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METABOLISM OF VITAMIN D_3 BY CYTOCHROMES P450

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1. ABSTRACT

The vitamin D_3 25-hydroxylase (CYP27A1), 25-hydroxyvitamin D_3 1alpha-hydroxylase (CYP27B1) and 1alpha,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1) are members of the cytochrome P450 superfamily, and key enzymes of vitamin D_3 metabolism. Using the heterologous expression in *E. coli*, enzymatic properties of the P450s were recently investigated in detail. Upon analyses of the metabolites of vitamin D_3 by the reconstituted system, CYP27A1 surprisingly produced at least seven forms of minor metabolites including 1alpha,25$(OH)_2D_3$ in addition to the major metabolite 25(OH)D_3. These results indicated that human CYP27A1 catalyzes multiple reactions involved in the vitamin D_3 metabolism. In contrast, CYP27B1 only catalyzes the hydroxylation at C-1alpha position of 25(OH)D_3 and 24*R*,25$(OH)_2D_3$. Enzymatic studies on substrate specificity of CYP27B1 suggest that the 1alpha-hydroxylase activity of CYP27B1 requires the presence of 25-hydroxyl group of vitamin D_3 and is enhanced by 24-hydroxyl group while the presence of 23-hydroxyl group greatly reduced the activity. Eight types of missense mutations in the CYP27B1 gene found in vitamin D-dependent rickets type I (VDDR-I) patients completely abolished the 1alpha-hydroxylase activity. A three-dimensional model of CYP27B1 structure simulated on the basis of the crystal structure of rabbit CYP2C5 supports the experimental data from mutagenesis study of CYP27B1 that the mutated amino acid residues may be involved in protein folding, heme-propionate binding or activation of molecular oxygen. CYP24A1 expressed in *E. coli* showed a remarkable metabolic processes of 25(OH)D_3 and 1alpha,25$(OH)_2D_3$. Rat CYP24A1 catalyzed six sequential monooxygenation reactions that convert 1alpha,25$(OH)_2D_3$ into calcitroic acid, a known final metabolite of C-24 oxidation pathway.

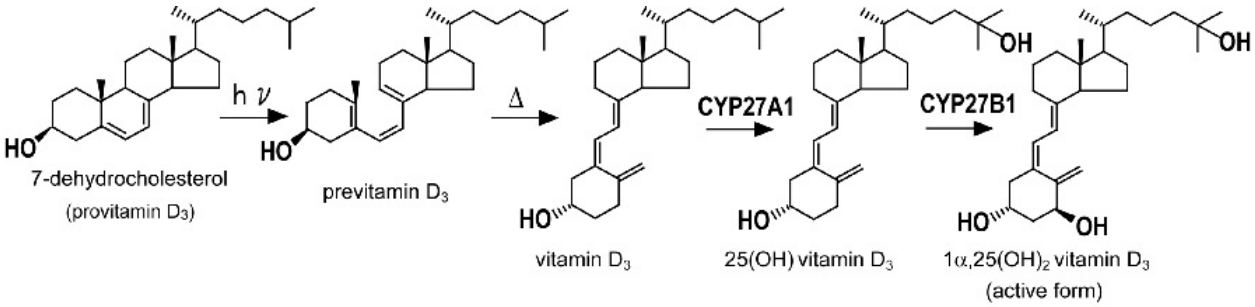

Figure 1. Synthetic pathway of active form of vitamin D_3. The schematic pathway of vitamin D_3 shows the conversion of 7-dehydrocholesterol to an active form of vitamin D_3 (1alpha,25$(OH)_2D_3$) by the action of light exposure ($h\nu$) in the skin, heat-induced isomerization (Δ) , CYP27A1 in the liver, and CYP27B1 in the kidney.

In addition to the C-24 oxidation pathway, human CYP24A1 catalyzed also C-23 oxidation pathway to produce 1alpha,25$(OH)_2D_3$-26,23-lactone. Surprisingly, more than 70 % of the vitamin D metabolites observed in a living body were found to be the products formed by the activities of CYP27A1, CYP27B1 and CYP24A1. The species-based difference was also observed in the metabolism of vitamin D analogs by CYP24A1, suggesting that the recombinant system for human CYP24A1 may be of great use for the prediction of the metabolism of vitamin D analogs in humans.

2. INTRODUCTION

In humans, vitamin D_3 (cholecalciferol) is formed from 7-dehydrocholesterol by the exposure to ultraviolet light in the skin (figure 1). Vitamin D_3 undergoes two steps of hydroxylation. The first step occurs at 25 position in the liver, producing 25-hydroxyvitamin D_3 (25$(OH)D_3$). The second step occurs at the 1alpha position in the kidney, producing 1alpha, 25-dihydroxyvitamin D_3 (1alpha,25$(OH)_2D_3$), the most potent form of vitamin D_3. This active form of vitamin D_3 plays a central role in calcium homeostasis by promoting absorption of calcium and phosphorous from the intestine, reabsorption of phosphate in the kidney, and release calcium and phosphate from the bone (1-4). In addition to the classically recognized functions, 1alpha,25$(OH)_2D_3$ has been also found to be capable of regulating cell proliferation and differentiation.

Since Horsting and DeLuca (5) reported that vitamin D_3 is hydroxylated at 25-position in rat liver homogenates, subcellular localization of the 25-hydroxylation of vitamin D_3 had long been a question. Easy cross contamination of microsomes and mitochondria by classical subcellular fractionation, multiplicity of microsomal P450s in the liver, and instability of the 25-hydroxylase activity for purification hampered the elucidation of subcellular localization and the identification of enzymes responsible for the activity. After intensive studies, both mitochondria and microsomes were found to be responsive for the vitamin D_3 25-hydroxylation although the majority of the 25-hydroxylation appears to undergo in mitochondria. In humans, we now believe that the vitamin D_3 25-hydroxylation is catalyzed by CYP27A1 in mitochondria and CYP2R1 (6) in microsomes although the microsomal 25-hydroxylase(s) may vary among species.

In the last two decades, extensive studies on characterization and purification of 25-hydroxyvitamin D_3 1alpha-hydroxylase have been carried out (7-9). However, no reports showing complete purification have been published presumably due to the extremely low expression level and instability of the enzyme. In spite of almost no information on the 1alpha-hydroxylase at the protein level, the cDNAs encoding 1alpha-hydroxylase were cloned by several groups. Takeyama *et al*. (10) cloned a cDNA encoding mouse 25-hydroxyvitamin D_3 1alpha-hydroxylase (CYP27B1) from the kidney of the vitamin D receptor (VDR) knock-out mice using a sophisticated expression cloning system. Rat by Shinki *et al.* (11) and human CYP27B1 cDNAs by Monkawa *et al*. (12) and Fu *et al.* (13) were also independently identified and reported in 1997.

A large number of vitamin D_3 metabolites have been identified and implicated in the metabolic pathways of vitamin D_3. Thus, the presence of a large number of enzymes had been assumed in the complicated vitamin D_3 metabolic map. However, many of the enzymes responsible for each metabolic step have not been identified, which puzzled us long time. The question was addressed by the studies on the sequential monooxygenation reactions by CYP24A1 (14-16) indicating that CYP24A1 has a central role in the extraordinary complicated metabolism of vitamin D_3. Combined with studies of CYP27A1 and CYP27B1, these results finally lead us to the conclusion that the complicated metabolism of vitamin D_3 is mainly originated from the activities of three mitochondrial P450s, CYP27A1, CYP27B1 and CYP24A1.

Vitamin D analogs are potentially useful for clinical treatments of type I rickets, osteoporosis, renal osteodystrophy, psoriasis, leukemia**,** and breast cancer (17). On the use of vitamin D analogs, the information of metabolism in target tissues such as kidney, small intestine**,** and bones is pharmacologically essential as reported by Komuro *et al.* (18). CYP24A1 is considered to be associated with the major metabolic pathways of the vitamin D analogs in these tissues (18, 19). Species-dependent metabolism of vitamin D_3 analogs appears to be

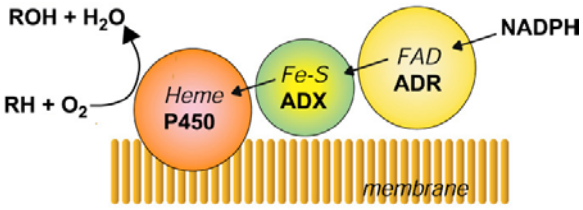

Figure 2. Electron transport chain of mitochondrial P450 monooxygenase. Electrons are transferred from NADPH through NADPH-adrenodoxin reductase (ADR) and adrenodoxin (ADX) to mitochondrial P450 to exhibit the monooxygenase activity. RH and ROH indicate the substrate and the product, respectively.

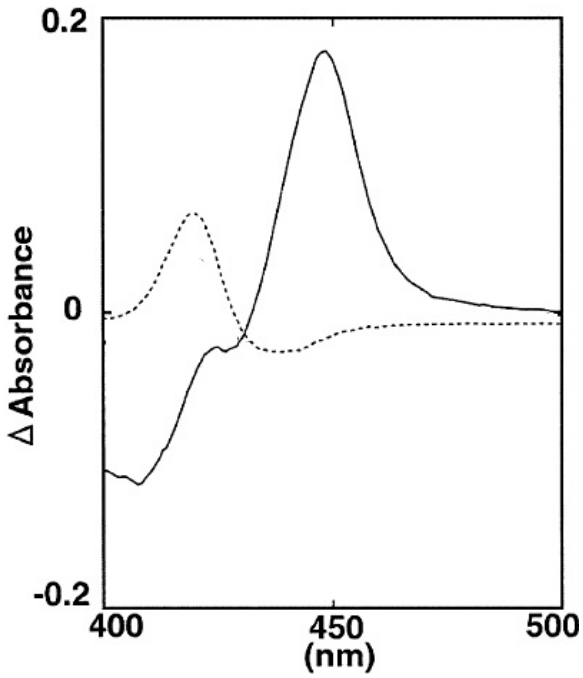

Figure 3. Reduced CO-difference spectra of expressed human CYP27A1.Reduced CO-difference spectra of membrane fractions from *E. coli* cells expressing human CYP27A1 (solid line), and those from the control *E. coli* cells (dot line) are presented.

originated from specificity of CYP24A1-dependent reactions. Since human kidney specimen is not easily obtained and substrate specificities of CYP24A1 vary among species, the development of an *in vitro* system containing human CYP24A1 was essential for the prediction of the drug metabolism in human kidney.

Since complete genome sequence analysis of *E. coli* K12 suggested the absence of P450 gene in *E. coli* K12 genome (20), many mammalian P450s have been investigated using the heterologous expression in *E. coli* (21, 22). In addition, because *E. coli* has no steroids in cell membranes, *E. coli* expression system has low backgrounds on kinetic studies of P450 related to metabolism of vitamin D_3. Mitochondrial P450s catalyze monooxygenase reaction utilizing molecular oxygen and reducing equivalents of electrons from NADPH via ferredoxin (adrenodoxin) and ferredoxin-reductase (adrenodoxin reductase) (figure 2). Therefore, analysis of mitochondrial P450 activity requires purified ferredoxin and ferredoxin-reductase for *in vitro* reconstitution or their coexpression in *E. coli* for whole cell assay system, both of which are now available. Our previous studies using these analytical methods demonstrated that the *E. coli* expression system was quite useful for the investigation of CYP27A1 (23), CYP27B1 (24-26) and CYP24A1 (27-29). In this review, we describe enzymatic properties of CYP27A1, CYP27B1 and CYP24A1, and discuss their physiological roles.

3. CYP27A1

3.1. Vitamin D_3 25-hydroxylases in mitochondria and microsomes

The 25-Hydroxylation of vitamin D_3 is catalyzed in the liver by mitochondrial CYP27A1, and a microsomal P450 whose identity in most species has not been determined. The existence of more than one enzyme raises the question of physiological significance between the mitochondrial enzyme and microsomal enzyme(s). The importance of the microsomal enzyme(s) is demonstrated by mutations in the CYP27A1 gene in humans and mice. The loss of CYP27A1 has profound effects on cholesterol metabolism and causes cerenbrotendinous xanthomatosis (CTX) (30). Large amounts of cholestanol, cholesterol, and bile alcohol are produced and accumulated in many tissues, whereas the vitamin D metabolism is normal in individuals (31). These findings indicate that the microsomal enzyme(s) can compensate for loss of CYP27A1 in vitamin D metabolism but not in bile acid synthesis. The microsomal enzymes were identified as CYP2C11 in male rats (32), and CYP2D25 in pigs (33). However, none of human P450s in CYP2C and 2D subfamilies showed the vitamin D 25-hydroxylasion activity. Recently, Cheng *et al.* (6) demonstrated that human CYP2R1 catalyzes 25-hydroxylasion of both vitamin D_2 and D_3, suggesting that the microsomal vitamin D 25-hydroxylase is CYP2R1 at least in humans. Relative contribution of the mitochondrial enzyme (CYP27A1) and the microsomal enzyme (CYP2R1) was estimated to be approximately 70 % and 30 %, respectively, by Cheng *et al*. (6) on the basis of the data reported by Bjorkhem and Holmberg (34).

3.2. Expression of human CYP27A1

After isolation of cDNA by Cali *et.al*. (35), the human CYP27A1 has been expressed in *E. coli* with an amino-terminal modification of the putative mature form because *E. coli* cells appears to have no processing machinery of mitochondrial precursor proteins (36). The mature form of CYP27A1 was efficiently expressed in *E. coli* at the approximate level of 1000 nmol/L culture estimated by the reduced CO-difference spectrum of the membrane fraction in our laboratory (figure 3), representing that the P450 content was approximately 5% of the membrane proteins. The addition of vitamin D_3 or 1alpha(OH)D_3 to the membrane fraction induced a Type I spectrum, indicating a change of the heme iron of human

Table 1. Kinetic parameters of human CYP27A1 for the metabolism of vitamin D_3 and 25-hydroxyvitamin D_3

Substrate	Hydroxylation Position	Km (μM)	Vmax (mol/min/mol)	Vmax/Km (x 10^{-3})
vitamin D_3	25	3.2±0.5	0.269±0.029	93
	27	4.6±2.1	0.036±0.002	8
25(OH)D_3	1alpha	3.5±0.4	0.021±0.002	6
	24	5.5±0.7	0.014±0.003	3
	26 (27)	2.9±0.7	0.054±0.008	19

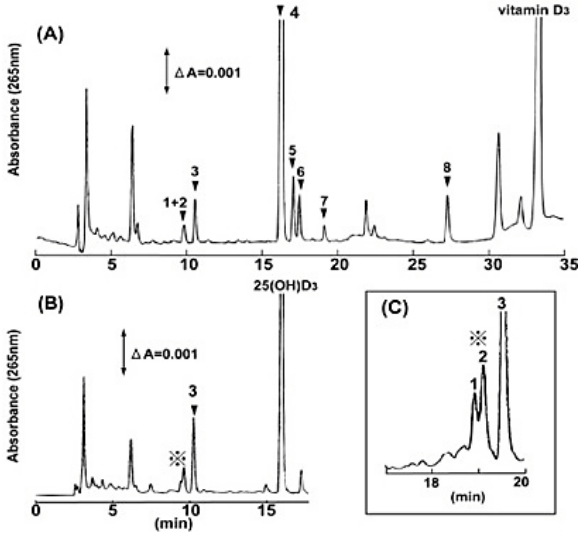

Figure 4. HPLC analyses of metabolites of vitamin D_3 by CYP27A1.The reaction products of vitamin D_3 (A) and 25(OH)D_3 metabolites (B) were displayed on HPLC. The peak marked in (B) was separated into two peaks under a different HPLC condition (C).

CYP27A1 from a low-spin state to a high-spin state upon the substrate binding, a characteristic spin shift by formation of substrate-P450 complex.

3.3. Metabolism of vitamin D_3 by CYP27A1

The reconstituted system containing bovine adrenodoxin (ADX), bovine adrenodoxin reductase (ADR), and the *E. coli* membrane fraction having CYP27A1, was used to investigate the metabolism of vitamin D_3. HPLC analysis of the reaction products demonstrated the presence of at least eight metabolites (figure 4A). The major peak (peak 4 in figure 4) was identified as 25(OH)D_3 by its retention time on HPLC and mass spectral analysis. The metabolite designated as peak 1+2 was clearly separated to two peaks whose retention times were identical with those of 24*R*,25(OH)$_2$D$_3$ and 1alpha,25(OH)$_2$D$_3$ (figure 4C) under another HPLC condition. Three metabolites 1, 2 and 3 with the same retention times as those in figure 4A were also detected when 25(OH)D_3 was used as a substrate (figure 4B). Combined with the results from mass spectral analysis, the metabolites 1 and 2 were strongly suggested to be 24*R*,25 (OH)$_2$D$_3$ and 1alpha,25(OH)$_2$D$_3$, respectively. Mass spectrum of the metabolite 3 indicates that it is also dihydroxylated product of vitamin D_3. Based on the report indicating that CYP27A1 secondly prefer the hydroxylation at C26/C27 position of vitamin D_3 (37), the metabolite 3 was postulated as 25,26(27)(OH)$_2$D$_3$. It should be noted that the positions of C26 and C27 were changed by the addition of hydroxyl group to C25. Therefore, 25,27(OH)$_2$D$_3$ would be also produced because a considerable amount of 26(OH)D_3 was produced from vitamin D_3. Furthermore, the metabolites, 1alpha,24*R*,25 (OH)$_3$D$_3$, the putative 1alpha,25,26 (OH)$_3$D$_3$ and 1alpha,25,27(OH)$_3$D$_3$ were observed when 1alpha,25 (OH)$_2$D$_3$ was used as a substrate. Thus, the metabolite 3 appears to contain 25,26(OH)$_2$D$_3$ and 25,27(OH)$_2$D$_3$. The metabolites 5 and 6 showed mass spectra with a molecular ion at m/z 401 (M+H), suggesting that they were monohydroxylated product of vitamin D_3. Based on the report by Dilwarth *et. al*. (38), the metabolite 5 appeared to be a mixture of 26(OH)D_3 and 27(OH)D_3. In our previous report (23), it was assumed that the metabolites 5 and 6 were 27(OH)D_3 and 26(OH)D_3, respectively. However, the fragmentation pattern of the metabolite 6 by mass spectral analysis resembles that of 25(OH)D_3 rather than the metabolite 5, suggesting that this assumption might be incorrect. The structure of the metabolite 6 has not yet been determined. The metabolite 7 (figure 4A) showed a mass spectrum with a molecular ion at m/z 399 (M+H), suggesting that the ketone was formed by the addition of another hydroxyl group at the same position to form a *gem*-diol intermediate, which spontaneously rearranges with loss of a water (14, 27). Since C25 position cannot form the ketone, the metabolite 7 was suggested to be 27-oxo-D_3. Mass spectrum of the metabolite numbered 8 strongly suggests that the metabolite is a dehydrogenated form of vitamin D_3.

3.4. Kinetic analysis of CYP27A1-dependent reactions

When the substrate concentration was varied, the reaction followed Michaelis-Menten type kinetics on 25-hydroxylation towards vitamin D_3 and 1alpha(OH)D_3. The K_m and V_{max} values were 3.2 μM and 0.27 (mol/min/mol P450) for vitamin D_3 and 6.9 μM and 0.79 (mol/min/mol P450) for 1alpha(OH)D_3, respectively (Table 1). In addition, the K_m and V_{max} values for 1alpha-hydroxylation, 24-hydroxylation and 26(27)-hydroxylation toward 25(OH)D_3 were also estimated. Although these hydroxylation reactions showed significantly small V_{max} values as compared with V_{max} value of the 25-hydroxylation of vitamin D_3, they showed similar K_m values. It is noted that the determined K_m value for 1alpha-hydroxylation toward 25(OH)D_3 was similar to the K_m value for the same reaction catalyzed by CYP27B1 (24, 25) as described in the next session.

To date, the metabolism of vitamin D_3 has not been sufficiently analyzed, whereas Berginer *et al*. (39) reported a physiological significance of human CYP27A1-dependent vitamin D_3 25-hydroxylation in patients with

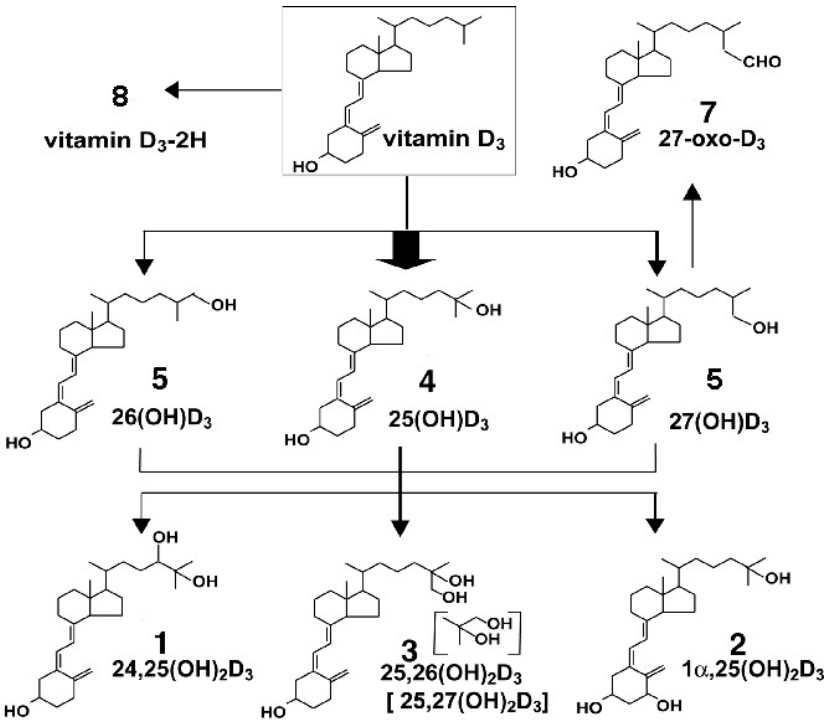

Figure 5. Putative metabolic pathway of vitamin D_3 by human CYP27A1.The metabolites produced by CYP27A1 were analyzed by HPLC and mass spectrometric analyses. The results were summarized as above. The numberings of metabolites correspond to those in figure 4.

cerebrotendinous xanthomatosis. Although Furster *et al*. (40) reported a K_m value for 1alpha(OH)D_3 25-hydroxylation catalyzed by rabbit CYP27A1, no kinetic studies showing K_m value for vitamin D_3 25-hydroxylation had been demonstrated. Our studies using *E. coli* expression system have provided insights into the enzymatic properties and physiological roles of human CYP27A1 in the vitamin D_3 metabolism. The K_m value of 3.2 μM obtained from our studies suggests that CYP27A1 is the physiologically essential vitamin D_3 25-hydroxylase in humans. Although the V_{max} value seems quite low, enough amounts of 25(OH)D_3 may be retained in blood circulation due to the long half-life of 25(OH)D_3 (41).

3.5. Metabolic pathway of vitamin D_3 by CYP27A1

The metabolic pathway of vitamin D_3 by CYP27A1 was summarized in figure 5 based on the chemical structures of the metabolites and the time courses of their amounts in the reaction system. The formation of the dehydrogenated form of vitamin D_3 seems to be an atypical reaction for P450, while further analysis is needed to determine its chemical structure. Although physiological significance of other reactions than 25-hydroxylation have not yet been well understood, the multiple reactions yield known compounds such as 1alpha,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$ that are physiologically important (42-44).

The first step of 1alpha,25$(OH)_2D_3$ production from vitamin D_3 appears to be the conversion from vitamin D_3 to 25(OH)D_3 judged by no detection of 1alpha(OH)D_3 as a metabolite of vitaminD_3. Thus, the 25-hydroxyl group may be essential for the 1alpha-hydroxylation catalyzed by CYP27A1 as CYP27B1 specifically catalyzes 1alpha-hydroxylation of vitamin D derivatives having 25-hydroxyl group (24, 25). Axen *et al*. (45), reported the 1alpha-hydroxylation of 25(OH)D_3 by CYP27A1 and indicated a possibility that CYP27A1 was a physiologically essential "25(OH)D_3 1alphahydroxylase". However, the cDNA cloning and functional expression of CYP27B1 (10) clearly demonstrated that CYP27B1 is the essential 25(OH)D_3 1alpha-hydroxylase. Recent study of the megalin-mediated transport of 25(OH)D_3 in proximal of kidney cell also supported the CYP27B1-dependent mechanism of the production of 1alpha,25$(OH)_2D_3$ (46). On the other hand, our previous work (47) demonstrated the presence of a patient with Vitamin D-dependent rickets type I (VDDR-I) with nearly normal serum 1alpha,25$(OH)_2D_3$ level in spite of the complete defects of CYP27B1 genes. Judging from V_{max}/K_m values of CYP27A1-dependent vitamin D_3 25-hydroxylation and 25(OH)D_3 1alpha-hydroxylation, 25(OH)D_3 1alpha-hydroxylation activity catalyzed by human CYP27A1 should not be neglected. The nearly normal 1alpha,25$(OH)_2D_3$ level in the serum of the patient mentioned above might be derived from CYP27A1-dependent 1alpha-hydroxylation (38).

4. CYP27B1

4.1. Identification of 1alpha-hydroxylase of 25-hydroxyvitamin D_3

The 1alpha-Hydroxylase of 25-hydroxyvitamin D_3 is a key enzyme in the determination of the serum level

Table 2. Kinetic parameters of mouse and human CYP27B1 for 1alpha-hydroxylation of 25(OH)D_3 and 24*R*,25(OH)$_2D_3$

Substrate	K_m (μM)	V_{max}[a]	V_{max}/K_m
mouse CYP27B1			
25(OH)D_3	2.7	9.5	3.5
24*R*,25(OH)$_2D_3$	1.3	16.7	12.3
human CYP27B1			
25(OH)D_3	2.7	3.9	1.4
24*R*,25(OH)$_2D_3$	1.1	3.2	2.9

The data previously published were summarized (24, 25).a, (pmol product/min/mg protein).

Table 3. Vitamin D_3 derivatives and 1α-hydroxylase activities of mouse and human CYP27B1

1α-Hydroxylase Activity (pmol product/min/mg protein)		
Substrate	mouse CYP27B1	human CYP27B1
vitamin D_3	＜0.05	＜0.05
25(OH)D_3	1.5	0.6
24*R*,25(OH)$_2D_3$	4.3	1.5
23*S*,25(OH)$_2D_3$	0.2	＜0.1
24-oxo-25(OH)	1.1	0.4
24-oxo-23*S*,25(OH)$_2D_3$	＜0.1	＜0.05
tetranor-23(OH)D_3	＜0.05	＜0.05

The data were obtained at a substrate concentration of 0.5 μM (24, 25).

of 1alpha,25-dihydroxyvitamin. In the last 20 years, characterization and purification of 25-hydroxyvitamin D_3 1alpha-hydroxylase have been extensively attempted (7-9). However, no reports demonstrated the complete purification presumably due to the extremely low expression level and instability of the enzyme.

Takeyama *et al*., (10) found that mice lacking the vitamin D receptor (VDR) developed an abnormally high serum concentration of 1alpha,25(OH)$_2D_3$, suggesting the excessive 1alpha-hydroxylase expression. Therefore, they attempted the cDNA cloning of 1alpha-hydroxylase with poly $(A)^+$ RNA prepared from the kidneys of VDR knockout mice using the nuclear receptor-mediated expression system. The isolated cDNA clone encoding 25(OH)D_3 1alpha-hydroxylase contained a complete open reading frame encoding 507 amino acids. The deduced amino acid sequence strongly suggests that this protein is a mitochondrial P450 with 42 % homology to rat CYP27A1 (48). Thus, the name CYP27B1 was given to the 1alpha-hydroxylase as a member of P450 superfamily (49). Using the mouse cDNA as a probe, human 1alpha-hydroxylase cDNAs were also isolated from normal and patients with pseudovitamin D-deficient rickets (50). Other groups also isolated the cDNA clones of rat and human 1alpha-hydroxylase (11-13).

Vitamin D-dependent rickets type I (VDDR-I) is a form of hereditary rickets inherited as an autosomal recessive trait (51, 52) and caused by defect in the activity of renal 25-hydroxyvitamin D_3 1alpha-hydroxylase (CYP27B1) (13, 50, 53, 54). Surprisingly, more than 20 types of mutations have been identified from patients with the rare VDDR-I disease (13, 50, 53, 54). Enzymatic analysis of human 1alpha-hydroxylase from normal subjects and patients with VDDR-I is indispensable to elucidate the relationship between residual activity of the mutants and severity of the disease.

4.2. Expression of 1alpha-hydroxylase (CYP27B1) in *E. coli*

The cleavage site of CYP27B1 for *in vivo* production of the mature form after import into mitochondria is not yet determined. Therefore, the putative cleavage site of murine CYP27B1 was determined on the basis of the alignment with rat 24-hydroxylase (55) to express CYP27B1 in *E. coli* as a mature form. The expression plasmid for the human CYP27B1 mature form containing amino-terminal 33-508 amino acid residues was also constructed in a manner similar to the murine isoform (24). Because of inefficient expression, the expression levels of mouse and human CYP27B1 were unable to be determined by reduced CO-difference spectra although the expression of both enzymes in *E. coli* were efficient enough for metabolic studies.

4.3. Kinetic analysis using *in vitro* reconstitution system

When 25(OH)D_3 concentration was varied, the reaction followed Michaelis-Menten type kinetics on 1alpha-hydroxylation. The K_m and V_{max} values of mouse CYP27B1 were 2.7μM and 9.5 pmol product/mg protein/min for 25(OH)D_3, and 1.3μM and 16.7 pmol product/mg protein/min for 24*R*, 25(OH)$_2D_3$, respectively (Table 2). On the other hand, the K_m and V_{max} values of human CYP27B1 were 2.7μM and 3.9 pmol product/mg protein/min for 25(OH)D_3, and 1.1 and 3.2 pmol product/mg protein/min for 24*R*, 25(OH)$_2D_3$, respectively. The difference of V_{max} between mouse CYP27B1 and human CYP27B1 may be due to the difference of their expression levels in *E. coli*. Physiological concentrations of those substrates may be much less than the corresponding K_m values. Thus, V_{max}/K_m is a physiologically important parameter. It was noticed that the V_{max}/K_m values of mouse and human CYP27B1 for 24*R*,25(OH)$_2D_3$ were higher than the corresponding V_{max}/K_m values for 25(OH)D_3. These results indicated that 24*R*,25(OH)$_2D_3$ is a better substrate than 25(OH)D_3 for both mouse and human CYP27B1. The preferential 1alpha-hydroxylation of 24*R*,25(OH)$_2D_3$ is not rationally understood at this time, since 1alpha,25(OH)$_2D_3$ is known to be the most potent form of vitamin D_3.

4.4. Substrate specificity of CYP27B1

The substrates, 23*S*,25(OH)$_2D_3$, 24-oxo-25(OH)D_3, 24-oxo-23,25(OH)$_2D_3$, and tetranor-23-(OH)D_3 which are known as metabolites of 25(OH)D_3 catalyzed by 24-hydroxylase (CYP24A1) (14, 15), were examined for the 1alpha-hydroxylase activity in the reconstituted system. Mouse and human CYP27B1 showed only trace and no detectable activity toward 23*S*,25(OH)$_2D_3$ (Table 3), respectively. On the substrate 24-oxo-25(OH)D_3, the product was observed at the same retention times as the authentic standard of 24-oxo-1alpha,25(OH)$_2D_3$. The 1alpha-hydroxylation of 24-oxo-25(OH)D_3 by CYP27B1 was catalyzed as efficiently as that of 25(OH)D_3,

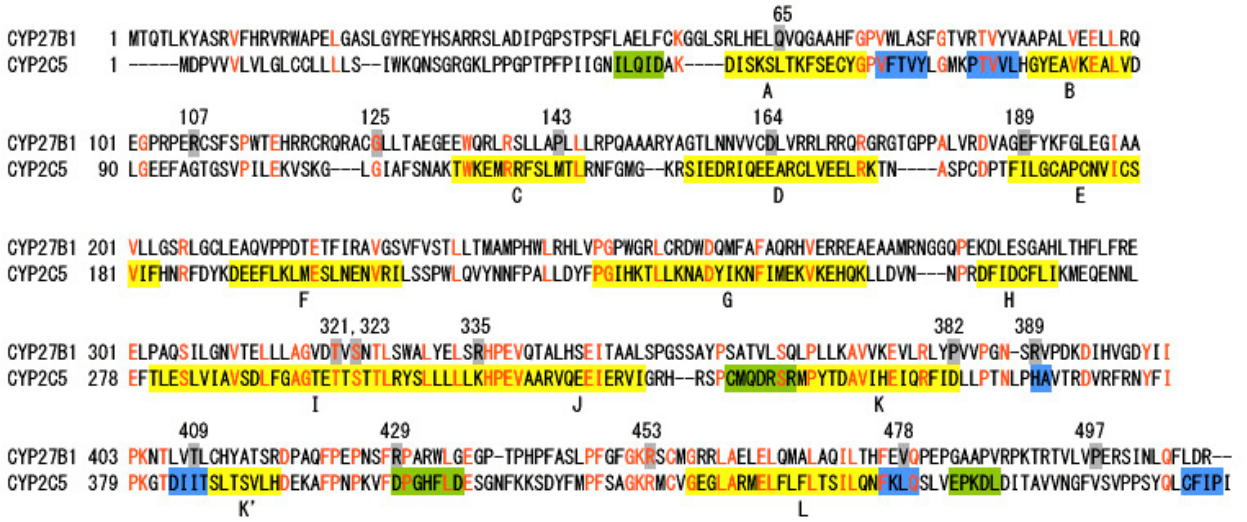

Figure 6. Sequence alignment of human CYP27B1 with rabbit CYP2C5. Yellow, green and blue boxes on CYP2C5 represent alpha-helix, 3/10 helix and beta-strand, respectively, in the crystal structure of CYP2C5 (58). The A to L helices are denoted by letters beneath the sequence of CYP2C5. Gray boxes and the above numbers show residues whose missense mutations cause VDDR-I. Red letters represent identical residues between both CYPs.

suggesting that 24-oxo group has little effect on the activity. The enzyme showed the 1alpha-hydroxylation of 24-oxo-23*S*,25(OH)$_2$D$_3$ too low to quantify. Both mouse and human CYP27B1 showed no detectable activity towards vitamin D$_3$ and tetranor-23(OH)D$_3$, both of which have no 25-hydroxyl group. These results suggest that the 25-hydroxyl group of vitamin D$_3$ is essential for the 1alpha-hydroxylase activity of both mouse and human CYP27B1. It is noted that the 24-hydroxyl group enhances the activity in contrast to the great reduction by the 23-hydroxyl group, while the 24-oxo group gives little effect on the activity of CYP27B1.

4.5. Coexpression of CYP27B1 with adrenodoxin (ADX) and adrenodoxin reductase (ADR)

Intriguingly, when the substrates 25(OH)D$_3$ and 24*R*,25(OH)$_2$D$_3$ were directly added into the cell culture, the 1alpha-hydroxylated metabolites were detected in a time-dependent manner in the *E. coli* cells expressing mouse CYP27B1 or human CYP27B1 in the absence of the coexpression of adrenodoxin and reductase. These results strongly indicate the presence of a redox partner of CYP27B1 in *E. coli* cells. Previously, the *E. coli* flavoproteins flavodoxin and NADPH-flavodoxin (ferredoxin) reductase were found to be able to serve as an electron transfer system for microsomal P450s (56). Therefore, flavodoxin (or ferredoxin) and NADPH-flavodoxin (ferredoxin) reductase in *E. coli* may transfer reducing equivalents of electrons to mitochondrial P450s. However, the electron transport from the *E. coli* redox partners to CYP27B1 does not seem to be very efficient. Therefore, the bovine ADX and ADR, the redox partners of mitochondrial P450, were coexpressed with CYP27B1 to efficiently analyze the 1alpha-hydroxylase activity in the recombinant *E. coli* cells utilizing a polycistronic mRNA method. The cDNAs encoding mature forms of CYP24A1, bovine ADX and bovine ADR each containing a ribosomal-binding sequence upstream of the translational start site were organized into a polycistronic transcription unit under the regulation of *tac* promoter.

When the substrate 25(OH)D$_3$ was added to cell culture of the recombinant *E. coli* cells, the production of 1alpha,25(OH)$_2$D$_3$ was remarkably enhanced by the coexpression of ADX and ADR. Thus, electrons appear to be efficiently transferred from NADPH through ADR and ADX to CYP27B1 in *E. coli* cells. This novel coexpression system will be quite useful for the elucidation of enzymatic properties of 1alpha-hydroxylase, and possibly useful as a bioreactor to produce 1alpha,25(OH)$_2$D$_3$.

4.6. Structure-function analysis of CYP27B1 based on the analysis of CYP27B1 mutants from VDDR-1

Kitanaka *et al*. [48] have cloned eight types of missense mutations and one nonsense mutation from Japanese VDDR-I patients (47, 50), and other groups identified nine missense mutations from the patients (13, 54). None of the CYP27B1 mutants expressed in mammalian cells (13, 50, 53, 54) and *E. coli* cells (25, 47) showed 1alpha-hydroxylase activity towards 25(OH)D$_3$. Thus, the mutated amino acid residues seemed to play important roles in the function of 1alpha-hydroxylase such as substrate binding, activation of molecular oxygen, interaction with adrenodoxin, and folding of the P450 structure. To investigate the mutations in depth, we generated various mutants of CYP27B1 related to the mutations found in the patients. In addition, the corresponding mutations were introduced to CYP27A1 that belongs to the same P450 family with CYP27B1. As CYP27A1 showed much higher expression level than CYP27B1 in *E. coli* cells, further analyses including heme-binding and substrate-binding were performed using CYP27A1 in place of CYP27B1. Spectral analyses including reduced CO-difference spectra and substrate-induced difference spectra, and enzymatic analyses of the mutant CYP27A1 gave information on structure-function relationships of both CYP27A1 and CYP27B1. Based on the sequence alignment (figure 6), Arg-107, Gly-

Table 4. Putative functions of the mutated amino acid residues of human CYP27B1 found in the patients with VDDR-I

Mutation	Location	Function
Q65H	A helix	H-bond with substrate
R107H	loop B-C	folding
G125E	loop B-C	folding
P143L	C helix (terminal)	folding (helix breaker)
D164L	D helix	folding (four-helix bundling)
E189L	E helix	folding
T321R	I helix	oxygen activation
S323Y	I helix	folding
R335P	I helix	folding
P382S	K helix (terminal)	folding (helix breaker)
R389C(H)	beta strand	heme propionate binding
T409I	beta strand	H-bond with substrate
R429P	3/10 helix	folding
R453C	loop K'-L	heme propionate binding
V478G	beta strand	folding
P497R	terminal loop	folding

The putative roles of the amino acid residues at the mutated points of CYP27B1 from VDDR-I patients were summarized (26).

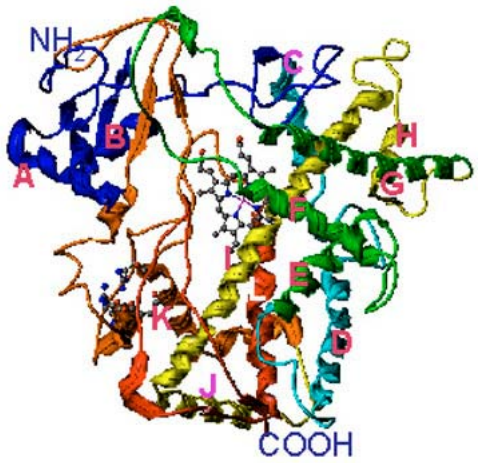

Figure 7. Three-dimensional structure model of CYP27B1. The structure was constructed by homology modeling technique. The overall folding of CYP27B1 is colored from blue at the N-terminal to red at the C-terminal and the heme is shown as ball and stick. The A to L helices are depicted by magenta letters.

25, and Pro-497 of CYP27B1 are postulated to be associated with substrate-binding. However, the expression of the mutants at protein levels was significantly reduced, and no hemoproteins were detected in R127K, G145A and P518R of CYP27A1 corresponding to R107K, G125A and P497R of CYP27B1. These experimental data strongly suggested that mutations of these amino acid residues destroyed the tertiary structure of the substrate-heme pocket. The CYP27A1 mutants R405K and R474K, which correspond to R389K and R453K of CYP27B1, also produced no detectable hemoproteins. The results are consistent with the assumption from alignment analysis that Arg-389 and Arg-453 of CYP27B1 were involved in heme-propionate binding.

The notable results were obtained on Asp-164 of CYP27B1. D164E showed the 1alpha-hydroxylase activity while D164N and D164Q of CYP27B1 abolished the activity, strongly suggesting the importance of the negative charge at position 164. The results from D183N, and the double mutant D184N/D183N of human CYP27A1 corresponding to D164N of CYP27B1 strongly suggest that at least one negative charge is necessary for CYP27A at 183 or 184 position of CYP27A1. The results are also supported by the alignment analyses that the rat CYP27A1 and rabbit CYP27A1 have conserved negative charged residues at 183 and 184 positions, respectively.

4.7. Molecular modeling of CYP27B1 by computer

According to the alignment of the three human P450s (CYP27A1, CYP27B1 and CYP24A1) and rabbit CYP2C5, we constructed a 3D model of human CYP27B1 by using SYBYL modeling soft, COMPOSER, and the atomic coordinates of the crystal structure of rabbit CYP2C5 as a template (57, 58). As seen in figure 7, the simulated 3D structure of CYP27B1 contains 17 helices (13 alpha-helices and four 3/10 helices) and six beta-strands, as does the template CYP2C5 (57). The molecular model of CYP27B1 may well explain the roles of amino acid residues at the mutated positions seen in VDDR-I patients as summarized in Table 4. Asp-164 of CYP27B1, and Asp-183 and Asp184 of CYP27A1 appear to be located in D-helix, which forms the four-helix bundle with E, I, and, J-helices (58), and exposed to the surface of the protein. The binding-affinity of the double mutant D184N/D183N to ADX was similar to the normal CYP27A1 (data not shown), suggesting that the Asp residues are not responsible for interaction with ADX. The drastic reduction of the expression level of D184N/D183N might suggest that each of the Asp residues stabilizes the four-helix bundle consisting of D, E, I, and J-helices possibly by forming a salt bridge. Thus, the mutations at 164-position in CYP27B1 and D184 and/or D183 in CYP27A1 may indirectly alter the conformation within the heme pocket. The sequence alignment suggests that Thr-321 of CYP27B1 and Thr-339 of CYP27A1 correspond to Thr-252 of P450cam that is responsible for activation of molecular oxygen. The mutations on Thr-321 of CYP27B1 and Thr-339 of CYP27A1 showed great reduction of hydroxylation activities of both enzymes, suggesting that these Thr residues in CYP27A1 and CYP27B1 may be involved in the activation of molecular oxygen similarly to Thr-252 of P450cam (CYP101).

5. CYP24A1

5.1. Identification of CYP24A1 and heterologous expression in *E. coli*

Since the 24-hydroxylase activity is remarkably induced by 1alpha,25(OH)D_3, Ohyama *et al*., (55, 59) purified and determined the amino-terminal 8 amino acid

sequence of vitamin D_3 24-hydroxylase from kidneys of rats treated with 1alpha,25(OH)D_3. The purified sample showed a high 24-hydroxylase activity toward 25(OH)D_3 with K_m value of 2.8 μM. Utilizing specific antibody raised against the purified 24-hydroxylase, the cDNA encoding the 24-hydroxylase was cloned from a rat kidney cDNA library (60). The isolated cDNA clone contained the open reading frame encoding 514 amino acids. The primary sequence showed less than 40 % homology with other known P450s. Thus, the new family name CYP24 was given to the 24-hydroxylase.

Comparison of the deduced amino acid sequence of CYP24A1 with the N-terminal sequence of the purified 24-hydroxylase from mitochondria suggested that the N-terminal 35 amino acids of CYP24A1 are removed to yield the mitochondrial mature form. Although the human CYP24A1 cDNA encoding 513 amino acids was reported (61), the human CYP24A1 cDNA isolated in our laboratory contained a coding sequence for 514 amino acids. The sequence of our clone GCG TAC CCG encoding the amino acid sequence Ala-Tyr-Pro at 124th-126th from the amino terminus corresponded to the sequence GTA CCC encoding Val-Pro reported by Chen et al. (61). Based on alignment analysis of CYP24A1 from other species and the human genomic sequence, the inconsistency was clarified that the CYP24A1 contains 514 amino acids in human as in other species.

Because of undetectable background levels of vitamin D_3 metabolism, the heterologous expression systems were very useful for the elucidation of enzymatic properties of CYP24A1. For instance, Beckman *et al*. (15) using *Baculovirus* expression system and HPLC analysis suggested the formation of 24,25,26,27-tetranor 23(OH)D_3 from 25(OH)D_3 by human CYP24A1. In our laboratory, the expression plasmids for mature forms (36-514 amino acid residues) of rat and human CYP24A1 were constructed and introduced into *E. coli* JM109 cells (14, 16). Both rat and human CYP24A1 were expressed in *E. coli* at levels of approximately 100 nmol/L culture determined by reduced CO-difference spectrum of the membrane fraction. Our *in vitro* studies using the membrane fraction of recombinant *E. coli* cells indicated that rat CYP24A1 is capable of catalyzing not only 24-hydroxylation of 25(OH)D_3 and 1alpha,25(OH)$_2D_3$ but also two steps of subsequent monooxygenation reactions (1). In this section, we describe a remarkable metabolism of 25(OH)D_3 and 1alpha,25(OH)$_2D_3$ by CYP24A1 and species-based difference of CYP24A1-dependent vitamin D metabolism between humans and rat.

5.2. Metabolism of 25(OH)D_3 and 1alpha,25(OH)$_2$ D_3 by CYP24A1

5.2.1. Rat CYP24A1

Using the *in vitro* reconstituted system with the membrane fraction, catalytic abilities of rat CYP24A1 were investigated for the metabolism of 25(OH)D_3 and 1alpha,25(OH)$_2$ D_3. The four metabolites, 24*R*,25(OH)$_2D_3$, 24-oxo-25(OH)D_3, 24-oxo-23*S*,25(OH)$_2D_3$ and tetranor-23(OH)D_3 were detected in the time-dependent manner when 25(OH)D_3 was used as a substrate. The detailed analyses of time course in the 25(OH)D_3 metabolism strongly suggest the sequential conversion of 25(OH)D_3 through 24*R*,25(OH)$_2D_3$, 24-oxo-25(OH)D_3, and 24-oxo-23*S*,25(OH) $_2D_3$ to tetranor-23(OH)D_3 by CYP24A1 (14, 27). This metabolic pathway by CYP24A1 was further supported by the investigation using 24*R*,25(OH)$_2D_3$, 24-oxo-25(OH)D_3, and 24-oxo-23*S*,25(OH) $_2D_3$ as substrates. It was noted that 24-oxo-23*S*,25(OH)$_2D_3$ and tetranor-23(OH)D_3 were detected even in the early time of the reaction when total conversion ratio of the four metabolites was less than 0.05. The early formation of these two products was increased by the addition of excess amounts of ADX and ADR (14). Rat CYP24A1 also catalyzed the metabolism of 1alpha,25(OH)$_2D_3$ and produced 1alpha,24*R*,25(OH)$_3$ D_3, 24-oxo-1alpha,25(OH)$_2D_3$, and 24-oxo-1alpha,23*S*,25(OH)$_3D_3$ together with tetranor-23-oxo-1alpha(OH)D_3, tetranor-1alpha,23(OH)$_2D_3$ and calcitroic acid (27). These results suggest that CYP24A1 catalyzes six-steps of sequential monooxygenation toward 1alpha,25(OH)$_2D_3$ to yield calcitroic acid. Utilizing tetranor-1alpha,23(OH)$_2D_3$ as a substrate, the last two steps to calcitroic acid were confirmed to be the CYP24A1-dependent reactions. Five compounds, 1alpha,24*R*,25(OH)$_3$ D_3, 24-oxo-1alpha,25(OH)$_2D_3$, 24-oxo-1alpha,23*S*,25(OH)$_3$ D_3, tetranor-1alpha,23(OH)$_2D_3$ and calcitroic acid were observed as metabolites of 1alpha,25(OH)$_2D_3$ in the kidney and the osteoblastic cell (62). Our results suggest a surprising conclusion that all five known metabolites are the products catalyzed by the single enzyme CYP24A1. In addition, tetranor-23-oxo-1alpha(OH)D_3 was found to be a metabolic intermediate which has not been observed in physiological conditions probably due to its instability. The metabolic pathway of 1alpha,25(OH)$_2D_3$ by CYP24A1 elucidated in our study would occur in physiological conditions to yield the final product calcitroic acid. Induction of CYP24A1 by 1alpha,25(OH)$_2D_3$ (63) and complete inactivation of 1alpha,25(OH)$_2D_3$ by CYP24A1 appears to be a rational mechanism for precisely regulating the level of the active form of vitamin D_3. Thus, the most significant role of CYP24A1 may be to degrade the active form of vitamin D_3.

Physiologically, 25(OH)D_3 is also an important substrate for CYP24A1 because the concentration of 25(OH)D_3 in serum is 100-1000-fold higher than 1alpha,25(OH)$_2D_3$, and the product 24*R*,25(OH)$_2D_3$ has physiological functions such as differentiation of cartilage and hatching (42-44). Thus, another important role of CYP24A1 appears to be the production of 24*R*,25(OH)$_2D_3$. As mentioned above, 24*R*,25(OH) $_2D_3$ is a better substrate for CYP27B1 with a higher value of V_{max} /K_m than 25(OH)D_3 (24). Since 1alpha-hydoxylated product of 24*R*,25(OH)$_2D_3$, 1alpha,24*R*,25(OH)$_3$ D_3, can function as a ligand of vitamin D_3 receptor, 24*R*,25(OH)$_2D_3$ is also valuable as a substrate of CYP27B1. The sequential monooxygenation reactions are necessary for the complete inactivation and excretion of 1alpha,25(OH)$_2D_3$, while a step of monooxygenation by CYP24A1 produces 24*R*,25(OH)$_2$ D_3 from 25(OH)D_3. Thus, the lower K_m and the several sequential monooxygenation steps for

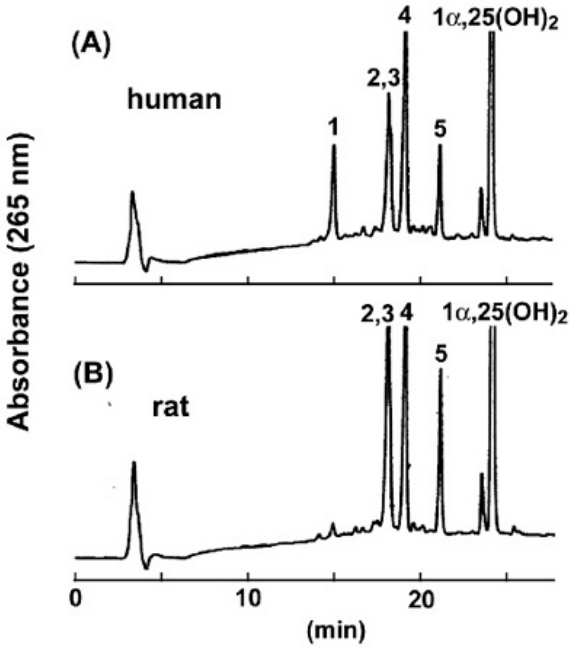

Figure 8. HPLC profiles of 1alpha,25$(OH)_2D_3$ and its metabolites by human CYP24A1 (A) and rat CYP24A1 (B). The metabolite peak 1, 1alpha,23*S*,25,26$(OH)_4D_3$; 2, 24-oxo-1alpha,23*S*,25$(OH)_3D_3$; 3, tetranor-1alpha,23$(OH)_2D_3$; 4, 1alpha,24*R*,25$(OH)_3D_3$ and 1alpha,23*S*,25$(OH)_3D_3$; 5, 24-oxo-1alpha,23*S*,25$(OH)_3D_3$.

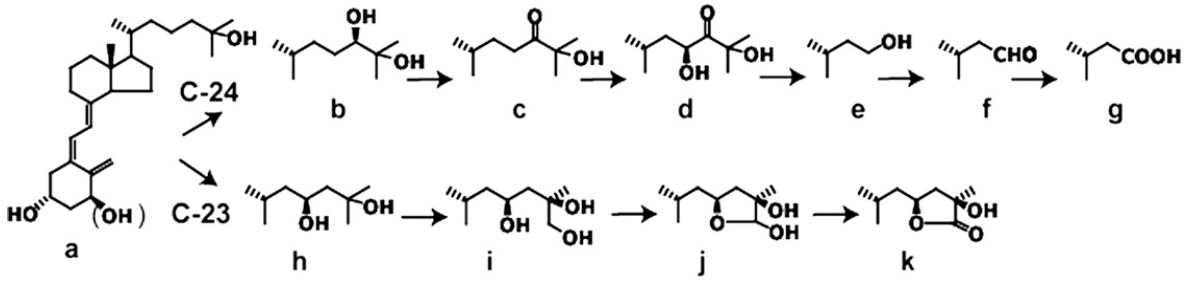

Figure 9. Matabolic pathways of 25$(OH)D_3$ and 1alpha,25$(OH)_2D_3$ by human CYP24A1. The scheme represents C-23 and C-24 consecutive hydroxylation pathways of 25$(OH)_2D_3$ and 1alpha,25$(OH)_2D_3$ catalyzed by a single P450 CYP24A1.

1alpha25$(OH)_2D_3$ as compared with 25$(OH)D_3$ may be physiologically rational. Further kinetic analysis with rapid quenching experiments (64) will provide insights into the mechanism of the sequential monooxygenation steps catalyzed by CYP24A1.

5.2.2. Human CYP24A1

Upon HPLC analysis, the metabolites of 1alpha,25$(OH)D_3$ by the human CYP24A1 showed five peaks as seen in figure 8. Of these, the retention times of four metabolites were identical with those of 1alpha,24*R*,25$(OH)_3D_3$, 24-oxo-1alpha,25$(OH)_2D_3$, 24-oxo-1alpha,23*S*, 25$(OH)_3D_3$ and 24,25,26,27-tetranor-1alpha,23$(OH)_2D_3$ as shown in the metabolites catalyzed by rat CYP24A1. However, the metabolite numbered 1 was not detected in the metabolites of 1alpha,25$(OH)_2D_3$ produced by rat CYP24A1 (27). The reverse phase HPLC peak with the same retention time as 1alpha,24*R*,25$(OH)_2D_3$ was separated into two peaks by the normal phase HPLC. The retention time of the metabolites coincided with those of authentic standards of 1alpha,23*S*,25$(OH)_3D_3$ and 1alpha,24*R*,25$(OH)_2D_3$. These results indicated that human CYP24A1 catalyzes both C-23-and C-24 hydroxylation pathways as reported by Beckman *et al*. (15) (figure 9). By mass spectrometry, the novel metabolite, peak 1 in figure 8A was analyzed to have fragment ions at 431 (M+H-H_2O), 415 (M+H-2 H_2O), 397 (M+H -3 H_2O), 379 (M+H -4 H_2O) and 361 (M+H -5 H_2O), suggesting that the metabolites was tetrahydroxylated product of vitamin D_3. It was produced from 1alpha,23*S*,25$(OH)_3D_3$ (h in figure 9) but not from 1alpha,24*R*,25$(OH)_3D_3$ (b in figure 9) (data not shown). These results strongly suggested that the metabolite may be 1alpha,23*S*,25,26$(OH)_3D_3$ (i in figure 9) which is the second metabolite of 1alpha,25$(OH)_2D_3$ in C-23 hydroxylation pathway (65).

Metabolism of 25$(OH)D_3$ by human CYP24A1 was also examined, and quite similar results were obtained (27). In order to examine the further metabolism of

23*S*,25,26(OH)$_3$D$_3$ by human CYP24A1, 23*S*,25,26(OH)$_3$D$_3$ (**i**) was added to the reconstituted system as a substrate. One metabolite was detected at the same retention time as 25(OH)D$_3$-26,23-lactone (k in figure 9). Unexpectedly, 25(OH)D$_3$-26,23-lactol (j) was not detected. When the chemically synthesized 25(OH)D$_3$-23,26-lactol was added to the reaction mixture and extracted with the organic solvent without reaction, most of 25(OH)D$_3$-23,26-lactol was lost probably due to unstable aldehyde formation of 25(OH)D$_3$-23,26-lactol (66). Finally, 25(OH)D$_3$-26,23-lactol was converted to 25(OH)D$_3$-26,23-lactone by human CYP24A1. These results strongly suggest that human CYP24A1 can catalyze all the steps of the C-23 hydroxylation pathway from 25(OH)D$_3$ through 23*S*,25(OH)$_2$D$_3$, 23*S*,25,26(OH)$_3$D$_3$ and 25(OH)D$_3$-26,23-lactol to 25(OH)D$_3$-26,23-lactone as seen in figure 9.

5.3. Kinetic studies of CYP24A1 for the metabolism of 25(OH)D$_3$ and 1alpha,25(OH)$_2$D$_3$

As described previously (27), CYP24A1 catalyzes sequential monooxygenation. To avoid the sequential monooxygenation, the activity was measured under very low concentrations of ADX and ADR. It should be noted that V_{max} value is strongly dependent on the concentration of ADX and ADR, and therefore apparently small activity of CYP24A1 is due to low concentrations of ADX and ADR. Under these conditions, only 24*R*-hydroxlation product and 23*S*-hydroxylation product by human CYP24A1 were detected as metabolites at a ratio of approximately 4 : 1. When the substrate concentration was varied, the reaction followed Michaelis-Menten type kinetics on 24*R*-hydroxylation. The K_m and V_{max} values of human CYP24A1 for 25(OH)D$_3$ determined with Hanes-Woolf plots were 0.16 μM and 0.088 (mol/min/mol P450). On the other hand, the K_m and V_{max} values for 1alpha,25(OH)$_2$D$_3$ were estimated to be 0.072 μM and 0.066 (mol/min/mol P450). Thus, physiologically essential parameter V_{max}/K_m value of human CYP24A1 for 1alpha,25(OH)$_2$D$_3$ was 1.7-fold higher than the V_{max}/K_m value for 25(OH)D$_3$ (27), which is similar to the results from rat CYP24A1 (14).

5.4. Metabolism of 25(OH)D$_3$ and 1alpha,25(OH)$_2$D$_3$ by CYP24A1 in living cell

On both 25(OH)D$_3$ and 1alpha,25(OH)$_2$D$_3$ substrates, the several metabolites by CYP24A1 were detected in *E. coli* cells expressing CYP24A1 without the coexpression of mitochondrial electron transfer partners. For instance, *E. coli* cells expressing rat CYP24A1 converted 25(OH)D$_3$ to the four metabolites 24*R*,25(OH)$_2$D$_3$, 24-oxo-25(OH)D$_3$, 24-oxo-23*S*,25(OH)$_2$D$_3$ and tetranor-23(OH)D$_3$. These results clearly indicated the existence of an electron donor to CYP24A1 in *E. coli* cells as in the case of CYP27B1.

For the enhancement of CYP24A1-dependent activity in living cells, we also constructed a coexpression plasmid for CYP24A1, bovine ADX and bovine ADR in a manner similar to CYP27B1. Upon the whole cell analyses of the 25(OH)D$_3$ metabolism using *E. coli* cells harboring human CYP24A1, bovine ADX and bovine ADR, the metabolites 23*S*,25(OH)$_2$D$_3$, 23*S*,25,26(OH)$_3$D$_3$, 25(OH)D$_3$-26,23-lactone in the C-23 hydroxylation pathway, and 24*R*,25(OH)$_2$D$_3$, 24-oxo-25(OH)D$_3$, 24-oxo-23*S*,25(OH)$_2$D$_3$ and 24,25,26,27-tetranor-23(OH)D$_3$ in the C-24 hydroxylation pathway were detected in the time-dependent manner in the engineered *E. coli* cells. The recombinant *E. coli* system also showed a series of 1alpha,25(OH)D$_3$ metabolites derived from the C-23 and C-24 pathways. Thus, this coexpression system appears to be quite useful because multiple metabolites of vitamin D analogs are readily obtained with the simple method.

5.5. Metabolism of vitamin D analogs by CYP24A1 and species-based difference between humans and rats

In this section, we introduce our recent studies on the metabolism of vitamin D analogs. These studies strongly suggest that the recombinant system harboring human CYP24A1 appears to be indispensable for prediction of the metabolism and efficacy of vitamin D analogs in human target tissues before clinical trials.

5.5.1. Metabolism of 26,26,26,27,27,27-hexafluoro-1alpha,25(OH)$_2$D$_3$

A vitamin D analog 26,26,26,27,27,27-hexafluoro-1alpha,25(OH)$_2$D$_3$ (F$_6$-1alpha,25(OH)$_2$D$_3$), which is now clinically used as a drug for secondary hyperparathyroidism, has been reported to be several times more potent than the parent compound at increasing intestinal calcium transport and bone calcium mobilization in vitamin D-deficient rats fed a low-calcium diet and at directly stimulating alkaline phosphatase activity in bone derived cells (67-69). Introduction of fluorine in the side chain increases the calcemic activity probably by increasing the half-life of the molecule. In the previous study (18), the distribution and metabolism of F$_6$-1alpha,25(OH)$_2$D$_3$ in bones of rats were compared with those of 1alpha,25(OH)$_2$D$_3$ by autoradiography and radio-HPLC. In the dosed groups, radioactivity was detected locally in the metaphysis, the modeling site in bones. Compared with the 1alpha,25(OH)$_2$D$_3$, F$_6$-1alpha,25(OH)$_2$D$_3$ was significantly retained in this site; moreover, it mainly persisted as the unchanged compound and F$_6$-1alpha,23*S*,25(OH)$_3$D$_3$.

Our previous study using *E. coli* expression system suggested that the metabolism of 1alpha,25(OH)$_2$D$_3$ in target tissues such as the kidney, small intestine, and bones may mostly depend on the activities of CYP24A1 (27, 28). Compared the metabolism of F$_6$-1alpha, 25(OH)$_2$D$_3$ in the *E. coli* cells co-expressing rat CYP24A1, bovine ADX and ADR with that in rat tissues, rat CYP24A1 clearly converted F$_6$-1alpha, 25(OH)$_2$D$_3$ to F$_6$-23-oxo-1alpha, 25(OH)$_2$D$_3$ (compound b) via F$_6$-1alpha, 23*S*,25(OH)$_3$D$_3$ (compound a in figure 10). The slower conversion rate from F$_6$-1alpha, 23*S*,25(OH)$_3$D$_3$ to F$_6$-23-oxo-1alpha, 25(OH)$_2$D$_3$ than that from F$_6$-1alpha, 25(OH)$_2$D$_3$ to F$_6$-1alpha, 23*S*,25(OH)$_3$D$_3$ caused the accumulation of F$_6$-1alpha, 23*S*,25(OH)$_3$D$_3$ (compound a). The reason for enhanced biologic activity in the kidney and small intestine appears to be related to F$_6$-1alpha,25(OH)$_2$D$_3$ metabolism to F$_6$-1alpha,23*S*,25(OH)$_3$D$_3$, a bioactive 23*S*-hydroxylated form

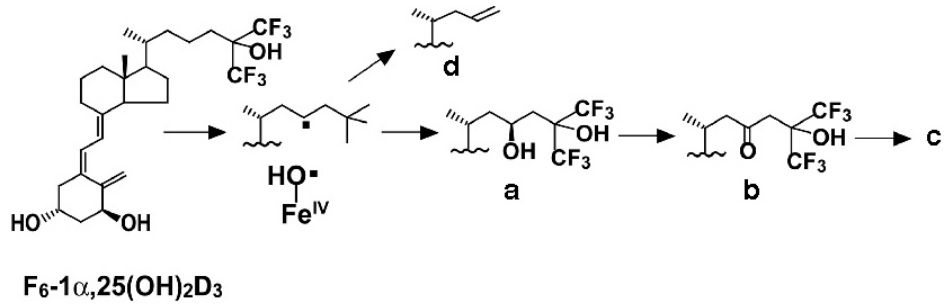

Figure 10. Metabolic pathway of F_6-1alpha,25$(OH)_2D_3$ by human CYP24A1 F_6-1alpha,25$(OH)_2D_3$ is converted to compound a, b, c, and d by human CYP24A1, whereas compound c is not detected in the metabolites by rat CYP24A1.

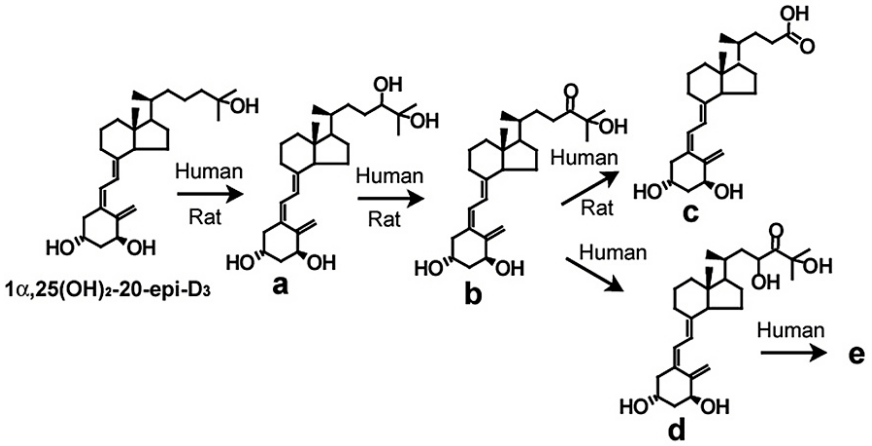

Figure 11. Species-dependent metabolism of 1alpha,25$(OH)_2$-20-epi-D_3 by CYP24A1. Metabolic pathways of 1alpha,25$(OH)_2$-20-epi-D_3 by rat and human CYP24A1 are schematically indicated. Compounds d and e were not detected in the metabolites by rat CYP24A1.

that is resistant to further metabolism (19, 70). In addition to two metabolites mentioned above, the putative ether compound (compound c in figure 10) with the same molecular mass as F_6-1alpha, 25$(OH)_2D_3$ was detected in the recombinant cells harboring human CYP24A1. The putative ether was not observed in the recombinant *E. coli* cells expressing rat CYP24A1. These results indicate the presence of species difference between human and rat CYP24A1 in the metabolism of F_6-1alpha, 25$(OH)_2D_3$. In addition, the metabolite (compound d in figure 10) with a cleavage at C_{24}-C_{25} bond of F_6-1alpha, 25$(OH)_2D_3$ was detected as a minor metabolite in both human and rat CYP24A1 (28). Although F_6-1alpha, 23*S*,25$(OH)_3D_3$ and F_6-23-oxo-1alpha, 25$(OH)_2D_3$ had a high affinity for vitamin D receptor, the side-chain cleaved metabolite and the putative ether showed extremely low affinity for vitamin D receptor. These findings indicate that human CYP24A1 has dual pathway for metabolic inactivation of F_6-1alpha,25$(OH)_2D_3$ while rat CYP24A1 has only one pathway. Judging from the fact that metabolism of F_6-1alpha, 25$(OH)_2D_3$ in rat CYP24A1-harboring *E. coli* cells is quite similar to that in the target tissues of rat, the metabolic profiles in human CYP24A1-harboring *E. coli* cells appear to be useful for the prediction of the metabolic pathways of the vitamin D analogs in human target tissues.

5.5.2 Metabolism of 20-epimer of 1alpha,25$(OH)_2D_3$

To date more than a thousand vitamin D analogs have been synthesized and their biological activity has been evaluated (17, 71, 72). Among them, one class of analogs, in which the stereochemistry at C-20 is inverted, have been particularly interested because of their unique biological property (73-81). Surprisingly, 1alpha,25$(OH)_2$-20-epi-D_3 showed higher affinity for VDR than the native 1alpha,25$(OH)_2D_3$ (78) in spite of significant change of the side-chain direction compared with native 1alpha,25$(OH)_2D_3$ (58, 82, 83). In addition, 1alpha,25$(OH)_2$-20-epi-D_3 have been shown 20-1000 fold enhanced ability to induce cell differentiation and growth inhibition, while their calcemic activity was only slightly elevated (73). We compared the human and rat CYP24A1-dependent metabolism of 1alpha,25$(OH)_2$-20-epi-D_3 by using the membrane fraction of the recombinant *E. coli* cells (29). The K_m values of rat CYP24A1 and human CYP24A1 for 1alpha,25$(OH)_2$-20-epi-D_3 were approximately 0.3 μM, which was significantly higher than those for 1alpha,25$(OH)_2D_3$. The V_{max}/K_m values of rat and human CYP24A1 for 1alpha,25$(OH)_2$-20-epi-D_3 were significantly reduced to 15% and 19% of those for 1alpha,25$(OH)_2D_3$ respectively. These results suggest that 1alpha,25$(OH)_2$-20-epi-D_3 would be metabolized more slowly than 1alpha,25$(OH)_2D_3$ in the body of rat and human. The rat CYP24A1 converted 1alpha,25$(OH)_2$-20-epi-D_3 to 25,26,27-trinor-1alpha(OH)-24(COOH)-20-epi-D_3 (c in figure 11) through 1alpha,24,25$(OH)_3$-20-epi-D_3 (a) and 1alpha,25$(OH)_2$-24-oxo-20-epi-D_3 (b in figure 11).

The binding affinity of trinor-1alpha(OH)-24(COOH)-20-epi-D_3 for vitamin D receptor (VDR) was less than 1/4,000 of that of 1alpha,25$(OH)_2$-20-epi-D_3. These results suggest that rat CYP24A1 can almost completely inactivate 1alpha,25$(OH)_2$-20-epi-D_3.

The human CYP24A1 converted 1alpha,25$(OH)_2$-20-epi-D_3 to 25,26,27-trinor-1alpha(OH)-24(COOH)-20-epi-D_3 (c in figure 11) and also to its putative demethylated compound (e in figure 11) via 1alpha,24,25$(OH)_3$-20-epi-D_3 (a), 1alpha,25$(OH)_2$-24-oxo-20-epi-D_3 (b) and 1alpha,23,25$(OH)_3$-24-oxo-20-epi-D_3 (d). It should be noted that (e) still has a considerable affinity for VDR, while (c) has no affinity for VDR. Thus, human CYP24A1 cannot completely inactivate 1alpha,25$(OH)_2$-20-epi-D_3. These results clearly demonstrate the species-based difference between human and rat on the CYP24A1-dependent metabolism of 1alpha,25$(OH)_2$-20-epi-D_3.

6. PERSPECTIVES

Recently, we have succeeded in the overexpression of mouse CYP27B1 at an expression level of 300 nmol/L culture by coexpression with Gro EL/ES, molecular chaperones involved in protein folding. The expression level is sufficient for preparation of large amounts of purified CYP27B1 hemoprotein to carry out structural analyses. Thus, structure-function study of CYP27B1 will make rapid progress by this expression system and the 3D model of CYP27B1 mentioned above.

We have found a novel metabolite of 1alpha,25$(OH)_2D_3$ by human CYP24A1 (84). The metabolite was identified as 25,26,27-trinor-23-ene-1alpha(OH)D_3 which appeared to be the same compound detected in the metabolism of F_6-1alpha, 25$(OH)_2D_3$. The C_{24}-C_{25} bond cleavage might occur by an unique reaction mechanism including radical rearrangement, because the metabolites contain no oxygen atoms at C-24 position. After hydrogen abstraction of C-23 position of 1alpha,25$(OH)_2D_3$, the radical intermediates seem to be partially converted into 25,26,27-trinor-23-ene-1alpha(OH)D_3, while the major portion of them may be converted into 1alpha,23*S*,25$(OH)_3D_3$. It should be noted that this metabolism is closely related to C-23 hydroxylation pathway and merely observed in the metabolism by rat CYP24A1. The combination of metabolic studies on series of vitamin D analogs, site-directed mutagenesis of CYP24A1, and molecular modeling of CYP24A1 by computer, would make it possible to understand substrate-recognition of CYP24A1 and species-based difference of CYP24A1 between humans and rats.

Recent X-ray analysis has revealed tertiary structure of mammalian microsomal CYPs (57, 85, 86). However, no reports on the tertiary structure of mitochondrial CYPs have yet been published. Thus, the most impressive study on CYP27A1, CYP27B1 and CYP24A1 appears to be their X-ray crystallographic analysis. In particular, the tertiary structure of CYP24A1 could provide useful information for the development of new vitamin D analogs for clinical use.

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Abbreviations: The abbreviations used are: VDR, vitamin D receptor; 25(OH)D_3, 25-hydroxyvitamin D_3; 1alpha,25(OH)D_3 , 1alpha,25-dihydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 23,25$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 24-oxo-25(OH)D_3 , 24-oxo-25-hydroxyvitamin D_3; 24-oxo-23,25$(OH)_2D_3$, 24-oxo-23,25-dihydroxyvitamin D_3; tetranor-23(OH)D_3, 24,25,26,27-tetranor-23-hydroxyvitamin D_3; ADX, adrenodoxin; ADR, NADPH-adrenodoxin reductase; P450, cytochrome P450.

Key Words: vitamin D, CYP27A1, CYP27B1, CYP24A1, cytochrome P450, coexpression, Reivew

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