

## METABOLISM OF VITAMIN D<sub>3</sub> BY CYTOCHROMES P450

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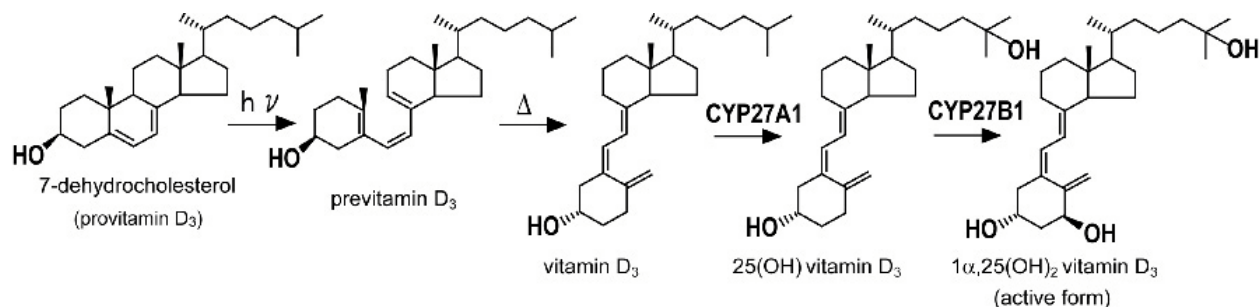
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### 1. ABSTRACT

The vitamin D<sub>3</sub> 25-hydroxylase (CYP27A1), 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase (CYP27B1) and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase (CYP24A1) are members of the cytochrome P450 superfamily, and key enzymes of vitamin D<sub>3</sub> 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<sub>3</sub> by the reconstituted system, CYP27A1 surprisingly produced at least seven forms of minor metabolites including 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in addition to the major metabolite 25(OH)D<sub>3</sub>. These results indicated that human CYP27A1 catalyzes multiple reactions involved in the vitamin D<sub>3</sub> metabolism. In contrast, CYP27B1 only catalyzes the hydroxylation at C-1 $\alpha$  position of 25(OH)D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>. Enzymatic studies on substrate specificity of CYP27B1 suggest that the 1 $\alpha$ -hydroxylase activity

of CYP27B1 requires the presence of 25-hydroxyl group of vitamin D<sub>3</sub> 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 1 $\alpha$ -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<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. Rat CYP24A1 catalyzed six sequential monooxygenation reactions that convert 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> into calcitric acid, a known final metabolite of C-24 oxidation pathway.

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**Figure 1.** Synthetic pathway of active form of vitamin D<sub>3</sub>. The schematic pathway of vitamin D<sub>3</sub> shows the conversion of 7-dehydrocholesterol to an active form of vitamin D<sub>3</sub> (1α,25(OH)<sub>2</sub>D<sub>3</sub>) by the action of light exposure (hν) 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 1α,25(OH)<sub>2</sub>D<sub>3</sub>-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<sub>3</sub> (cholecalciferol) is formed from 7-dehydrocholesterol by the exposure to ultraviolet light in the skin (figure 1). Vitamin D<sub>3</sub> undergoes two steps of hydroxylation. The first step occurs at 25 position in the liver, producing 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>). The second step occurs at the 1α position in the kidney, producing 1α, 25-dihydroxyvitamin D<sub>3</sub> (1α,25(OH)<sub>2</sub>D<sub>3</sub>), the most potent form of vitamin D<sub>3</sub>. This active form of vitamin D<sub>3</sub> 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, 1α,25(OH)<sub>2</sub>D<sub>3</sub> has been also found to be capable of regulating cell proliferation and differentiation.

Since Horsting and DeLuca (5) reported that vitamin D<sub>3</sub> is hydroxylated at 25-position in rat liver homogenates, subcellular localization of the 25-hydroxylation of vitamin D<sub>3</sub> 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<sub>3</sub> 25-hydroxylation although the majority of the 25-hydroxylation appears to undergo in mitochondria. In humans, we now believe that the vitamin D<sub>3</sub> 25-hydroxylation is catalyzed by CYP27A1 in

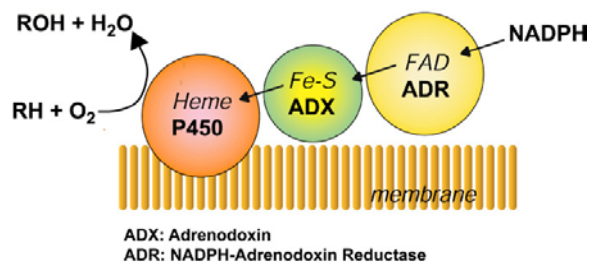
mitochondria and CYP2R1 (6) in microsomes although the microsomal 25-hydroxylase(s) may vary among species.

In the last two decades, extensive studies on characterization and purification of 25-hydroxyvitamin D<sub>3</sub> 1α-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 1α-hydroxylase at the protein level, the cDNAs encoding 1α-hydroxylase were cloned by several groups. Takeyama *et al.* (10) cloned a cDNA encoding mouse 25-hydroxyvitamin D<sub>3</sub> 1α-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.

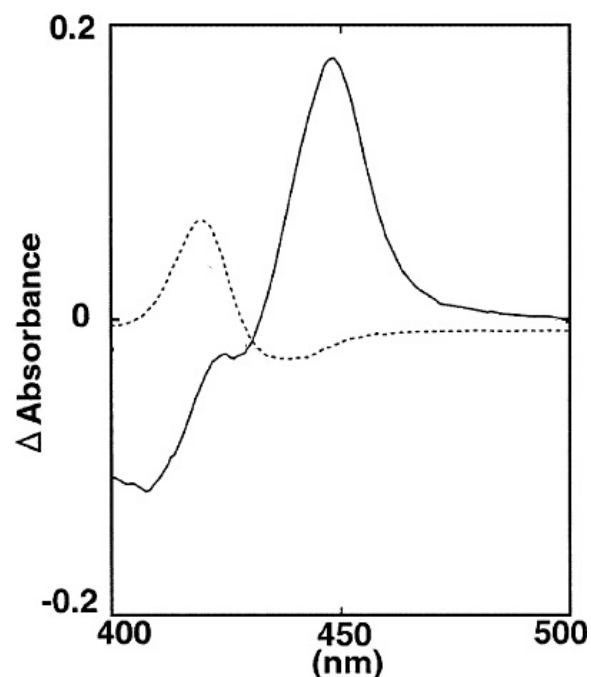
A large number of vitamin D<sub>3</sub> metabolites have been identified and implicated in the metabolic pathways of vitamin D<sub>3</sub>. Thus, the presence of a large number of enzymes had been assumed in the complicated vitamin D<sub>3</sub> metabolic map. However, many of the enzymes responsible 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<sub>3</sub>. Combined with studies of CYP27A1 and CYP27B1, these results finally lead us to the conclusion that the complicated metabolism of vitamin D<sub>3</sub> 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<sub>3</sub> analogs appears to be

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**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<sub>3</sub>. 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<sub>3</sub> 25-hydroxylases in mitochondria and microsomes

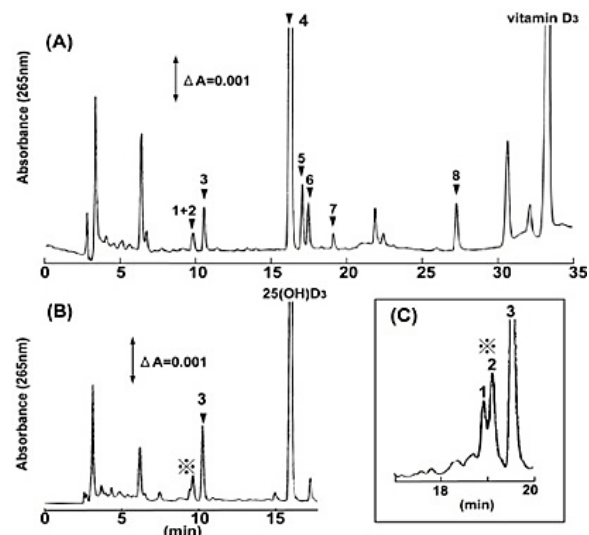
The 25-Hydroxylation of vitamin D<sub>3</sub> is catalyzed in the liver by mitochondrial CYP27A1, and a microsomal P450 whose identity in most species has not been determined. The existence of more than one enzyme raises the question of physiological significance between the mitochondrial enzyme and 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 cerebrotendinous 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-hydroxylation activity. Recently, Cheng *et al.* (6) demonstrated that human CYP2R1 catalyzes 25-hydroxylation of both vitamin D<sub>2</sub> and D<sub>3</sub>, 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<sub>3</sub> or 1 $\alpha$ (OH)D<sub>3</sub> 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<sub>3</sub> and 25-hydroxyvitamin D<sub>3</sub>

| Substrate              | Hydroxylation Position | K <sub>m</sub> (μM) | V <sub>max</sub> (mol/min/mol) | V <sub>max</sub> /K <sub>m</sub> (x 10 <sup>-3</sup> ) |
|------------------------|------------------------|---------------------|--------------------------------|--|
| vitamin D <sub>3</sub> | 25                     | 3.2±0.5             | 0.269±0.029                    | 93   |
|                        | 27                     | 4.6±2.1             | 0.036±0.002                    | 8  |
| 25(OH)D <sub>3</sub>   | 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<sub>3</sub> by CYP27A1. The reaction products of vitamin D<sub>3</sub> (A) and 25(OH)D<sub>3</sub> 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<sub>3</sub> by CYP27A1

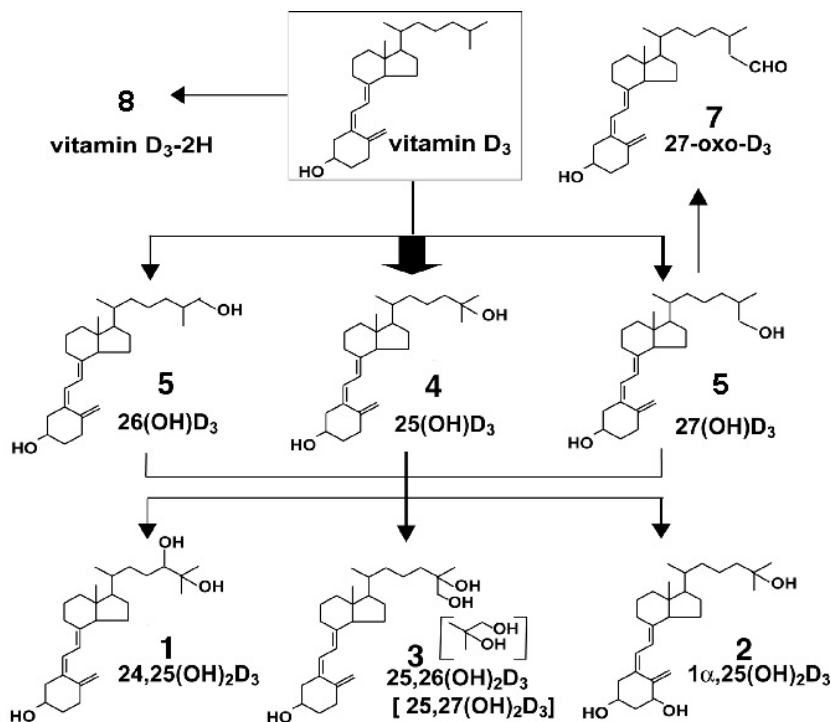
The reconstituted system containing bovine adrenodoxin (ADX), bovine adrenodoxin reductase (ADR), and the *E. coli* membrane fraction having CYP27A1, was used to investigate the metabolism of vitamin D<sub>3</sub>. HPLC analysis of the reaction products demonstrated the presence of at least eight metabolites (figure 4A). The major peak (peak 4 in figure 4) was identified as 25(OH)D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> and 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> (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<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> and 1alpha,25(OH)<sub>2</sub>D<sub>3</sub>, respectively. Mass spectrum of the metabolite 3 indicates that it is also dihydroxylated product of vitamin D<sub>3</sub>. Based on the report indicating that CYP27A1 secondly prefer the hydroxylation at C26/C27 position of vitamin D<sub>3</sub> (37), the metabolite 3 was postulated as 25,26(27)(OH)<sub>2</sub>D<sub>3</sub>. 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)<sub>2</sub>D<sub>3</sub> would be also produced because a considerable amount of 26(OH)D<sub>3</sub> was produced from vitamin D<sub>3</sub>. Furthermore, the metabolites, 1alpha,24*R*,25(OH)<sub>3</sub>D<sub>3</sub>, the putative 1alpha,25,26(OH)<sub>3</sub>D<sub>3</sub> and 1alpha,25,27(OH)<sub>3</sub>D<sub>3</sub> were observed when 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> was used as a substrate. Thus, the metabolite 3 appears to contain 25,26(OH)<sub>2</sub>D<sub>3</sub> and 25,27(OH)<sub>2</sub>D<sub>3</sub>. 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<sub>3</sub>. Based on the report by Dilwarth *et al.* (38), the metabolite 5 appeared to be a mixture of 26(OH)D<sub>3</sub> and 27(OH)D<sub>3</sub>. In our previous report (23), it was assumed that the metabolites 5 and 6 were 27(OH)D<sub>3</sub> and 26(OH)D<sub>3</sub>, respectively. However, the fragmentation pattern of the metabolite 6 by mass spectral analysis resembles that of 25(OH)D<sub>3</sub> 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<sub>3</sub>. Mass spectrum of the metabolite numbered 8 strongly suggests that the metabolite is a dehydrogenated form of vitamin D<sub>3</sub>.

### 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<sub>3</sub> and 1alpha(OH)D<sub>3</sub>. The *K<sub>m</sub>* and *V<sub>max</sub>* values were 3.2 μM and 0.27 (mol/min/mol P450) for vitamin D<sub>3</sub> and 6.9 μM and 0.79 (mol/min/mol P450) for 1alpha(OH)D<sub>3</sub>, respectively (Table 1). In addition, the *K<sub>m</sub>* and *V<sub>max</sub>* values for 1alpha-hydroxylation, 24-hydroxylation and 26(27)-hydroxylation toward 25(OH)D<sub>3</sub> were also estimated. Although these hydroxylation reactions showed significantly small *V<sub>max</sub>* values as compared with *V<sub>max</sub>* value of the 25-hydroxylation of vitamin D<sub>3</sub>, they showed similar *K<sub>m</sub>* values. It is noted that the determined *K<sub>m</sub>* value for 1alpha-hydroxylation toward 25(OH)D<sub>3</sub> was similar to the *K<sub>m</sub>* value for the same reaction catalyzed by CYP27B1 (24, 25) as described in the next session.

To date, the metabolism of vitamin D<sub>3</sub> has not been sufficiently analyzed, whereas Berginer *et al.* (39) reported a physiological significance of human CYP27A1-dependent vitamin D<sub>3</sub> 25-hydroxylation in patients with



**Figure 5.** Putative metabolic pathway of vitamin D<sub>3</sub> 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 1 $\alpha$ (OH)D<sub>3</sub> 25-hydroxylation catalyzed by rabbit CYP27A1, no kinetic studies showing  $K_m$  value for vitamin D<sub>3</sub> 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<sub>3</sub> metabolism. The  $K_m$  value of 3.2  $\mu$ M obtained from our studies suggests that CYP27A1 is the physiologically essential vitamin D<sub>3</sub> 25-hydroxylase in humans. Although the  $V_{max}$  value seems quite low, enough amounts of 25(OH)D<sub>3</sub> may be retained in blood circulation due to the long half-life of 25(OH)D<sub>3</sub> (41).

### 3.5. Metabolic pathway of vitamin D<sub>3</sub> by CYP27A1

The metabolic pathway of vitamin D<sub>3</sub> 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<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub> that are physiologically important (42-44).

The first step of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> production from vitamin D<sub>3</sub> appears to be the conversion from vitamin D<sub>3</sub> to 25(OH)D<sub>3</sub> judged by no detection of 1 $\alpha$ (OH)D<sub>3</sub> as a metabolite of vitamin D<sub>3</sub>. Thus, the 25-hydroxyl group

may be essential for the 1 $\alpha$ -hydroxylation catalyzed by CYP27A1 as CYP27B1 specifically catalyzes 1 $\alpha$ -hydroxylation of vitamin D derivatives having 25-hydroxyl group (24, 25). Axen *et al.* (45), reported the 1 $\alpha$ -hydroxylation of 25(OH)D<sub>3</sub> by CYP27A1 and indicated a possibility that CYP27A1 was a physiologically essential "25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase". However, the cDNA cloning and functional expression of CYP27B1 (10) clearly demonstrated that CYP27B1 is the essential 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase. Recent study of the megalin-mediated transport of 25(OH)D<sub>3</sub> in proximal of kidney cell also supported the CYP27B1-dependent mechanism of the production of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> level in spite of the complete defects of CYP27B1 genes. Judging from  $V_{max}/K_m$  values of CYP27A1-dependent vitamin D<sub>3</sub> 25-hydroxylation and 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylation, 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylation activity catalyzed by human CYP27A1 should not be neglected. The nearly normal 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> level in the serum of the patient mentioned above might be derived from CYP27A1-dependent 1 $\alpha$ -hydroxylation (38).

## 4. CYP27B1

### 4.1. Identification of 1 $\alpha$ -hydroxylase of 25-hydroxyvitamin D<sub>3</sub>

The 1 $\alpha$ -Hydroxylase of 25-hydroxyvitamin D<sub>3</sub> is a key enzyme in the determination of the serum level

**Table 2.** Kinetic parameters of mouse and human CYP27B1 for 1 $\alpha$ -hydroxylation of 25(OH)D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>

| Substrate                              | K <sub>m</sub> (μM) | V <sub>max</sub> <sup>a</sup> | V <sub>max</sub> /K <sub>m</sub> |
|--|---------------------|-------------------------------|----------------------------------|
| mouse CYP27B1                          |                     |                               |                                  |
| 25(OH)D <sub>3</sub>                   | 2.7                 | 9.5                           | 3.5                              |
| 24R,25(OH) <sub>2</sub> D <sub>3</sub> | 1.3                 | 16.7                          | 12.3                             |
| human CYP27B1                          |                     |                               |                                  |
| 25(OH)D <sub>3</sub>                   | 2.7                 | 3.9                           | 1.4                              |
| 24R,25(OH) <sub>2</sub> D <sub>3</sub> | 1.1                 | 3.2                           | 2.9                              |

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

**Table 3.** Vitamin D<sub>3</sub> derivatives and 1 $\alpha$ -hydroxylase activities of mouse and human CYP27B1

| 1 $\alpha$ -Hydroxylase Activity<br>(pmol product/min/mg protein) |                  |                  |
|---|------------------|------------------|
| Substrate   | mouse<br>CYP27B1 | human<br>CYP27B1 |
| vitamin D <sub>3</sub>  | <0.05            | <0.05            |
| 25(OH)D <sub>3</sub>  | 1.5              | 0.6              |
| 24R,25(OH) <sub>2</sub> D <sub>3</sub>                            | 4.3              | 1.5              |
| 23S,25(OH) <sub>2</sub> D <sub>3</sub>                            | 0.2              | <0.1             |
| 24-oxo-25(OH)   | 1.1              | 0.4              |
| 24-oxo-23S,25(OH) <sub>2</sub> D <sub>3</sub>                     | <0.1             | <0.05            |
| tetranor-23(OH)D <sub>3</sub>                                     | <0.05            | <0.05            |

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

of 1 $\alpha$ ,25-dihydroxyvitamin. In the last 20 years, characterization and purification of 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, suggesting the excessive 1 $\alpha$ -hydroxylase expression. Therefore, they attempted the cDNA cloning of 1 $\alpha$ -hydroxylase with poly (A)<sup>+</sup> RNA prepared from the kidneys of VDR knockout mice using the nuclear receptor-mediated expression system. The isolated cDNA clone encoding 25(OH)D<sub>3</sub> 1 $\alpha$ -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 1 $\alpha$ -hydroxylase as a member of P450 superfamily (49). Using the mouse cDNA as a probe, human 1 $\alpha$ -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 1 $\alpha$ -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<sub>3</sub> 1 $\alpha$ -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 1 $\alpha$ -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 1 $\alpha$ -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.

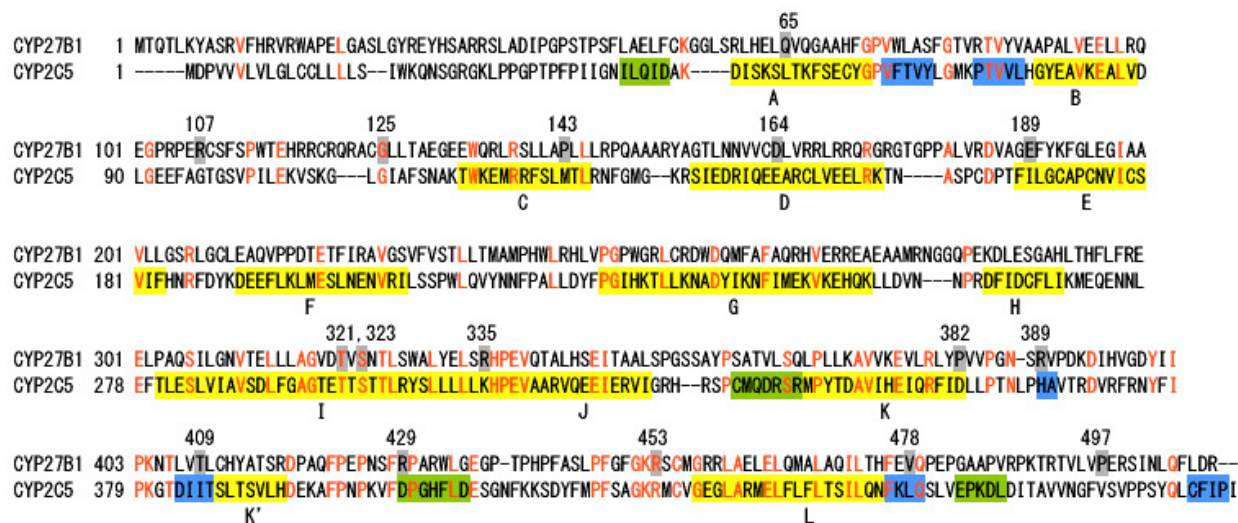
#### 4.3. Kinetic analysis using *in vitro* reconstitution system

When 25(OH)D<sub>3</sub> concentration was varied, the reaction followed Michaelis-Menten type kinetics on 1 $\alpha$ -hydroxylation. The K<sub>m</sub> and V<sub>max</sub> values of mouse CYP27B1 were 2.7 μM and 9.5 pmol product/mg protein/min for 25(OH)D<sub>3</sub>, and 1.3 μM and 16.7 pmol product/mg protein/min for 24R, 25(OH)<sub>2</sub>D<sub>3</sub>, respectively (Table 2). On the other hand, the K<sub>m</sub> and V<sub>max</sub> values of human CYP27B1 were 2.7 μM and 3.9 pmol product/mg protein/min for 25(OH)D<sub>3</sub>, and 1.1 and 3.2 pmol product/mg protein/min for 24R, 25(OH)<sub>2</sub>D<sub>3</sub>, respectively. The difference of V<sub>max</sub> 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<sub>m</sub> values. Thus, V<sub>max</sub>/K<sub>m</sub> is a physiologically important parameter. It was noticed that the V<sub>max</sub>/K<sub>m</sub> values of mouse and human CYP27B1 for 24R,25(OH)<sub>2</sub>D<sub>3</sub> were higher than the corresponding V<sub>max</sub>/K<sub>m</sub> values for 25(OH)D<sub>3</sub>. These results indicated that 24R,25(OH)<sub>2</sub>D<sub>3</sub> is a better substrate than 25(OH)D<sub>3</sub> for both mouse and human CYP27B1. The preferential 1 $\alpha$ -hydroxylation of 24R,25(OH)<sub>2</sub>D<sub>3</sub> is not rationally understood at this time, since 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is known to be the most potent form of vitamin D<sub>3</sub>.

#### 4.4. Substrate specificity of CYP27B1

The substrates, 23S,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25(OH)D<sub>3</sub>, 24-oxo-23,25(OH)<sub>2</sub>D<sub>3</sub>, and tetranor-23-(OH)D<sub>3</sub>, which are known as metabolites of 25(OH)D<sub>3</sub> catalyzed by 24-hydroxylase (CYP27A1) (14, 15), were examined for the 1 $\alpha$ -hydroxylase activity in the reconstituted system. Mouse and human CYP27B1 showed only trace and no detectable activity toward 23S,25(OH)<sub>2</sub>D<sub>3</sub> (Table 3), respectively. On the substrate 24-oxo-25(OH)D<sub>3</sub>, the product was observed at the same retention times as the authentic standard of 24-oxo-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. The 1 $\alpha$ -hydroxylation of 24-oxo-25(OH)D<sub>3</sub> by CYP27B1 was catalyzed as efficiently as that of 25(OH)D<sub>3</sub>,

## Metabolism of vitamin D by P450



**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-23S,25(OH)<sub>2</sub>D<sub>3</sub> too low to quantify. Both mouse and human CYP27B1 showed no detectable activity towards vitamin D<sub>3</sub> and tetranor-23(OH)D<sub>3</sub>, both of which have no 25-hydroxyl group. These results suggest that the 25-hydroxyl group of vitamin D<sub>3</sub> 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<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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<sub>3</sub> was added to cell culture of the recombinant *E. coli* cells, the production of 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub>.

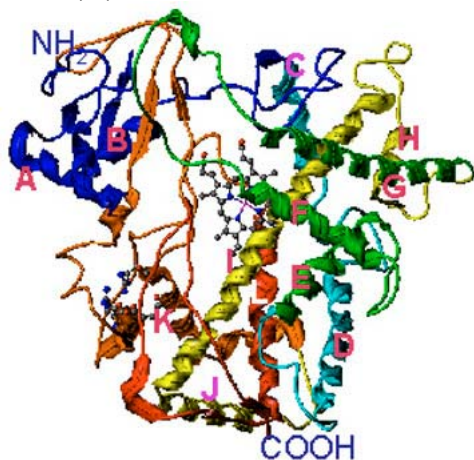
### 4.6. Structure-function analysis of CYP27B1 based on the analysis of CYP27B1 mutants from VDDR-I

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<sub>3</sub>. 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 1 $\alpha$ -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 Asp-184 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 1 $\alpha$ ,25(OH) $_2$ D $_3$ , Ohyama *et al.*, (55, 59) purified and determined the amino-terminal 8 amino acid



sequence of vitamin D<sub>3</sub> 24-hydroxylase from kidneys of rats treated with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. The purified sample showed a high 24-hydroxylase activity toward 25(OH)D<sub>3</sub> with  $K_m$  value of 2.8  $\mu$ M. 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<sub>3</sub> 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<sub>3</sub> from 25(OH)D<sub>3</sub> 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<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> but also two steps of subsequent monooxygenation reactions (1). In this section, we describe a remarkable metabolism of 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by CYP24A1 and species-based difference of CYP24A1-dependent vitamin D metabolism between humans and rat.

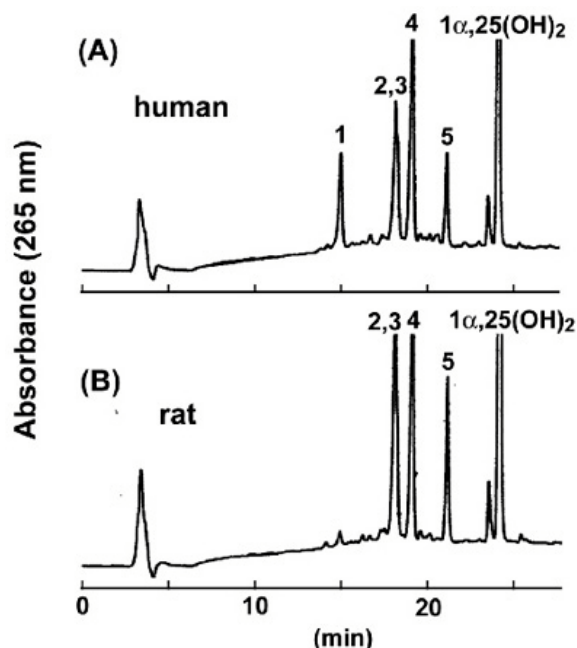
## 5.2. Metabolism of 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by CYP24A1

### 5.2.1. Rat CYP24A1

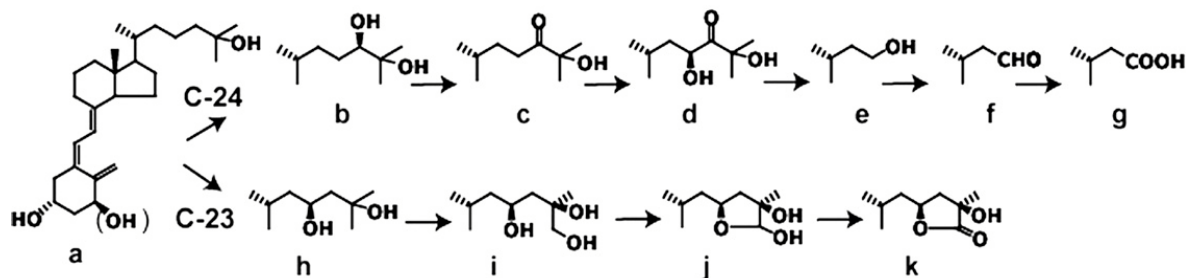
Using the *in vitro* reconstituted system with the membrane fraction, catalytic abilities of rat CYP24A1 were investigated for the metabolism of 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. The four metabolites, 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25(OH)D<sub>3</sub>, 24-oxo-23 $S$ ,25(OH)<sub>2</sub>D<sub>3</sub> and tetranor-23(OH)D<sub>3</sub> were detected in the time-dependent manner

when 25(OH)D<sub>3</sub> was used as a substrate. The detailed analyses of time course in the 25(OH)D<sub>3</sub> metabolism strongly suggest the sequential conversion of 25(OH)D<sub>3</sub> through 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25(OH)D<sub>3</sub>, and 24-oxo-23 $S$ ,25(OH)<sub>2</sub>D<sub>3</sub> to tetranor-23(OH)D<sub>3</sub> by CYP24A1 (14, 27). This metabolic pathway by CYP24A1 was further supported by the investigation using 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25(OH)D<sub>3</sub>, and 24-oxo-23 $S$ ,25(OH)<sub>2</sub>D<sub>3</sub> as substrates. It was noted that 24-oxo-23 $S$ ,25(OH)<sub>2</sub>D<sub>3</sub> and tetranor-23(OH)D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and produced 1 $\alpha$ ,24 $R$ ,25(OH)<sub>3</sub>D<sub>3</sub>, 24-oxo-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, and 24-oxo-1 $\alpha$ ,23 $S$ ,25(OH)<sub>3</sub>D<sub>3</sub> together with tetranor-23-oxo-1 $\alpha$ (OH)D<sub>3</sub>, tetranor-1 $\alpha$ ,23(OH)<sub>2</sub>D<sub>3</sub> and calcitroic acid (27). These results suggest that CYP24A1 catalyzes six-steps of sequential monooxygenation toward 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> to yield calcitroic acid. Utilizing tetranor-1 $\alpha$ ,23(OH)<sub>2</sub>D<sub>3</sub> as a substrate, the last two steps to calcitroic acid were confirmed to be the CYP24A1-dependent reactions. Five compounds, 1 $\alpha$ ,24 $R$ ,25(OH)<sub>3</sub>D<sub>3</sub>, 24-oxo-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-1 $\alpha$ ,23 $S$ ,25(OH)<sub>3</sub>D<sub>3</sub>, tetranor-1 $\alpha$ ,23(OH)<sub>2</sub>D<sub>3</sub> and calcitroic acid were observed as metabolites of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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-1 $\alpha$ (OH)D<sub>3</sub> was found to be a metabolic intermediate which has not been observed in physiological conditions probably due to its instability. The metabolic pathway of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by CYP24A1 elucidated in our study would occur in physiological conditions to yield the final product calcitroic acid. Induction of CYP24A1 by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (63) and complete inactivation of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by CYP24A1 appears to be a rational mechanism for precisely regulating the level of the active form of vitamin D<sub>3</sub>. Thus, the most significant role of CYP24A1 may be to degrade the active form of vitamin D<sub>3</sub>.

Physiologically, 25(OH)D<sub>3</sub> is also an important substrate for CYP24A1 because the concentration of 25(OH)D<sub>3</sub> in serum is 100-1000-fold higher than 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, and the product 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub>. As mentioned above, 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub> is a better substrate for CYP27B1 with a higher value of  $V_{max}/K_m$  than 25(OH)D<sub>3</sub> (24). Since 1 $\alpha$ -hydroxylated product of 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,24 $R$ ,25(OH)<sub>3</sub>D<sub>3</sub>, can function as a ligand of vitamin D<sub>3</sub> receptor, 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub> is also valuable as a substrate of CYP27B1. The sequential monooxygenation reactions are necessary for the complete inactivation and excretion of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, while a step of monooxygenation by CYP24A1 produces 24 $R$ ,25(OH)<sub>2</sub>D<sub>3</sub> from 25(OH)D<sub>3</sub>. Thus, the lower  $K_m$  and the several sequential monooxygenation steps for



**Figure 8.** HPLC profiles of  $1\alpha,25(\text{OH})_2\text{D}_3$  and its metabolites by human CYP24A1 (A) and rat CYP24A1 (B). The metabolite peak 1,  $1\alpha,23\text{S},25,26(\text{OH})_4\text{D}_3$ ; 2,  $24\text{-oxo-}1\alpha,23\text{S},25(\text{OH})_3\text{D}_3$ ; 3, tetranor- $1\alpha,23(\text{OH})_2\text{D}_3$ ; 4,  $1\alpha,24\text{R},25(\text{OH})_3\text{D}_3$  and  $1\alpha,23\text{S},25(\text{OH})_3\text{D}_3$ ; 5,  $24\text{-oxo-}1\alpha,23\text{S},25(\text{OH})_3\text{D}_3$ .



**Figure 9.** Metabolic pathways of  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  by human CYP24A1. The scheme represents C-23 and C-24 consecutive hydroxylation pathways of  $25(\text{OH})_2\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  catalyzed by a single P450 CYP24A1.

$1\alpha,25(\text{OH})_2\text{D}_3$  as compared with  $25(\text{OH})\text{D}_3$  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  $1\alpha,25(\text{OH})_2\text{D}_3$  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  $1\alpha,24\text{R},25(\text{OH})_3\text{D}_3$ ,  $24\text{-oxo-}1\alpha,25(\text{OH})_2\text{D}_3$ ,  $24\text{-oxo-}1\alpha,23\text{S},25(\text{OH})_3\text{D}_3$  and  $24,25,26,27\text{-tetranor-}1\alpha,23(\text{OH})_2\text{D}_3$  as shown in the metabolites catalyzed by rat CYP24A1. However, the metabolite numbered 1 was not detected in the metabolites of  $1\alpha,25(\text{OH})_2\text{D}_3$  produced by rat CYP24A1 (27). The reverse phase HPLC peak with the same retention time as  $1\alpha,24\text{R},25(\text{OH})_2\text{D}_3$  was separated into two peaks by the normal phase HPLC. The retention time of the metabolites

coincided with those of authentic standards of  $1\alpha,23\text{S},25(\text{OH})_3\text{D}_3$  and  $1\alpha,24\text{R},25(\text{OH})_2\text{D}_3$ . 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 ( $\text{M}+\text{H}-\text{H}_2\text{O}$ ), 415 ( $\text{M}+\text{H}-2\text{H}_2\text{O}$ ), 397 ( $\text{M}+\text{H}-3\text{H}_2\text{O}$ ), 379 ( $\text{M}+\text{H}-4\text{H}_2\text{O}$ ) and 361 ( $\text{M}+\text{H}-5\text{H}_2\text{O}$ ), suggesting that the metabolites was tetrahydroxylated product of vitamin  $\text{D}_3$ . It was produced from  $1\alpha,23\text{S},25(\text{OH})_3\text{D}_3$  (h in figure 9) but not from  $1\alpha,24\text{R},25(\text{OH})_3\text{D}_3$  (b in figure 9) (data not shown). These results strongly suggested that the metabolite may be  $1\alpha,23\text{S},25,26(\text{OH})_4\text{D}_3$  (i in figure 9) which is the second metabolite of  $1\alpha,25(\text{OH})_2\text{D}_3$  in C-23 hydroxylation pathway (65).

Metabolism of  $25(\text{OH})\text{D}_3$  by human CYP24A1 was also examined, and quite similar results were obtained (27). In order to examine the further metabolism of

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23*S*,25,26(OH)<sub>3</sub>D<sub>3</sub> by human CYP24A1, 23*S*,25,26(OH)<sub>3</sub>D<sub>3</sub> (i) was added to the reconstituted system as a substrate. One metabolite was detected at the same retention time as 25(OH)D<sub>3</sub>-26,23-lactone (k in figure 9). Unexpectedly, 25(OH)D<sub>3</sub>-26,23-lactol (j) was not detected. When the chemically synthesized 25(OH)D<sub>3</sub>-23,26-lactol was added to the reaction mixture and extracted with the organic solvent without reaction, most of 25(OH)D<sub>3</sub>-23,26-lactol was lost probably due to unstable aldehyde formation of 25(OH)D<sub>3</sub>-23,26-lactol (66). Finally, 25(OH)D<sub>3</sub>-26,23-lactol was converted to 25(OH)D<sub>3</sub>-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<sub>3</sub> through 23*S*,25(OH)<sub>2</sub>D<sub>3</sub>, 23*S*,25,26(OH)<sub>3</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>-26,23-lactol to 25(OH)D<sub>3</sub>-26,23-lactone as seen in figure 9.

### 5.3. Kinetic studies of CYP24A1 for the metabolism of 25(OH)D<sub>3</sub> and 1alpha,25(OH)<sub>2</sub>D<sub>3</sub>

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*-hydroxylation 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<sub>3</sub> determined with Hanes-Woolf plots were 0.16  $\mu$ M and 0.088 (mol/min/mol P450). On the other hand, the  $K_m$  and  $V_{max}$  values for 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> were estimated to be 0.072  $\mu$ M and 0.066 (mol/min/mol P450). Thus, physiologically essential parameter  $V_{max}/K_m$  value of human CYP24A1 for 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> was 1.7-fold higher than the  $V_{max}/K_m$  value for 25(OH)D<sub>3</sub> (27), which is similar to the results from rat CYP24A1 (14).

### 5.4. Metabolism of 25(OH)D<sub>3</sub> and 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> by CYP24A1 in living cell

On both 25(OH)D<sub>3</sub> and 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> 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<sub>3</sub> to the four metabolites 24*R*,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25(OH)D<sub>3</sub>, 24-oxo-23*S*,25(OH)<sub>2</sub>D<sub>3</sub> and tetranor-23(OH)D<sub>3</sub>. 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<sub>3</sub> metabolism using *E. coli* cells harboring human CYP24A1, bovine ADX and bovine ADR, the

metabolites 23*S*,25(OH)<sub>2</sub>D<sub>3</sub>, 23*S*,25,26(OH)<sub>3</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>-26,23-lactone in the C-23 hydroxylation pathway, and 24*R*,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25(OH)D<sub>3</sub>, 24-oxo-23*S*,25(OH)<sub>2</sub>D<sub>3</sub> and 24,25,26,27-tetranor-23(OH)D<sub>3</sub> 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<sub>3</sub> 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.

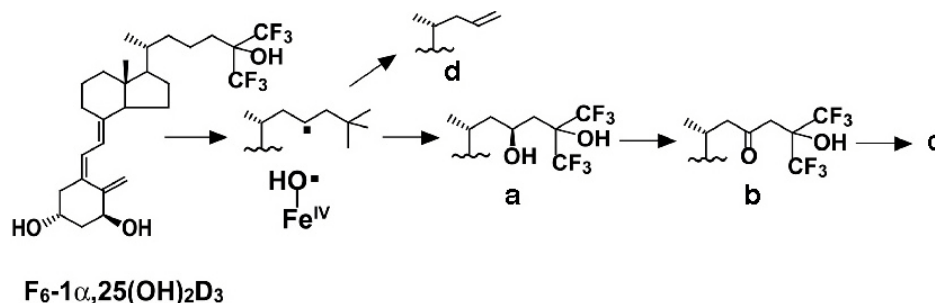
### 5.5. Metabolism of vitamin D analogs by CYP24A1 and species-based difference between humans and rats

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.

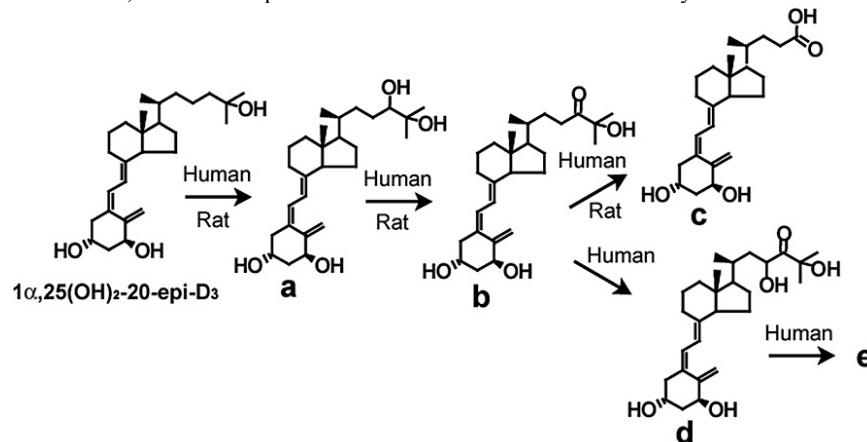
#### 5.5.1. Metabolism of 26,26,26,27,27,27-hexafluoro-1alpha,25(OH)<sub>2</sub>D<sub>3</sub>

A vitamin D analog 26,26,26,27,27,27-hexafluoro-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> (F<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub>), 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<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> in bones of rats were compared with those of 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub>, F<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> was significantly retained in this site; moreover, it mainly persisted as the unchanged compound and F<sub>6</sub>-1alpha,23*S*,25(OH)<sub>3</sub>D<sub>3</sub>.

Our previous study using *E. coli* expression system suggested that the metabolism of 1alpha,25(OH)<sub>2</sub>D<sub>3</sub> 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<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> in the *E. coli* cells co-expressing rat CYP24A1, bovine ADX and ADR with that in rat tissues, rat CYP24A1 clearly converted F<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> to F<sub>6</sub>-23-oxo-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> (compound b) via F<sub>6</sub>-1alpha,23*S*,25(OH)<sub>3</sub>D<sub>3</sub> (compound a in figure 10). The slower conversion rate from F<sub>6</sub>-1alpha,23*S*,25(OH)<sub>3</sub>D<sub>3</sub> to F<sub>6</sub>-23-oxo-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> than that from F<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> to F<sub>6</sub>-1alpha,23*S*,25(OH)<sub>3</sub>D<sub>3</sub> caused the accumulation of F<sub>6</sub>-1alpha,23*S*,25(OH)<sub>3</sub>D<sub>3</sub> (compound a). The reason for enhanced biologic activity in the kidney and small intestine appears to be related to F<sub>6</sub>-1alpha,25(OH)<sub>2</sub>D<sub>3</sub> metabolism to F<sub>6</sub>-1alpha,23*S*,25(OH)<sub>3</sub>D<sub>3</sub>, a bioactive 23*S*-hydroxylated form



**Figure 10.** Metabolic pathway of F<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by human CYP24A1. F<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> by CYP24A1. Metabolic pathways of 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> 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<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. In addition, the metabolite (compound d in figure 10) with a cleavage at C<sub>24</sub>-C<sub>25</sub> bond of F<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> was detected as a minor metabolite in both human and rat CYP24A1 (28). Although F<sub>6</sub>-1 $\alpha$ ,23S,25(OH)<sub>3</sub>D<sub>3</sub> and F<sub>6</sub>-23-oxo-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> while rat CYP24A1 has only one pathway. Judging from the fact that metabolism of F<sub>6</sub>-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>

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, 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> showed higher affinity for VDR than the native 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (78) in spite of significant change of the side-chain direction compared with native 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (58, 82, 83). In addition, 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> by using the membrane fraction of the recombinant *E. coli* cells (29). The *K<sub>m</sub>* values of rat CYP24A1 and human CYP24A1 for 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> were approximately 0.3  $\mu$ M, which was significantly higher than those for 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. The *V<sub>max</sub>*/*K<sub>m</sub>* values of rat and human CYP24A1 for 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> were significantly reduced to 15% and 19% of those for 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> respectively. These results suggest that 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> would be metabolized more slowly than 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in the body of rat and human. The rat CYP24A1 converted 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> to 25,26,27-trinor-1 $\alpha$ (OH)-24(COOH)-20-epi-D<sub>3</sub> (c in figure 11) through 1 $\alpha$ ,24,25(OH)<sub>3</sub>-20-epi-D<sub>3</sub> (a) and 1 $\alpha$ ,25(OH)<sub>2</sub>-24-oxo-20-epi-D<sub>3</sub> (b in figure 11).

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The binding affinity of trinor-1 $\alpha$ (OH)-24(COOH)-20-epi-D<sub>3</sub> for vitamin D receptor (VDR) was less than 1/4,000 of that of 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub>. These results suggest that rat CYP24A1 can almost completely inactivate 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub>.

The human CYP24A1 converted 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub> to 25,26,27-trinor-1 $\alpha$ (OH)-24(COOH)-20-epi-D<sub>3</sub> (c in figure 11) and also to its putative demethylated compound (e in figure 11) via 1 $\alpha$ ,24,25(OH)<sub>3</sub>-20-epi-D<sub>3</sub> (a), 1 $\alpha$ ,25(OH)<sub>2</sub>-24-oxo-20-epi-D<sub>3</sub> (b) and 1 $\alpha$ ,23,25(OH)<sub>3</sub>-24-oxo-20-epi-D<sub>3</sub> (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 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub>. These results clearly demonstrate the species-based difference between human and rat on the CYP24A1-dependent metabolism of 1 $\alpha$ ,25(OH)<sub>2</sub>-20-epi-D<sub>3</sub>.

## 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by human CYP24A1 (84). The metabolite was identified as 25,26,27-trinor-23-ene-1 $\alpha$ (OH)D<sub>3</sub> which appeared to be the same compound detected in the metabolism of F<sub>6</sub>-1 $\alpha$ , 25(OH)<sub>2</sub>D<sub>3</sub>. The C<sub>24</sub>-C<sub>25</sub> bond cleavage might occur by a 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, the radical intermediates seem to be partially converted into 25,26,27-trinor-23-ene-1 $\alpha$ (OH)D<sub>3</sub>, while the major portion of them may be converted into 1 $\alpha$ ,23,25(OH)<sub>3</sub>D<sub>3</sub>. 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<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>; 1alpha,25(OH)D<sub>3</sub>, 1alpha,25-dihydroxyvitamin D<sub>3</sub>; 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25-dihydroxyvitamin D<sub>3</sub>; 23,25(OH)<sub>2</sub>D<sub>3</sub>, 23,25-dihydroxyvitamin D<sub>3</sub>; 24-oxo-25(OH)D<sub>3</sub>, 24-oxo-25-hydroxyvitamin D<sub>3</sub>; 24-oxo-23,25(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-23,25-dihydroxyvitamin D<sub>3</sub>; tetranor-23(OH)D<sub>3</sub>, 24,25,26,27-tetranor-23-hydroxyvitamin D<sub>3</sub>; ADX, adrenodoxin; ADR, NADPH-adrenodoxin reductase; P450, cytochrome P450.

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

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