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EIGHT CYTOCHROME P450S CATALYZE VITAMIN D METABOLISM

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1. ABSTRACT

Vitamin D_3 plays a central role in calcium and phosphate homeostasis and is essential for the proper development and maintenance of bone. To exert its biological activities, vitamin D_3 has to receive enzymatic transformation to the active form, 1,25-dihydroxyvitamin D_3. The first step is the 25-hydroxylation reaction in the liver that produces 25-hydroxyvitamin D_3, the major circulating form of vitamin D_3. The 25-hydroxylation reaction is the prerequisite step for the subsequent 1α-hydroxylation and 24-hydroxylation reactions in the kidney. The 1α-hydroxylation reaction produces the active form of vitamin D_3, whereas 24-hydroxylation reaction leads to inactivation. Both reactions are strictly controlled by parathyroid hormone, 1,25-dihydroxyvitamin D_3, and calcium in a reciprocal manner in the kidney. At present, six cytochrome P450s (CYP2C11, 27A1, 2D25, 2R1, 3A4, and 2J3) are found to exhibit vitamin D 25-hydroxylation activities, and CYP27B1 and CYP24 are proved to be 1α-hydroxylase and 24-hydroxylase, respectively. The main focus of this review is to summarize the properties of individual P450 in light of their catalytic activities to understand their physiological significance.

2. INTRODUCTION

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), regulates calcium homeostasis, controls cell growth and differentiation, and modifies immune responses. The action of 1,25-$(OH)_2D_3$ is mediated primarily through interaction with the intracellular vitamin D receptor in the target cells, which modulates the expression of specific genes involved in the action of vitamin D_3 (1).

Vitamin D has been identified as an anti-rickets factor and established as an essential nutrient. However, vitamin D_3 is also produced in the skin by an ultraviolet light-induced photolytic conversion of 7-dehydrocholesterol to previtamin D_3 followed by thermal isomerization to vitamin D_3 (Figure 1). Vitamin D_3 is inert and must be activated to exert its biological function. The first hydroxylation occurs at the C-25 position in the liver to produce 25-hydroxyvitamin D_3 (25-OH-D_3) (15-60 ng/ml), which is the major circulating form of vitamin D_3 bound to vitamin D binding protein. The subsequent hydroxylation of 25-OH-D_3 at C-1α occurs mainly in the kidney and results in the synthesis of the hormonally active 1,25-$(OH)_2D_3$ (20-60 pg/ml) (2) (Figure 2). The finding of production of vitamin D_3 in the skin along with the metabolic activation by specific enzymes and the interaction with a cognate receptor of the active metabolite has changed the view of vitamin D from a vitamin to a group of steroid hormones.

All the processes in the vitamin D_3 metabolism are catalyzed by certain cytochrome P450 enzymes (Figure 2). The reactions that are most tightly regulated are 1α-hydroxylation by CYP27B1 and 24-hydroxylation by CYP24 in the kidney, the major organ of CYP27B1 and CYP24 expression. *CYP27B1* gene expression is stimulated by low serum calcium, low serum phosphorus, and parathyroid hormone (PTH), but is suppressed by high levels of 1,25-$(OH)_2D_3$. In contrast, PTH, low serum calcium, and low serum phosphorus suppress *CYP24* gene expression and 1,25-$(OH)_2D_3$ strongly induces *CYP24* gene expression (2, 3).

Cytochrome P450s, members of the CYP gene superfamily, are found in most organisms such as bacteria, plants, and animals, According to the nomenclature system

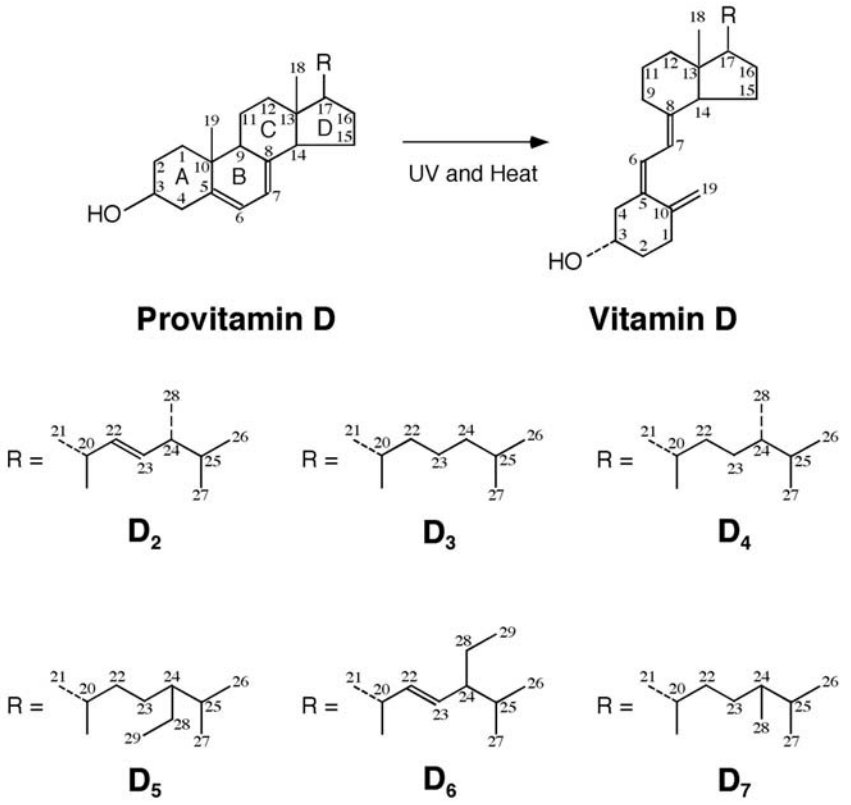

Figure 1. Structure of vitamin D. Vitamin D (9,10-secosterol) is derived from provitamin D (sterol) through the chemical reactions by UV light and heat. Six vitamin D compounds (D_2-D_7) with different side chain structures have been isolated. Vitamin D_2 and vitamin D_3 are the compounds showing biological activities on calcium homeostasis.

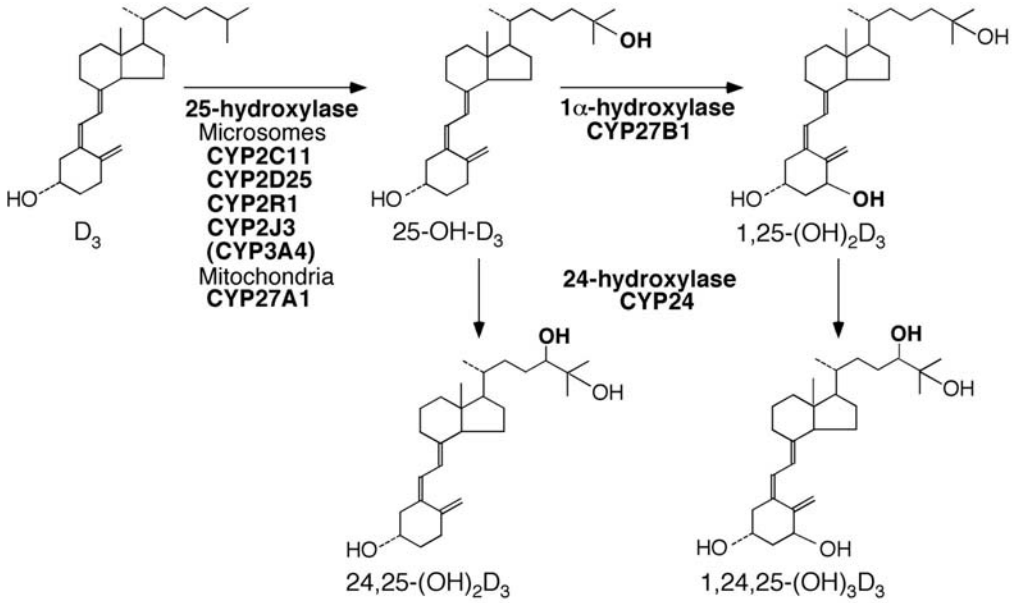

Figure 2. Cytochrome P450s involved in vitamin D_3 metabolism. Conversion of vitamin D_3 to 25-OH-D_3 by 25-hydroxylases is the initial step in the activation process. 25-OH-D_3 is subsequently hydroxylated at C-1α by 1α-hydroxylase in the kidney. CYP2C11 (male rat microsomes), CYP2D25 (pig microsomes), CYP2R1 (microsomes), CYP3A4 (human microsomes), CYP2J3 (rat microsomes), and CYP27A1 (mitochondria) have been reported as vitamin D 25-hydroxylases. CYP27B1 (mitochondria) catalyzing 1α-hydroxylation reaction of 25-OH-D_3 is the essential enzyme in the activation process of vitamin D_3. In contrast, CYP24 (mitochondria) catalyzes 24-hydroxylation of 25-OH-D_3 and 1,25-$(OH)_2D_3$, leading to deactivation. *CYP27B1* and *CYP24* gene expression are reciprocally regulated in the kidney by calcium, 1,25-$(OH)_2D_3$, PTH, calcitonin, and phosphate.

based on the amino acid identity that proposed by the Committee for a Standardized Cytochrome P450 Nomenclature, a P450 protein sequence from one gene family usually is defined as having under 40% amino acid identity to a P450 protein from any other family, and P450s having more than 55% homology are in the same subfamily (4). For example, in the case of CYP27B1, CYP represents cytochrome P450, the following Arabic number, 27, denotes the family, a letter, B, designates the subfamily, and an Arabic numeral, 1, represents the individual gene within the subfamily. To establish the identity of each P450, we used here the CYP formula but not terms such as vitamin D_3 25-hydroxylase and 25-hydroxyvitamin D_3 1α-hydroxylase.

This review focuses on the catalytic properties of the P450s that have been characterized as vitamin D hydroxylases, *i.e.* vitamin D_3 25-hydroxylase (CYP2C11, CYP27A1, CYP2D25, CYP2R1, CYP3A4, and CYP2J3), 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1), and 25-hydroxyvitamin D_3 24-hydroxylase (CYP24) with a brief note for the history of their isolations. For physiological aspects and regulation that are not covered here, readers should be directed to recent reviews (1-3, 5, 6).

3. P450S EXHIBITING 25-HYDROXYLATION ACTIVITY

3.1. Short view

Vitamin D_3 25-hydroxylation reaction is a prerequisite step for the synthesis of 1,25-$(OH)_2D_3$. It has been established that the liver is the major site of vitamin D_3 25-hydroxylation in the rat, since the hepatectomized rat failed to synthesize 25-OH-D_3 (7). Both mitochondrial and microsomal fractions in the liver contain 25-hydroxylation activities although early studies reported contradict results for subcellular localization of 25-hydroxylase owing to very low activities in the fractions (8, 9).

A vitamin D_3 derivative, 1α-OH-D_3 (a therapeutic compound for chronic renal failure), is usually used as a preferable substrate to vitamin D_3 itself for the accurate measurement of 25-hydroxylation activity because of efficient 25-hydroxylation reaction (10). The 25-hydroxylation activity of vitamin D_2 is also detected in the rat and human livers although vitamin D_2, which derived from ergosterol, is not synthesized in body (11).

Recently, CYP2R1, CYP3A4, and CYP2J3 have been reported as microsomal vitamin D 25-hydroxylases. Therefore, there exist total six P450s together with previously identified CYP2C11, CYP27A1, and CYP2D25, complicating the biological significance of the 25-hydroxylation step. Comparison of their catalytic properties is probably useful to estimate the significance of individual P450s.

3.2. CYP2C11

CYP2C11 is the first P450 that has been identified as a vitamin D_3 25-hydroxylase. Anderson *et al*. (12) and Hayashi *et al*. (13) purified a P450 from male rat liver microsomes following vitamin D_3 25-hydroxylation activity independently. Both groups determined the amino-terminal sequence to identify the P450s (14, 15). The N-terminal sequences were identical with CYP2C11 (old names; P450h, P450M-1, P45016α, P450UT-A, P450RLM5) (16). CYP2C11 is expressed in only male rat liver microsomes but not in female ones. Expression of *CYP2C11* gene is hormonally regulated and increases with development. CYP2C11 is known as the most abundant P450 isoform in adult male liver microsomes and characterized by high testosterone 2α- and 16α-hydroxylation activities. As expected, the purified P450 as a vitamin D_3 25-hydroxylase showed strong testosterone hydroxylation activities.

The 25-hydroxylation activities of the purified P450 were 0.21 and 1.73 nmol/min/nmol P450 for vitamin D_3 and 1α-OH-D_3, respectively, with activities for 2α- and 16α-hydroxylation of testosterone (9.34 and 8.36 nmol/min/nmol P450, respectively), indicating relatively lower activity toward vitamin D (15). It should be noted that 1α-OH-D_3 is a better substrate than vitamin D_3 itself for 25-hydroxylation reaction. No vitamin D_2 25-hydroxylation activity was observed in the purified P450 fraction (12). The relatively low activity of the purified P450 for vitamin D_3 had raised a question if the final preparation is not homogeneous and is contaminated with CYP2C11. To clarify this point, Hayashi *et al*. carried out two different experiments. The first was the inhibition experiment with polyclonal and three monoclonal antibodies (17). These antibodies inhibited both vitamin D_3 and testosterone hydroxylation reactions with similar efficiencies, suggesting that a common P450 catalyzes both reactions. The second experiment was the analysis of the substrate specificity of CYP2C11 prepared by expression of CYP2C11 cDNA in yeast cells (18). Surprisingly, the expressed CYP2C11 showed no 25-hydroxylase activities for vitamin D_3 and 1α-OH-D_3, nevertheless showing significant 2α- and 16α-hydroxylation activities for testosterone. They finally concluded that a very similar but different cytochrome P450 from CYP2C11 catalyzes vitamin D_3 hydroxylation reaction. However, the testosterone 2α- and 16α-hydroxylation activities of the CYP2C11 partially purified from yeast cells were one order magnitude lower than those of the CYP2C11 purified from rat liver microsomes, suggesting the presence of an unknown inhibitor in the preparation from yeast cells. Therefore, there have left ambiguity whether CYP2C11 is a vitamin D_3 25-hydroxylase or not. Final conclusion should be waited until the highly purified CYP2C11 expressed in a heterologous system is fully characterized.

3.3. CYP27A1

P450 enzymes have been purified as mitochondrial vitamin D_3 25-hydroxylases from rat and rabbit livers (19, 20). Detailed characterization using the purified P450s revealed strong 27-hydroxylation activities for 5β-cholestane-3α,7α,12α-triol, an intermediate of bile acid synthesis. The 27-hydroxylation activity (turnover number of the rat P450, 36 nmol/min/nmol P450) was about 100 times higher than the vitamin D_3 25-hydroxylation activity (turnover number, 0.36 nmol/min/nmol P450) (19, 21). Subsequently, the cDNAs

of the rat and rabbit P450s were isolated and expressed in COS cells to establish their identities (22-24). The P450s were named CYP27A1 after its strong 27-hydroxylation activity for 5β-cholestane-3α,7α,12α-triol. It should be noted that the term of "26-hydroxylation" is often used instead of "27-hydroxylation" when the stereochemistry at the C-25 of the hydroxylated product is not considered (25). *CYP27A1* gene is conserved across vertebrate species (4).

Further characterizations were carried out using CYP27A1 enzymes expressed in culture cells and in *E. coli* (26-29). These studies revealed 26(27)-hydroxylation activities for both vitamin D_3 and 1α-OH-D_3 in addition to the 25-hydroxylation activity (26, 28). Interestingly, CYP27A1 catalyzed 24- and 26(27)-hydroxylation reactions toward vitamin D_2 but not 25-hydroxylation reaction (26). These activities were significant but lower than 25-hydroxylation activity toward vitamin D_3. CYP27A1 was also found to have 1α-hydroxylation activity of 25-OH-D_3 (27) although this activity was 1000 times lower than the 27-hydroxylase activity toward 5β-cholestane-3α,7α,12α-triol (29). The hydroxylation activity of CYP27A1 at C-1α of 25-hydroxyvitamin D_3 led to an early hypothesis that 1α-hydroxylation activity was resident in the CYP27A1. However, later the genuine 1α-hydroxylase has been cloned.

CYP27A1 is an essential enzyme involved in bile acid biosynthesis and is a gene responsible for the rare inherited disease cerebrotendinous xanthomatosis (CTX), which shows xanthomas, premature atherosclerosis, accumulation of cholestanol, and excretion of great amounts of bile alcohols (30-32). The physiological function of CYP27A1 in cholesterol metabolism and relatively low activities toward vitamin D_3 compounds raised the question whether CYP27A1 functions as a vitamin D_3 25-hydroxylase or not *in vivo*. Mice disrupted in *Cyp27a1* gene showed no significant change of the 25-OH-D_3 and 1,25-$(OH)_2D_3$ concentrations in the serum, although a marked change in the composition of bile acids (33, 34). Therefore, CYP27A1 seems to be of little importance for 25-hydroxylation of vitamin D_3 in mice. Disturbances in vitamin D metabolism have been described in a few cases of CTX (30), but this does not seem to be a general findings (33). Taken together, the major physiological role of CYP27A1 is likely in the cholesterol metabolism but not vitamin D_3 activation. However, there is no evidence that CYP27A1 does not contribute 25-hydroxylation of vitamin D_3 *in vivo*.

3.4. CYP2D25

In pigs, vitamin D_3 25-hydroxylation activity was found not only in the liver but also in the kidney (35, 36). Wikvall and co-workers have focused on vitamin D_3 metabolism in pigs. All data discussed here are brought out from the extensive research of his group. In 1997, Postlind *et al*. (37) has reported the cDNA cloning of a cytochrome P450 on the basis of amino acid sequence of the P450 purified from pig liver microsomes as a vitamin D_3 25-hydroxylase. The P450 shows more than 70% identities to CYP2D subfamily members, and was named CYP2D25. The isolated cDNA demonstrated by Northern blotting analysis that CYP2D25 mRNA is expressed primarily in the liver and significantly in the kidney.

CYP2D25 exhibited 25-hydroxylation activity toward 1α-OH-D_3 (760 pmol/min/nmol P450), vitamin D_3 (200 pmol/min/nmol P450), and vitamin D_2 (110 pmol/min/nmol P450), indicating that vitamin D_2 is also a good substrate (38). Interestingly, CYP2D25 showed significant 1α-hydroxylation activity (12 pmol/min/nmol P450) and 26-hydroxylation activity (45 pmol/min/nmol P450) of 25-hydroxyvitamin D_3 (39). CYP2D25 has a high affinity for vitamin D_3 based on the *Km* value of 0.1 μM, whereas CYP2C11 and CYP27A1 have been reported to have 5-10 μM of *Km* values (38).

Human has one CYP2D subfamily member, namely CYP2D6, exhibiting 77% identity with CYP2D25. To examine whether CYP2D6 has vitamin D_3 25-hydroxylation activity or not, recombinant CYP2D6 were subjected to an analysis of the catalytic activities (38). CYP2D6 showed no vitamin D_3 25-hydroxylation activity. Furthermore there is no correlation between the 25-hydroxylation activity toward 1α-OH-D_3 and the content of CYP2D6 in different human liver microsomes, indicating that an enzyme different from CYP2D6 catalyzes 25-hydroxylation of vitamin D_3 in humans.

3.5. CYP2R1

Rat CYP2C11 and pig CYP2D25 have been identified as microsomal vitamin D_3 25-hydroxylases. However, both of them are species-specific P450s and the orthologous genes of them have not assigned in other mammals. In 2003, Cheng *et al*. (40) have reported CYP2R1 as a microsomal vitamin D_3 25-hydroxylase in mice and humans. They expertly identified CYP2R1 by an expression cloning method from a *Cyp27a1*-ablated mouse cDNA library and vitamin D_3 25-hydroxylase activity of a recombinant CYP2R1 was confirmed. The *CYP2R1* gene has been found in human and pufferfish genomes, indicating conservation of *CYP2R1* genes throughout vertebrates (41). We have searched for rat CYP2R1 in DNA EST database and found a partial cDNA sequence highly homologous to the murine CYP2R1 cDNA.

The 25-hydroxylation activities of CYP2R1 for vitamin D_3 and 1α-OH-D_3 were comparable to those of CYP27A1 on the basis of a vitamin D receptor-based, ligand-dependent reporter activation assay (40). Human CYP2R1 enzymes hydroxylated both vitamin D_3 and vitamin D_2 at C-25 with similar efficiency, whereas human CYP27A1 shows a preference for vitamin D_3 over vitamin D_2 as a substrate. We have expressed the rat CYP2R1 using *E. coli* expression system and confirmed 25-hydroxylation activity toward 1α-OH-D_3 with a partially purified rat CYP2R1 (Aiba *et al*., unpublished data).

CYP2R1 mRNA was most abundant in the liver and testis, and expressed without sexual dimorphism in mouse livers (40). The enzymatic properties of CYP2R1 have not been studied except for vitamin D 25-hydroxylation activity. To assess the physiological function

of CYP2R1 protein as a vitamin D_3 25-hydroxylase, further study will focus on determination of precise enzyme activities for vitamin D related compounds. One of the most direct strategies for the evaluation must be generation of knockout mice for *Cyp2r1* gene to analyze the vitamin D_3 metabolism. Recently Cheng *et al*. (42) have reported that a patient with low circulation levels of 25-hydroxyvitamin D_3 was found to be homozygous for a transition mutation in exon 2 of the *CYP2R1* gene.

3.6. CYP3A4

Gupta *et al*. (43) have recently reported that CYP3A4, a human member of CYP3A subfamily, exhibits 25-hydroxylation activities for 1α-OH-D_2, 1α-OH-D_3, and vitamin D_2 on the basis of the screening of the major sixteen human hepatic P450s expressed in baculovirus-infected insect cells. The 25-hydroxylation activities of CYP3A4 were 1.33, and 0.144 nmol/min/nmol P450 for 1α-OH-D_2 and 1α-OH-D_3, respectively. The activity for 1α-OH-D_2 was the highest among P450s known as 25-hydroxylases, whereas the activity for 1α-OH-D_3 was one order magnitude lower than that of CYP27A1. The activity for vitamin D_2 was 1.58 nmol/nmol P450/1.5 hour. However, no activity was detected for vitamin D_3.

Kamachi *et al*. (10) had similarly examined using major fourteen human P450s prepared in lymphoblastoid cells whether they have the 25-hydroxylation activity for 1α-OH-D_3. No activity was detected in all tested P450s including CYP3A4. The discordance of the observations is not clear. The different results may be attributable to the source of P450s, *i.e.* baculovirus-infected insect cells or lymphoblastoid cells, since P450 content and coexistent proteins in the membrane fractions may differ each other. As mentioned in the section CYP2C11, the purified CYP2C11 clearly showed hydroxylation activities for vitamin D_3 and testosterone, although the crude CYP2C11 prepared from yeast cells showed no and weak hydroxylation activities for vitamin D_3 and testosterone, respectively. Therefore, purified CYP3A4 may show higher activity than crude fraction.

CYP3A4 is the most abundant cytochrome P450 expressed in adult human liver and intestine, and contributes to the metabolism of approximately a half of the drugs in use today. It is well known that CYP3A4 is induced by xenobiotics such as rifampicin via pregnane X receptor. Therefore, some drugs affecting *CYP3A4* gene expression may modulate vitamin D_3 activation. Recent studies have shown that 1,25-$(OH)_2D_3$ can induce CYP3A4 via vitamin D receptor (VDR) (44, 45). Interestingly, pregnane X receptor and VDR mediate the induction of CYP3A4 through common enhancer elements of the proximal ER6 (everted repeats of the half-site with a spacer of 6 bp) and the distal DR3 (direct repeats with a spacer of 3 bp, refer to Figure 3) in the promoter. It should be noted that the expression level of vitamin D receptor is quite lower in the liver than in the classical target tissues such as intestine. The findings present the possibilities that 1,25-$(OH)_2D_3$ stimulates vitamin D activation and also enhances detoxification of xenobiotics and carcinogens by inducing CYP3A4 in the intestine.

3.7. CYP2J3

CYP2C11 is a male rat specific P450 and is scarcely detected in the female rat liver. However, female rat liver microsomes show substantial vitamin D_3 25-hydroxylation activity (14, 46). The enzyme in the female rat liver has remained unidentified. To determine the P450 isoform exhibiting the 25-hydroxylation activity, Yamasaki *et al*. (47) has purified the enzyme from female rat livers following 1α-OH-D_3 25-hydroxylation activities and identified CYP2J3, a rat member of CYP2J subfamily, in partially purified fraction by using MALDI-TOF mass spectrometry.

They have prepared a recombinant CYP2J3 enzyme in *E. coli* and characterized its activities. Purified recombinant CYP2J3 showed strong 25-hydroxylase activities toward vitamin D_3 and 1α-OH-D_3 with turnover numbers (Vmax) of 3.3 and 22, respectively. The values were markedly higher than those of CYP2C11, CYP2D25, CYP27A1, and CYP3A4 (Table 1). CYP2J3 did not hydroxylate 25-OH-D_3 at any position, although CYP27A1 and CYP2D25 showed 26-, 24-, and 1α-hydroxylation activities. Unlike CYP2C11, real time PCR analysis of CYP2J3 expression level in livers showed no remarkable sexual dimorphism, although the level is 2-fold higher in the male rat, suggesting the importance of CYP2J3 as a microsomal vitamin D_3 25-hydroxylase on the basis of its catalytic activity and expression pattern.

CYP2J3 has been recognized as a P450 that is involved in the oxidation of arachidonic acid in the rat heart (48). The reported activity of CYP2J3 for arachidonic acid is about 10 times lower than that for vitamin D_3. Expression level of CYP2J3 mRNA is 2 orders of magnitudes higher in the liver than in the heart (47). Therefore, the major physiological function of CYP2J3 in the liver is very likely vitamin D_3 25-hydroxylation rather than arachidonic acid oxidation.

3.8. Perspectives

Five microsomal and one mitochondrial P450 enzymes have been reported as vitamin D 25-hydroxylases (Tables 1 and 2). CYP2R1 is likely to be the most promising P450 of all as a physiologically important microsomal vitamin D_3 25-hydroxylase since it is conserved from pufferfishes to humans. To establish the importance of CYP2R1, it is essential to characterize enzyme specificities of CYP2R1. More detailed assessment of the role of CYP2R1 in vitamin D_3 metabolism should wait for generation of *Cyp2r1* gene-ablated mice.

On the other hand, purification from male and female rats based on the activity led to CYP2C11 and CYP2J3, respectively, and purification from pig reached CYP2D25. The results suggest that the CYP2C11, CYP2J3, and CYP2D25 are the major contributors to vitamin D_3 25-hydroxylation reaction in each animal. In light of these considerations, it seems that different species use different P450 molecular species for vitamin $D_3$25-hydroxylation. Consistent with the notion, there is no clear evidence

Table 1. Comparison of turnover numbers of vitamin D_3 25-hydroxylases

25-Hydroxylase	Turnover number	
	Vitamin D_3	1α-OH-D_3
	nmol/min/nmol P450	
CYP2C11 (male rat)[1]	0.21 (100 μM)[2]	1.7 (100 μM)
CYP2D25 (pig)[3]	0.30 (100 μM)	1.6 (100 μM)
CYP2R1 (human)[4]	—[5]	—
CYP3A4 (human)[6]	ND[7]	0.14 (V_{max})
CYP2J3 (rat)[8]	2.7 (20 μM)	18 (20 μM)
CYP27A1 (rat)[9]	0.31 (100 μM)	1.4 (20 μM)

[1] Ref. 15, [2] Substrate concentrations used in the measurements are denoted in the parenthesis, [3] Ref. 36, [4] Ref. 40, [5] No data, but probably comparable activities with CYP27A1 according to Ref. 40, [6] Ref. 43, [7] not detected, [8] Ref. 47, [9] Ref. 20

Table 2. Characteristic of vitamin D_3 25-hydroxylases

	CYP2C11	CYP2D25	CYP2J3	CYP2R1	CYP27A1
Species	rat (male specific)	pig	rat	human, rat, mouse, fugu	human, rat, mouse, rabbit, fugu
Tissue	liver	liver, kidney	liver, heart	liver, testis	liver, kidney, lung, ovary, keratinocytes, macrophages
Cellular distribution	microsomes	microsomes	microsomes	microsomes	mitochondria
Amino acid size[1]	500	500	502	501 (human, mouse)	531 (human) 533 (rat, mouse) 535 (rabbit)
Molecular weight[1]	57181	56511	57969	57356 (human) 57310 (mouse)	60234 (human) 60733 (rat) 60720 (mouse) 60254 (rabbit)
Other activities	testosterone 2α-hydroxylation 16α-hydroxylation		arachidonic acid epoxygenation ω-1 hydroxylation		sterol 27-hydroxylation
GenBank™/ EMBL Data Bank accession no.	J02657	Y16417	U39943	AY323817 (human) AY323818 (mouse)	M62401 (human) Y07534 (rat) AK004977 (mouse) J04717 (rabbit)
Swiss-Plot accession no.	P08683	O46658	P51590	(no data)	Q02318 (human) P17178 (rat) Q9DBG1 (mouse) P17177 (rabbit)

[1] The values are for the unprocessed precursor of CYP

that the 25-hydroxylation activity of vitamin D_3 is regulated by certain factors concerning calcium homeostasis. The levels of 25-OH-D_3 simply increase in proportion to vitamin D_3 intake, and for this reason, plasma 25-OH-D_3 levels are commonly used as an indicator of vitamin D_3 status. Therefore, vitamin D binding protein, which binds to 25-OH-D_3 and circulates with it in blood stream, seems to be a key protein that controls availability of 25-OH-D_3 at the storage and the supply levels via megalin-mediated endocytosis for the subsequent 1α-hydroxylation in the kidney (3).

4. P450 EXHIBITING 1α-HYDROXYLATION ACTIVITY: CYP27B1

The formation of 1,25-$(OH)_2D_3$ from 25-OH-D_3 occurs mainly in the kidney. The 1α-hydroxylation step is the most tightly regulated step in vitamin D metabolism. Because of the physiological importance of 1,25-$(OH)_2D_3$, the enzyme that catalyzes 1α-hydroxylation of 25-OH-D_3 has long been the research target in vitamin D endocrine system. A large effort for purification from chicken and rat kidneys has revealed that 1α-hydroxylase is a mitochondrial P450 enzyme with a turnover number of 4.4 nmol/min/nmol P450 (49) and the molecular masses were determined 54-55 kDa (49, 50). The cDNA cloning was suddenly reported in 1996 by St-Arnaud *et al*. at the annual meeting of American Society for Bone and Mineral Research using homology with a mitochondrial P450, CYP24. In 1997, four different groups have reported the cDNA cloning for mouse, rat, and human 1α-hydroxylases by using expression cloning technology and PCR degenerate primers corresponding to the heme-binding domains of CYP24 and CYP27A1 (51-54). Since the identity of the cloned cDNA to CYP27A1 was more than 40%, this gene was designated *CYP27B1* (53). The achievement of CYP27B1 cDNA cloning has accelerated researches on regulation and an inherited disorder of vitamin D_3 metabolism.

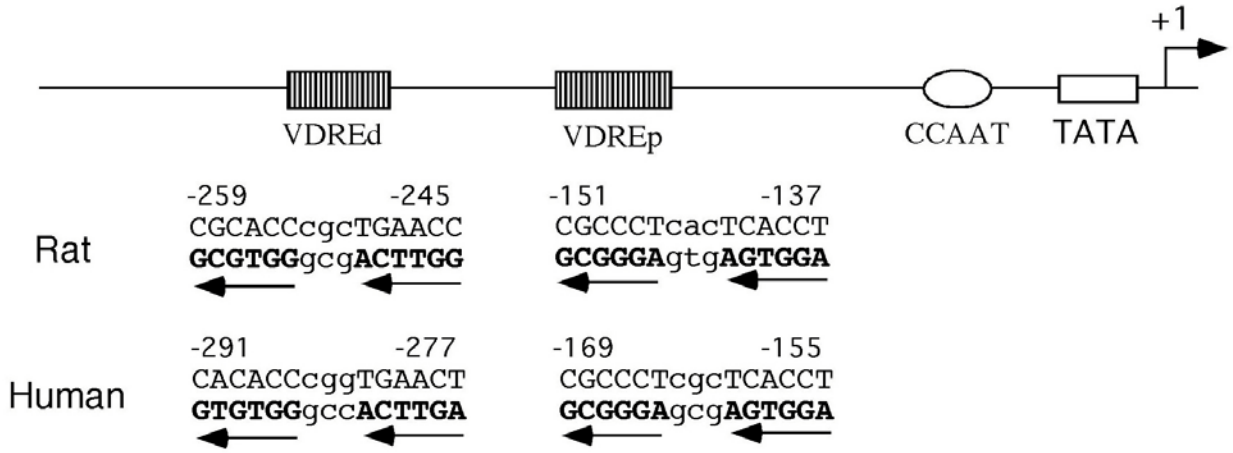

Figure 3. Conserved vitamin D responsive elements (VDRE) of rat and human *CYP24* genes. Two VDREs that consist of direct repeats of half-sites separated by 3 bp (DR3) are in the anti-strand in the both genes. Arrows indicates the sequences resemble to AGGTCA half-site and its orientation. The numbers above the sequences indicate the positions from the transcriptional start sites (+1). Two VDREs mediates vitamin D_3-dependent stimulation of the transcription synergistically (or additively).

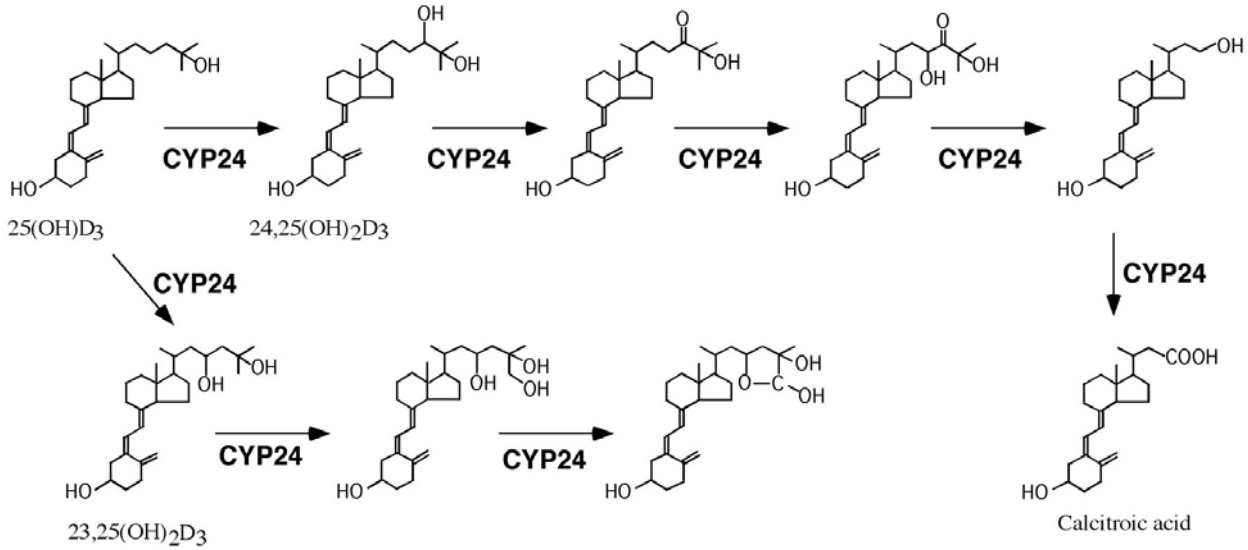

Figure 4. Successive side-chain oxidations of 25-OH-D_3 by CYP24 in the degradation process. C-24 and C-23 oxidation pathways for 25-OH-D_3 are catalyzed by a single CYP24 enzyme. In addition to 25-OH-D_3, 1,25-$(OH)_2D_3$ is also a good substrate for this successive oxidations. The catalytic activities of CYP24 were investigated using rat and human recombinant CYP24 proteins.

Enzymatic characterization has been carried out using recombinant mouse and human CYP27B1 enzymes expressed in *E. coli*. CYP27B1 of both mouse and human catalyzes 1α-hydroxylation toward not only 25-OH-D_3 but also 24,25-$(OH)_2D_3$ with *Km* values of 2.7 μM and 1.1-1.3 μM, respectively, indicating the preference for 24,25-$(OH)_2D_3$ (55, 56). However, the turnover numbers were not reported because the expression levels were too low to measure the P450 content by the reduced CO-differential spectra. We recently achieved a high expression of rat CYP27B1 of 78 nmol/liter culture with coexpression of GroES and GroEL chaperone proteins in *E. coli* system (Aiba *et al*., unpublished results). The expressed rat CYP27B1 showed a turnover number of 5.6 nmol/min/nmol P450 toward 25-OH-D_3; the value is comparable to that of purified 1α-hydroxylase enzyme from rat (49).

The expression of *CYP27B1* gene receives very tight regulations by PTH, calcitonin, and 1,25-$(OH)_2D_3$ to maintain calcium homeostasis (2). PTH and calcitonin are up-regulators of the gene expression. PTH induces CYP27B1 in the renal proximal convoluted tubules under hypocalcemic conditions (57). Analysis of 5'-upstream region of mouse *Cyp27b1* gene using luciferase reporter assay system revealed PTH-dependent stimulation of *CYP27B1* gene expression in a pig kidney cell line, AOK-B50. Three potential cAMP-responsive elements in the promoter seem to be involved in the induction mechanism (58). Calcitonin is found to induce the expression of *CYP27B1* gene at distal area of the nephron in normocalcemic rats (57). It should be noted that PTH and calcitonin function in different calcium status and at different area of nephron. In contrast, 1,25-$(OH)_2D_3$ is known to be a

negative regulator of *CYP27B1* expression. The promoter analysis of the human gene showed that a region down stream of –0.9 kb mediates negative responsiveness by 1,25-$(OH)_2D_3$ (59). However, the molecular mechanism of transcriptional repression by 1,25-$(OH)_2D_3$ through VDR remains to be determined.

Vitamin D-dependent rickets type I (also known as pseudo-vitamin D deficiency rickets) is an autosomal recessive disorder characterized by low serum calcium, secondary hyperparathyroidism, and low circulating levels of 1,25-$(OH)_2D_3$. Hence, it has been suspected if *CYP27B1* mutation causes this inherited disorder. Genome analyses of patients suffering the inherited disorder revealed several types of mutations (missense mutations, deletions, and insertions) in *CYP27B1* gene (54, 60-62). *Cyp27b1* knockout mice showed similar characteristics to vitamin D-dependent rickets type I, in which no circulating 1,25-$(OH)_2D_3$ could be detected (63, 64). Therefore, CYP27B1 is the sole enzyme responsible for the biosynthesis of 1,25-$(OH)_2D_3$ from 25-OH-D_3.

5. P450 EXHIBITING 24-HYDROXYLATION ACTIVITY: CYP24

The 25-hydroxyvitamin D_3 24-hydroxylase has an essential role in the catabolism of vitamin D_3 compounds. Ohyama *et al*. (65) purified the enzyme based on the 25-hydroxyvitamin D_3 24-hydroxylation activities from kidney mitochondria of vitamin D-treated rats. Subsequently, its cDNA was isolated using specific antibodies against the purified enzyme and named CYP24 after the hydroxylation position (66). Comparison of the deduced amino acid sequence with the N-terminal sequence of the purified protein revealed a typical mitochondria targeting signal sequence rich in amino acids with positive charges (67).

The *CYP24* gene is expressed in many vitamin D target tissues, and is strongly stimulated by 1,25-$(OH)_2D_3$, particularly in the kidney and intestine. Promoter analyses of rat and human CYP24 genes demonstrated that the two vitamin D responsive elements localizing around –150 bp (proximal; VDREp) and –250 bp (distal; VDREd) are essential to the induction by 1,25-$(OH)_2D_3$ via VDR (68-73) (Figure 3). Additionally, trans-acting factors other than VDR and cis-elements involved in the regulation have been reported (74-77), although the principal induction mechanism is through the two VDREs. In contrast to 1,25-$(OH)_2D_3$, PTH suppresses 24-hydroxyalse activity in the kidney. A possible mechanism has been proposed from *in vivo* experiments using rats that PTH down-regulates VDR expression and in turn a reduced VDR level diminishes the *CYP24* gene expression (78, 79).

CYP24 catalyzes 24-hydroxylation not only of 25-OH-D_3 (22 nmol/min/nmol P450) but also of 1,25-$(OH)_2D_3$ (6.9 nmol/min/nmol P450) as the initial steps of degradation (67). Apparent *Km* value of CYP24 for 1,25-$(OH)_2D_3$ (0.23 μM) is ten times lower than that for 25-OH-D_3 (3.1 μM), indicating a preference for 1,25-$(OH)_2D_3$ as a substrate (80). Hence, it is conceivable that the physiological function of CYP24 is the degradation of 1,25-$(OH)_2D_3$ rather than 25-OH-D_3. It was demonstrated using recombinant CYP24 proteins expressed in *E. coli* that CYP24 enzyme has 23-hydroxylation activity as well as 24-hydroxylation activity depending on animals. For example, rat CYP24 catalyzes mainly 24-hydroxylation, whereas human CYP24 shows significant 23-hydroxylation activity as well as 24-hydroxylation activity (81, 82). CYP24 is responsible for the further oxidation of the side-chain to calcitroic acid or 26,23-lactone (Figure 4). Rat CYP24 also catalyzed 24-hydroxylation of 1,25-$(OH)_2D_2$ but did not catalyze further oxidation reactions leading to calcitroic acid, suggesting that either the double bond at C-22 or C-24 methyl group blocks the metabolism (83) (see Figure 1).

To assess the physiological significance of CYP24, *Cyp24*-knockout mice were generated. Deletion of CYP24 activity resulted in a high level of circulating 1,25-$(OH)_2D_3$ due to the decreased capacity of degradation, and consequently caused impairment of intramembranous bone formation (84). This result indicates that the oxidation pathway catalyzed by CYP24 is crucial for the degradation of 1,25-$(OH)_2D_3$.

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Abbreviations: CYP, cytochrome P450; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3; PTH, parathyroid hormone; CTX, cerebrotendinous xanthomatosis; VDR, vitamin D receptor; MALDI-TOF, matrix-assisted laser adsorption ionization/time of flight; PCR, polymerase chain reaction; VDRE, vitamin D responsive element

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