism of vitamin D using a yeast expression system, and compared vitamin D metabolism by human CYP2R1 with that by human CYP27A1.

## Materials and methods

*Materials*. Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). Primer DNAs were purchased from Proligo Japan KK (Kyoto, Japan). Human Lung cDNA library No. 104 was purchased from Takara Bio. (Shiga, Japan). The DNA sequencing kit was purchased from Amersham–Pharmacia Biotech (Buckinghamshire, England). Vitamin $D_3$, vitamin $D_2$, and $1\alpha(OH)D_3$ were purchased from Wako (Osaka, Japan). $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ were purchased from Funakoshi (Tokyo, Japan). $1\alpha(OH)D_2$ was purchased from Calbiochem (La Jolla, CA, USA). $25(OH)D_2$ and $1\alpha,25(OH)_2D_2$ were kindly given by Dr. K. Yamamoto of Tokyo Medical and Dental University. Recombinant human CYP2J2 coexpressed in baculovirus-infected insect cells with human cytochrome P450 reductase and cytochrome $b_5$ (supersomes) was purchased from BD Gentest (Worburn, MA, USA). NADPH was purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of the best commercially available grade.

*cDNA cloning of human CYP2R1*. Human CYP2R1 cDNA was obtained from human Lung cDNA library No. 104 (Takara Bio, Shiga, Japan) with PCR methods. Two sets of primers (i) 5′-CTC GAG AAA AAA ATG TGG AAG CTT TGG AGA GCT GAA GAG-3′ and 5′-AGC ATC AAC AAA ATG CTG AGG TAG CTG AGG-3′ for N-terminal region of CYP2R1 and (ii) 5′-TAA TGC CTT TCC ATG GAT TGG CAT CC-3′ and 5′-CTC GAG TCA GCG TCT TTC AGC ACA GAT GAG-3′ for C-terminal region of CYP2R1 were used for PCR on the basis of the CYP2R1 cDNA sequence as described by Cheng et al. [9]. The PCR amplifications were performed in the presence of 20 mM Tris–HCl, pH 8.3, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/ml BSA, 1 μM of forward and reverse primers, 200 μM dNTPs, and 0.5 U of *Pyrobest* DNA polymerase (Takara Shuzo, Kyoto, Japan). The samples were subjected to 30 cycles of 20 s at 98 °C, 30 s at 57 °C, and 70 s at 72 °C. All the nucleotide sequence of the human CYP2R1 cDNA was confirmed by DNA sequencing.

*Construction of expression plasmid*. The expression plasmid for CYP2R1 was constructed as follows. The PCR fragment (0.85 kbp) encoding the N-terminal region of human CYP2R1 with *Xho*I and *Hin*cII site at each side was subcloned into pUC19 digested with *Hin*cII. The resultant plasmid was digested with *Xho*I and *Hin*cII to yield a *Xho*I–*Hin*cII fragment (0.8 kbp). Likewise, the fragment (0.75 kbp) encoding the C-terminal region was subcloned into pUC19, and digested with *Xho*I and *Hin*cII to yield a *Hin*cII–*Xho*I fragment (0.7 kbp). The two fragments were doubly inserted into *Xho*I site of pBluescript II SK(+) (Stratagene, Amsterdam, The Netherlands). The plasmid containing the mature form of human CYP2R1 was digested with *Xho*I, the *Xho*I fragment was cloned into the coexpression vector for P450, and yeast NADPH-P450 reductase, pGYR-NX, was digested with the same restriction enzyme [13].

*Preparation of microsomal fractions from the recombinant Saccharomyces cerevisiae cells*. The recombinant *S. cerevisiae* cells expressing human CYP2R1 were cultivated in a synthetic minimal medium containing 8% glucose, 5.4% yeast nitrogen base without amino acids, and 160 mg/L histidine at 30 °C. Microsomal fraction was prepared from recombinant S. *cerevisiae* cells as described previously [14].

*Preparation of subcellular fractions from the recombinant Escherichia coli cells expressing human CYP27A1*. The subcellular fraction of *E. coli* cells was carried out basically according to our previous study [15]. One hundred millimolar Tris–HCl, pH 7.4, buffer was used for suspension of the membrane fraction.

*Measurement of reduced CO difference spectra and substrate-induced difference spectra*. The reduced CO-difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously [16,17]. The concentrations of human CYP2R1 and human CYP27A1 were determined from the reduced CO-difference spectrum using a difference of the extinction coefficients at 446 and 490 nm of 91 $mM^{-1}\,cm^{-1}$ [18]. The substrate-induced difference spectra of the microsomal fraction containing 0.13 μM P450 were measured in the presence of 20 μM vitamin $D_3$, vitamin $D_2$, $1\alpha(OH)D_2$, and $25(OH)D_3$. The solution contains ethanol at a final concentration of 2.0%.

*Measurement of enzyme activity of CYP2R1*. The substrates, vitamin $D_3$, $25(OH)D_3$, $1\alpha(OH)D_3$, vitamin $D_2$, and $1\alpha(OH)D_2$, were each dissolved in ethanol. The reaction mixture contains each of substrates (0–10 μM) and the microsomes containing 30 nM of human CYP2R1 in 50 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by the addition of NADPH at a final concentration of 0.5 mM at 37 °C. Aliquots of the reaction mixture were collected after varying time intervals and were extracted with four volumes of chloroform/methanol (3:1, v/v). The organic phase was recovered and dried up. The resulting residue was solubilized with acetonitrile and applied to HPLC performed on a YMC-Pack ODS-AM [4.6 × 300 mm] (YMC, Tokyo, Japan). A linear gradient of 70–100% acetonitrile aqueous solution for 15 min followed by 100% acetonitrile for 25 min was used as a mobile phase. The flow-rate was 1.0 ml/min, and the elution was detected by measuring absorption at 265 nm. The column temperature was maintained at 40 °C.

*Measurement of enzyme activity of human CYP27A1*. The activity of human CYP27A1 was measured in the recombinant system

containing the membrane fraction containing 0–10 μM substrate, 0.5 μM CYP27A1, 0.5 μM NADPH–adrenodoxin reductase, 5 μM adrenodoxin, 1.0 mM NADPH, 100 mM Tris–HCl, pH 7.4, and 1 mM EDTA at 37 °C as described previously [7]. The reaction mixture was extracted with four volumes of chloroform–methanol (3:1, v/v) and analyzed as described above.

*Mass spectrometric analysis of the metabolites*. Isolated metabolites from HPLC effluents were subjected to LC–mass spectrometric analysis using a Finnegan mat TSQ-70 with atmospheric pressure chemical ionization (APCI), positive mode. The conditions of LC were described below: column; reverse phase ODS column ($6 \times 150$ mm) (μBondapak C18, Waters); mobile phase; 90% methanol aqueous solution; flow rate; 1.0 ml/min; and UV detection; 265 nm.

*Other methods*. The concentrations of vitamin $D_3$ derivatives were estimated by their molar extinction coefficient of $1.8 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 264 nm [19]. Protein concentration was determined by the method of Lowry et al. [20], using bovine serum albumin as a standard. The content of NADPH P450 reductase was estimated from cytochrome *c* reduction activity as described previously [21].

## Results

### *cDNA cloning of human CYP2R1*

The amino acid sequence deduced from the cloned CYP2R1 cDNA was identical with that reported by Cheng et al. [9], although one silent mutation was observed.

### *Expression of human CYP2R1 in the recombinant S. cerevisiae cells*

The P450 content in the microsomal fraction prepared from the recombinant yeast cells was estimated to be 21 pmol/mg protein on the basis of reduced CO difference spectrum (Fig. 1). The content of NADPH-P450 reductase in the microsomes was estimated to be 27 pmol/mg protein on the basis of the cytochrome *c* reductase activity of the purified yeast NADPH-P450 reductase sample [21,22]. Addition of 1α(OH)$D_2$ to the microsomal fraction containing CYP2R1 induced a typical type I spectrum (data not shown), indicating a change of the heme iron of CYP2R1 from a low-spin state to a high-spin state upon binding of 1α(OH)$D_2$. The addition of vitamin $D_3$ and $D_2$ also induced typical type I spectra. On the other hand, no detectable spectrum change was observed by the addition of 25(OH)$D_3$.

### *Human CYP2R1-dependent metabolism of vitamin D*

On the metabolism of vitamin $D_3$ and 1α(OH)$D_3$, CYP2R1 showed metabolites at the same retention time as 25(OH)$D_3$, and 1α,25(OH)$_2D_3$, respectively (Figs. 2A and E). The metabolites collected from HPLC effluents were subjected to LC–mass spectrometric analysis. The mass spectrum of the metabolite 1 (M1) showed a molecular ion at *m*/*z* 401 (M + H), and fragment ions at 383 (401 − $H_2O$), 365 (401 − $2H_2O$) (Fig. 3A). The ion at *m*/*z* 430 (M + H + 29) was also observed [7]. The spectrum coincided with that of authentic standard of 25(OH)$D_3$, suggesting that M1 is 25(OH)$D_3$ (Fig. 3B). Likewise, the metabolite 2 (M2) from 1α(OH)$D_3$ showed nearly the same mass spectrum as authentic standard of 1α,25(OH)$_2D_3$ (Figs. 3C and D).

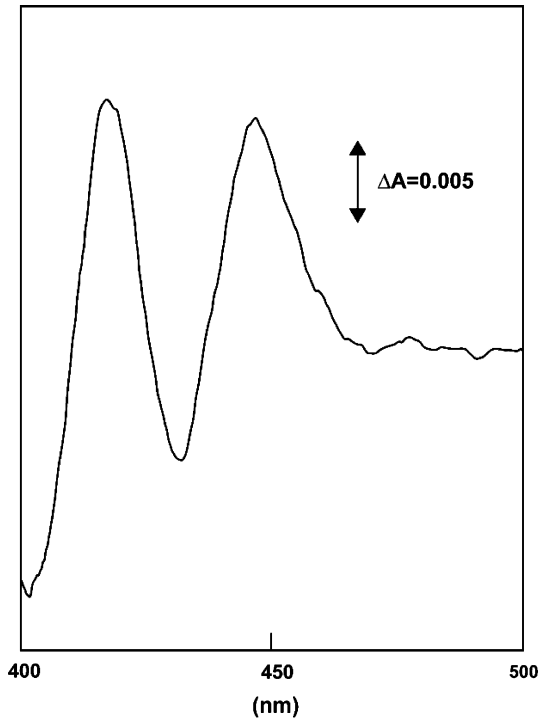


Fig. 1. Reduced CO-difference spectrum of microsomal fraction prepared from *S. cerevisiae* cells expressing human CYP2R1.

On the metabolism of vitamin $D_2$ and 1α(OH)$D_2$, CYP2R1 showed metabolites at the same retention time as 25(OH)$D_2$ and 1α,25(OH)$_2D_2$, respectively (Figs. 2C and F). As shown in Figs. 3E and G, the metabolite 3 (M3) showed a molecular ion at *m*/*z* 413 (M + H), and fragment ions at 395 (413 − $H_2O$), 377 (413 − $2H_2O$) (Fig. 3E). The spectrum coincided with that of authentic standard of 25(OH)$D_2$ (Fig. 3F). These results strongly suggested that the M2 is 25(OH)$D_2$. The mass spectrum of the metabolite 4 (M4) was quite similar to that of authentic standard of 1α,25(OH)$_2D_2$, suggesting that M4 is 1α,25(OH)$_2D_2$. On the other hand, CYP2R1 showed no detectable catalytic activity toward 25(OH)$D_3$.

### *Human CYP27A1-dependent metabolism of vitamin D*

Human mitochondrial CYP27A1 is known as a vitamin $D_3$ 25-hydroxylase and used to be considered as the physiologically important [23,24]. As reported previously, the main metabolite of vitamin $D_3$ by CYP27A1 was 25(OH)$D_3$ (Fig. 2B). CYP27A1 also showed 25-hydroxylation activity toward 1α(OH)$D_3$ (data not shown). Moreover, CYP27A1 showed 1α-hydroxylation activity

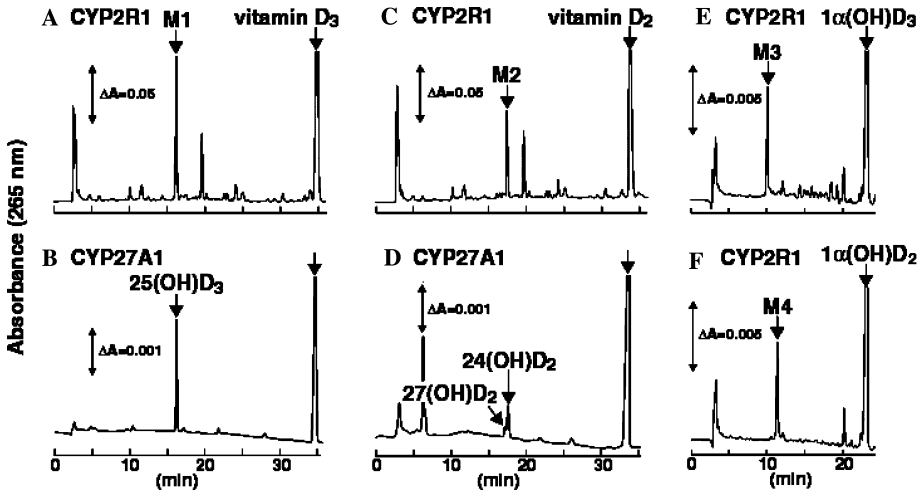


Fig. 2. HPLC profiles of vitamin $D_3$ and its metabolites by CYP2R1 (A) and CYP27A1 (B), those of vitamin $D_2$ and its metabolites by CYP2R1 (C) and CYP27A1 (D), that of $1\alpha(OH)D_3$ and its metabolites by CYP2R1 (E), and that of $1\alpha(OH)D_2$ and its metabolites by CYP2R1 (F). After incubation with each of 10 μM substrate for 20 min (A,C,E,F) or 30 min (B,D), the reaction mixture was examined and analyzed by HPLC as described in Materials and methods. $25(OH)D_3$ (B) indicates the elution position of authentic standard of $25(OH)D_3$. $24(OH)D_2$ and $27(OH)D_2$ (D) indicate the putative elution positions of $24(OH)D_2$ and $27(OH)D_2$ based on Guo et al. [8].

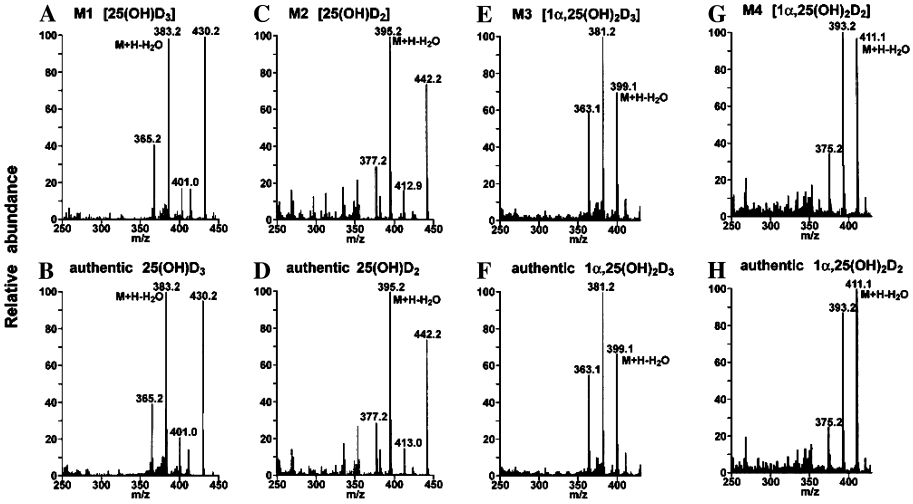


Fig. 3. Mass spectra of the metabolites M1 (A), M2 (C), M3 (E), and M4 (G) in Fig. 2, and authentic standards of $25(OH)D_3$ (B), $25(OH)D_2$ (D), $1\alpha,25(OH)_2D_3$ (F), and $1\alpha,25(OH)_2D_2$ (H).

toward $25(OH)D_3$ (data not shown), suggesting that CYP27A1 has an ability to convert vitamin $D_3$ to $1\alpha,25(OH)_2D_3$. On the other hand, the mass spectra of the metabolites from vitamin $D_2$ by CYP27A1 were different from that of $25(OH)D_2$. Judging from the results described by Guo et al. [8] the major metabolites were considered to be $24(OH)D_2$ and $27(OH)D_2$ (Fig. 2D). Likewise, CYP27A1 showed 24- and 27-hydroxylation activity toward $1\alpha(OH)D_2$.

*Comparison of vitamin D hydroxylation activity between CYP2R1 and CYP27A1*

Although the reconstituted system of mitochondrial CYP27A1 contains membrane fraction of *E. coli*, the reconstituted system of CYP2R1 contains microsomal fraction of yeast. Thus, it is difficult to compare their enzymatic properties directly. Table 1 summarizes the hydroxylation activity of CYP2R1 and CYP27A1 toward vitamin $D_3$, $1\alpha(OH)D_3$, $25(OH)D_3$, vitamin $D_2$, and $1\alpha(OH)D_2$ at a substrate concentration of 10 μM. The 25-hydroxylation activity of CYP2R1 toward vitamin $D_3$ was 5-fold higher than that of CYP27A1. The addition of 1α-hydroxyl group to the substrate increased the 25-hydroxylation activity of CYP27A1. On the other hand, the 25-hydroxylation activity of CYP2R1 toward $1\alpha(OH)D_3$ and $1\alpha(OH)D_2$ was nearly the same as that toward vitamin $D_3$ and vitamin $D_2$, respectively. On the metabolism of $25(OH)D_3$, CYP27A1 catalyzes not only 1α-hydroxylation but also 26(27)-hydroxylation,

Table 1
Comparison of vitamin D hydroxylation activity between human CYP2R1 and human CYP27A1

| Substrate | Position | CYP2R1 activity (mol/min/mol p450) | CYP27A1 activity (mol/min/mol p450) |
|---|---|---|---|
| $VD_3$ | 25 | $1.20 \pm 0.05$ | $0.20 \pm 0.03$ |
| $1\alpha(OH)D_3$ | 25 | $0.80 \pm 0.08$ | $0.47 \pm 0.04$ |
| $25(OH)D_3$ | 1α | — | $0.012 \pm 0.005$ |
| $VD_2$ | 24 | — | $0.033 \pm 0.004$ |
| | 25 | $0.84 \pm 0.09$ | — |
| | 27 | — | $0.014 \pm 0.001$ |
| $1\alpha(OH)D_2$ | 24 | — | $0.11 \pm 0.02$ |
| | 25 | $0.75 \pm 0.04$ | — |
| | 27 | — | $0.11 \pm 0.02$ |

Each of the hydroxylation activity toward vitamin $D_3$, $1\alpha(OH)D_3$, $25(OH)D_3$, vitamin $D_2$, and $1\alpha(OH)D_2$ was measured in the presence of 30 nM of CYP2R1, 10 μM of a substrate and 0.5 mM NADPH at 37 °C for 2 min. The activity of CYP27A1 was measured in the presence of 0.5 μM CYP27A1, 10 μM of a substrate, 0.5 μM NADPH–adrenodoxin reductase, and 5 μM adrenodoxin at 37 °C for 15, 30, or 60 min as described in Materials and methods.

and 24-hydroxylation [7]. Although we have revealed that the 25-hydroxylation activity and the 1α-hydroxylation activity are closely linked together [25,26], CYP2R1 showed no detectable 1α-hydroxylation activity toward $25(OH)D_3$. CYP2R1 showed 25-hydroxylation activity toward vitamin $D_2$ and $1\alpha(OH)D_2$. On the other hand, CYP27A1 catalyzes not 25-hydroxylation but 24- and 27-hydroxylation toward vitamin $D_2$ and $1\alpha(OH)D_2$. Based on these results, metabolic pathways of vitamin $D_2$ by CYP2R1, and CYP27A1 are summarized in Fig. 4.

*Kinetic analysis of CYP2R1-dependent 25-hydroxylation of vitamin $D_3$ and vitamin $D_2$*

The kinetic parameters, $K_m$ and $k_{cat}$, for the CYP2R1-dependent 25-hydroxylation activity toward vitamin $D_3$ were estimated to be 0.45 μM and 0.97 $min^{-1}$, respectively, and those toward vitamin $D_2$ were estimated to be 0.67 μM and 0.61 (mol/min/mol P450), respectively (Table 2). On the other hand, the $K_m$ and $k_{cat}$ values for CYP27A1-dependent 25-hydroxylation activity toward vitamin $D_3$ were estimated to be 3.2 μM and 0.27 (mol/min/mol P450), respectively (Table 2). The $k_{cat}/K_m$ value of CYP2R1 for vitamin $D_3$ 25-hydroxylation was 26-fold higher than that of CYP27A1 (Table 2).

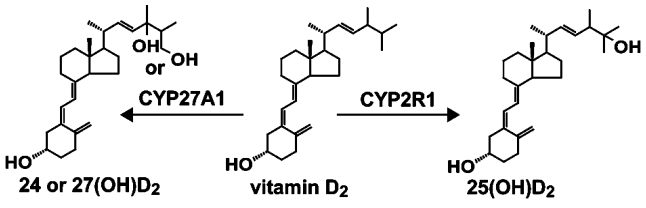


Fig. 4. Putative metabolic pathways of vitamin $D_2$ by CYP2R1 and CYP27A1.

Table 2
Kinetic parameters of human CYP2R1 and human CYP27A1 for vitamin D 25-hhydroxylation

| CYP | Substrate | $K_m$ (μM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ |
|---|---|---|---|---|
| 2R1 | $VD_2$ | $0.67 \pm 0.12$ | $0.61 \pm 0.04$ | 0.91 |
| 2R1 | $VD_3$ | $0.45 \pm 0.16$ | $0.97 \pm 0.05$ | 2.16 |
| 27A1[a] | $VD_3$ | $3.2 \pm 0.5$ | $0.27 \pm 0.03$ | 0.084 |

[a] The values were reported in our previous study [7].

## Discussion

First, vitamin $D_3$ is converted to $25(OH)D_3$ in the liver, and next $25(OH)D_3$ is converted to $1\alpha,25(OH)_2D_3$ in the kidney. Although CYP27B1 is well known to be a physiologically important 25-hydroxyvitamin D 1α-hydroxylase, it remains unclear which enzyme is a physiologically important vitamin D 25-hydroxylase. In this study, detailed enzymatic properties of human CYP2R1 were revealed using a yeast expression system. Recombinant CYP2R1 showed 25-hydroxylation activity toward vitamin $D_3$, as reported previously [9]. CYP2R1 also showed 25-hydroxylation activity toward $1\alpha(OH)D_3$, indicating that its enzymatic properties are similar those of to CYP27A1. However, clear substrate specificity differences were observed between CYP2R1 and CYP27A1. Although CYP27A1 showed 1α-hydroxylation activity toward $25(OH)D_3$, CYP2R1 showed no detectable activity toward $25(OH)D_3$ (Table 1). On the metabolism of vitamin $D_2$, CYP2R1 catalyzed 25-hydroxylation, while CYP27A1 catalyzed 24- and 27-hydroxylation (Table 1, Fig. 4). Thus, the binding mode of vitamin $D_2$ to the substrate-heme pocket of CYP27A1 appears to be different from vitamin $D_3$. On the other hand, CYP2R1 may recognize vitamins $D_3$ and $D_2$ in a similar manner.

One of our interests is which enzyme mainly contributes to the activation of vitamin D in humans. Previous studies [27–29] have shown that mitochondrial hydroxylase is a low affinity, high capacity enzyme, whereas the microsomal hydroxylase is a high affinity, low capacity enzyme. In this study, 25-hydroxylation activity toward vitamin $D_3$ of CYP2R1 was 3.6-fold higher than CYP27A1, and CYP2R1 showed 7-fold higher affinity for vitamin $D_3$ than CYP27A1. Thus, our results appear consistent with previous studies. In addition, considering that the $k_{cat}/K_m$ value of CYP2R1 for vitamin $D_3$ 25-hydroxylation is 26-fold higher than CYP27A1 (Table 2), it appears that CYP2R1 contributes significantly to the activation of vitamin $D_3$.

Vitamin $D_2$, which is created from ergosterol and uptaken from the diet, is converted to the active form,$1\alpha,25(OH)_2D_2$, in the same manner as vitamin $D_3$ in humans [30]. As described above, CYP2R1 showed 25-hydroxylation activity toward vitamin $D_2$, while CYP27A1 showed 24- and 27-hydroxylation activity (Table 1, Fig. 4). Based on the fact that the serum level of

25(OH)$D_2$ is much higher than those of 24(OH)$D_2$ and 27(OH)$D_2$, vitamin $D_2$ 25-hydroxylase is more important than 24- and 27-hydroxylase (CYP27A1). Thus, it is possible to assume that CYP2R1 is much more essential than CYP27A1 for the activation of vitamin $D_2$. The $K_m$ and $k_{cat}$ values for CYP2R1-dependent 25-hydroxylation activity toward vitamin $D_2$ were estimated to be 0.67 μM and 0.61 $min^{-1}$, respectively (Table 2), indicating that CYP2R1 binds vitamin $D_2$ with a similar affinity to vitamin $D_3$. These results demonstrate the importance of CYP2R1 as a vitamin $D_2$ 25-hydroxylase. CYP2R1 may mainly contribute not only to the activation of vitamin $D_3$, but also to that of vitamin $D_2$ in humans.

Recently, Yamasaki et al. [5] reported that rat CYP2J3 showed high 25-hydroxylation activity toward vitamin $D_3$ and 1α(OH)$D_3$. We examined the metabolism of vitamin $D_3$, 1α(OH)$D_3$, vitamin $D_2$, and 1α(OH)$D_2$ by recombinant CYP2J2, a putative human ortholog of rat CYP2J3, expressed in baculovirus-infected insect cells. CYP2J2 showed 25-hydroxylation activity towards 1α(OH)$D_3$, vitamin $D_2$, and 1α(OH)$D_2$ but not toward vitamin $D_3$. 25-Hydroxylation activity toward 1α(OH)$D_3$, vitamin $D_2$, and 1α(OH)$D_2$ (at a substrate concentration of 10 μM) was $0.060 \pm 0.006$, $0.020 \pm 0.003$, and $0.860 \pm 0.003$ (mol/min/mol P450), respectively. To date, CYP2J2 is known as one of the enzymes responsible for the epoxidation of endogenous arachidonic acid pools in the human heart [31]. The catalytic activity toward arachidonic acid by CYP2J2 is 26- and 8-fold higher than those towards vitamins $D_3$ and $D_2$, respectively [32]. Moreover, 25-hydroxylation activity towards vitamin $D_3$ and $D_2$ by CYP2J2 is significantly lower compared to those by CYP2R1. These results suggest that human CYP2J2 does not play a central role in the activation of vitamin D in humans.

A recent report by Cheng et al. [9], which showed that the mutation of CYP2R1 causes selective 25(OH)$D_3$ deficiency [10], strongly suggests that CYP2R1 is a physiologically important vitamin D 25-hydroxylase. This study confirmed that human CYP2R1 is a physiologically important vitamin D 25-hydroxylase on the basis of comparisons of enzymatic properties of CYP2R1 with those of CYP27A1. Although Cheng et al. [9] reported that mouse CYP2R1 mRNA was most abundant in the liver and testis, and present in lower levels in numerous other tissues, there are no reports showing the expression level and the tissue distribution of human CYP2R1 at the protein level. Thus, further studies are needed to quantitatively evaluate the contribution of CYP2R1.

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