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# Metabolism of Vitamin $D_3$ by Human CYP27A1[1]

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**Human vitamin $D_3$ 25-hydroxylase (CYP27A1) cDNA was expressed in *Escherichia coli,* and its enzymatic properties were revealed. The reconstituted system containing the membrane fraction prepared from the recombinant *E. coli* cells was examined for the metabolism of vitamin $D_3$. Surprisingly, at least eight forms of metabolites including the major product 25(OH)$D_3$ were observed. HPLC analysis and mass spectrometric analysis suggested that those metabolites were 25(OH)$D_3$, 26(OH)$D_3$, 27(OH)$D_3$, 24*R*,25(OH)$_2D_3$, 1$\alpha$,25(OH)$_2D_3$, 25,26(OH)$_2D_3$ (25,27(OH)$_2D_3$), 27-oxo-$D_3$ and a dehydrogenated form of vitamin $D_3$. These results suggest that human CYP27A1 catalyzes multiple reactions and multiple-step metabolism toward vitamin $D_3$. The $K_m$ and $V_{max}$ values for vitamin $D_3$ 25-hydroxylation and 25(OH)$D_3$ 1$\alpha$-hydroxylation were estimated to be 3.2 $\mu$M and 0.27 (mol/min/mol P450), and 3.5 $\mu$M and 0.021 (mol/min/mol P450), respectively. These kinetic studies have made it possible to evaluate a physiological meaning of each reaction catalyzed by CYP27A1.** 

***Key Words:*** **P450; CYP27A1; vitamin $D_3$; vitamin D 25-hydroxylase; *E. coli*.**

The active form of vitamin $D_3$, 1$\alpha$,25(OH)$_2D_3$ plays essential roles in calcium homeostasis, immunology and cell differentiation. It is well-known that vitamin $D_3$ is firstly converted to 25(OH)$D_3$ in liver, and secondly 25(OH)$D_3$ is converted to 1$\alpha$,25(OH)$_2D_3$ in kidney. In humans, mitochondrial CYP27A1 appears to be the most essential enzyme for vitamin $D_3$ 25-hydroxylation, judging from serum 25(OH)$D_3$ levels in the patients with cerebrotendinous xanthomatosis CTX (1). However, *in vitro* studies with the purified CYP27A1 sample showed only a small 25-hydroxylation activity of CYP27A1 toward vitamin $D_3$ (2, 3). To our knowledge, no reports showing the $K_m$ value of human CYP27A1 for vitamin $D_3$ 25-hydroxylation have been published. Our previous studies (4–7) indicated that *E. coli* expression system was quite useful for elucidation of enzymatic properties of mammalian steroidogenic P450s because *E. coli* has no cytochrome P450 gene (8) and no steroids. The reconstituted system containing the membrane fraction of the recombinant *E. coli* cells expressing each of CYP24 (4, 5) and CYP27B1 (6, 7) showed the remarkable activity. In this study, the *E. coli* membrane fraction containing human CYP27A1 was used for the metabolism of vitamin $D_3$. As expected, a remarkable metabolism was observed. Several metabolites were identified by HPLC analysis and mass spectrometric analysis. Kinetic analysis revealed $K_m$ and $V_{max}$ values for vitamin $D_3$ 25-hydroxylation and 25(OH)$D_3$ 1$\alpha$-hydroxylation (9). These results may explain the serum 1$\alpha$,25(OH)$_2D_3$ level of patients with pseudovitamin D-deficient rickets (PDDR) without functional CYP27B1 genes (10). In this article, we describe multiple reactions including novel reactions by human CYP27A1, and discuss their physiological meanings.

## MATERIALS AND METHODS

*Materials.* DNA modifying enzymes, restriction enzymes and DNA sequencing kit were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Linker and primer DNAs were purchased from Japan Bio-Service (Tokyo, Japan). *Escherichia coli* DH5$\alpha$ (Takara Shuzo Co.) was used as a host strain. Human liver cDNA library HL1145y was purchased from Clontech (California). Vitamin $D_3$, 25(OH)$D_3$, 1$\alpha$,25(OH)$_2D_3$ and 24*R*,25(OH)$_2D_3$ were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). 1$\alpha$,24,25-TrihydroxyvitaminD$_3$ was kindly donated by Chugai Pharm. Co. (Tokyo, Japan). NADPH

Abbreviations used: 24(OH)$D_3$, 24-hydroxyvitamin $D_3$; 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 26(OH)$D_3$, 26-hydroxyvitamin $D_3$; 27(OH)$D_3$, 27-hydroxyvitamin $D_3$; 27-oxo-$D_3$, 27-oxo-vitamin $D_3$; 1$\alpha$,24(OH)$_2D_3$, 1 $\alpha$,24-dihydroxyvitamin $D_3$; 1$\alpha$,25(OH)$_2D_3$,1 $\alpha$,25-dihydroxyvitamin $D_3$; 1$\alpha$,26(OH)$_2D_3$,1 $\alpha$,26-dihydroxyvitamin $D_3$; 1$\alpha$,27(OH)$_2D_3$,1 $\alpha$,27-dihydroxyvitamin $D_3$; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin $D_3$; 25,27(OH)$_2D_3$, 25,27-dihydroxyvitamin $D_3$; 1 $\alpha$,24,25(OH)$_3D_3$, 1 $\alpha$,24,25-trihydroxyvitamin $D_3$; 1 $\alpha$,25,27(OH)$_3D_3$, 1 $\alpha$,25,27-trihydroxyvitamin $D_3$.

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was purchased from Oriental Yeast Co. (Tokyo, Japan). Other chemicals used were of the highest quality commercially available.

*Recombinant DNA procedures.* Recombinant DNA procedures and transformation of *E. coli* was performed as described previously (4). Polymerase chain reaction (PCR) was carried out with a PC2000 apparatus (Perkin Elmer Cetus, Foster City, U.S.A.) as described (11). Sequencing of PCR fragment and synthesized linker DNAs was performed by using an ABI PRISM dye terminater cycle sequencing ready reaction kit (Perkin Elmer, Foster City, USA). Recombinant *E. coli* cells were cultivated in TB-broth medium as described previously (4).

*cDNA cloning of human CYP27A1.* Human CYP27A1 (9) was obtained from human liver cDNA library HL1145y (Clontech, California) with PCR methods. Two sets of primers (i) GGA AAA AAA CAT ATG GCT CTT CCA TCT GAT AAA GCC AAG CTA CCG GA and TAT AGA GCG CTG CTT CCG CT for N-terminal region of CYP27A1 and (ii) AAG GAG CAC CGG GAC AGC ACG ACC and CCAGGAACCACTGTGCCAGGAGCTGG for C-terminal region of CYP27A1 were used for PCR on the basis of the human CYP27A1 cDNA sequence as described by Cali *et al.* (12).

*Construction of expression plasmids.* The expression plasmid for human CYP27A1 was constructed as described below. The PCR fragment (0.12 kbp) encoding the N-terminal region of the putative mature form of human CYP27A1 with NdeI and KpnI site at each side was obtained. As described by Pikuleva *et al.* (2), the sequence containing translational start codon was modified without amino acid change. The PCR fragment (1.38 kbp) encoding the C-terminal region of human CYP27A1 was ligated with pUC19 digested with HincII. The resultant plasmid was partially digested with HindIII and then digested with KpnI to yieled a KpnI-HindIII fragment (1.40 kbp). The NdeI-KpnI fragment (0.12 kbp) and the KpnI-HindIII fragment (1.40 kbp) were doubly inserted into NdeI and HindIII sites of pKSNdl which was derived from pKK223-3 as described previously (7) to yield the expression plasmid pKH27A1.

*Cultivation of the recombinant E. coli cells.* Recombinant *E. coli* cells were grown in TB broth (4) containing 50 $\mu$g/ml ampicillin at 29°C under good aeration. The induction of transcription of CYP27A1 cDNA under the tac promoter was initiated by addition of isopropyl-thio-$\beta$-D-galactopyranoside (IPTG) at a final concentration of 1 mM when the cell density (O.D.660) reached 0.5. $\delta$-Aminolevulinic acid and chloramphenicol were also added at final concentrations of 0.5 mM and 1 $\mu$g/ml, respectively. The recombinant cells were gently shaken at 29°C under good aeration by bubbling.

*Preparation of subcellular fractions.* Subcellular fractionation of *E. coli* cells was carried out basically according to our previous study (4). 100 mM Tris-HCl (pH 7.4) buffer was used for suspension of the membrane fraction.

*Measurement of reduced CO difference spectra and substrate-induced difference spectra.* Reduced CO difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously (13). The concentration of CYP27A1 was determined from the reduced CO-difference spectrum using a difference of the extinction coefficients at 446 nm and 490 nm of 91 $mM^{-1}$ $cm^{-1}$ (14). The substrate-induced difference spectra of the membrane fraction containing 3.2 mg protein/ml were measured in the presence of 0 to 1.0 $\mu$M of 1$\alpha$(OH)$D_3$.

*Measurement of enzyme activity of CYP27A1.* Towards vitamin $D_3$, 1$\alpha$(OH)$D_3$ and 25(OH)$D_3$, the activity was measured in the reconstituted system consisting of the membrane fraction containing 0.5 $\mu$M CYP27A1, 5.0 $\mu$M of adrenodoxin (ADX), 0.5 $\mu$M of NADPH-adrenodoxin reductase (ADR), 5.0 $\mu$M of substrate, 0.5 mM of NADPH, 100 mM Tris-HCl (pH 7.4) and 1 mM EDTA at 37°C. For the determination of $K_m$ and $V_{max}$ values for 25-hydroxylation towards vitamin $D_3$ and 1$\alpha$(OH)$D_3$, and 1$\alpha$, 24 and 26(27)-hydroxylation reactions toward 25(OH)$D_3$, the concentrations of CYP27A1, ADX and ADR were reduced to 0.1 $\mu$M, 2.0 $\mu$M and 0.2 $\mu$M, respectively.

The reaction was initiated by addition of NADPH. Aliquots of the reaction mixture were collected after varying time intervals and extracted with four volumes of chloroform-methanol (3:1). The organic phase was recovered and dried up. The resulting residue was solubilized with acetonitrile and applied to HPLC under the following conditions: column, YMC-Pack ODS-AM (4.6 × 300 mm) (YMC Co., Tokyo, Japan); UV detection, 265 nm; flow rate, 1.0 ml/min; column temperature, 40°C; mobile phase, a linear gradient of 70–100% acetonitrile aqueous solution for 15 min for analysis of metabolites of vitamin $D_3$, 1$\alpha$(OH)$D_3$ and 25(OH)$D_3$. For the analysis of metabolites of 25(OH)$D_3$ (peaks 1 and 2), the mobile phase of 50% acetonitrile for 5 min followed by a linear gradient of 50–100% acetonitrile aqueous solution per 15 min was applied.

*Mass spectrometric analysis of the metabolites.* Isolated metabolites from HPLC effluents were subjected to mass spectrometric analysis using a Finnegan mat TSQ-70 with atmospheric pressure chemical ionization (APCI), positive mode. The conditions of LC were described below: column; reverse phase ODS column (6 × 150 mm) ($\mu$Bondapak C18, Waters); mobile phase, 20–100% acetonitrile aqueous solution per 25 min; flow-rate, 1.0 ml/min, UV detection, 265 nm.

*Other methods.* The concentrations of vitamin $D_3$ derivatives were estimated by their molar extinction coefficient of $1.80 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 264 nm (15). Protein concentration was determined by the method of Lowry *et al.* (16), using bovine serum albumin as a standard.

## RESULTS

*cDNA cloning of human CYP27A1.* The cloned CYP27A1 cDNA contained the same sequence as the sequence reported by Cali *et al.* (12). The DNA sequence coding for amino-terminal seven amino acid residues of the putative mature form was modified as described by Pikuleva *et al.* (2).

*Expression of human CYP27A1 in E. coli.* The recombinant *E. coli* JM109/pKH27A1 cells were lysed and the membrane fraction was prepared. Reduced CO-difference spectrum of the membrane fraction showed a peak at around 446 nm (data not shown) while the membrane fraction from the control JM109/pKSNdl cells showed no peak, suggesting the production of human CYP27A1 hemoprotein. The P450 content in the membrane fraction was calculated to be 1.1 nmol/mg protein.

*Substrate-induced spectra of human CYP27A1.* The addition of 1$\alpha$(OH)$D_3$ to the membrane fraction prepared from JM109/pKH27A1 cells induced a type I spectrum (data not shown), indicating a change of the heme iron of human CYP27A1 from a low-spin state to a high-spin state upon the substrate binding. However, neither vitamin $D_3$ nor 25(OH)$D_3$ caused the spectral change.

*HPLC analysis of metabolites of vitamin $D_3$, 1$\alpha$(OH)$D_3$, 25(OH)$D_3$ and 1$\alpha$,25(OH)$_2D_3$.* The reconstituted system containing the membrane fraction prepared from the recombinant *E. coli* cells, adrenodoxin and adrenodoxin reductase was examined for the metabolism of vitamin $D_3$. HPLC analysis demonstrated the presence of at least eight metabolites (Figs. 1A and 1C). The retention time of a major peak (peak 3 in Fig.

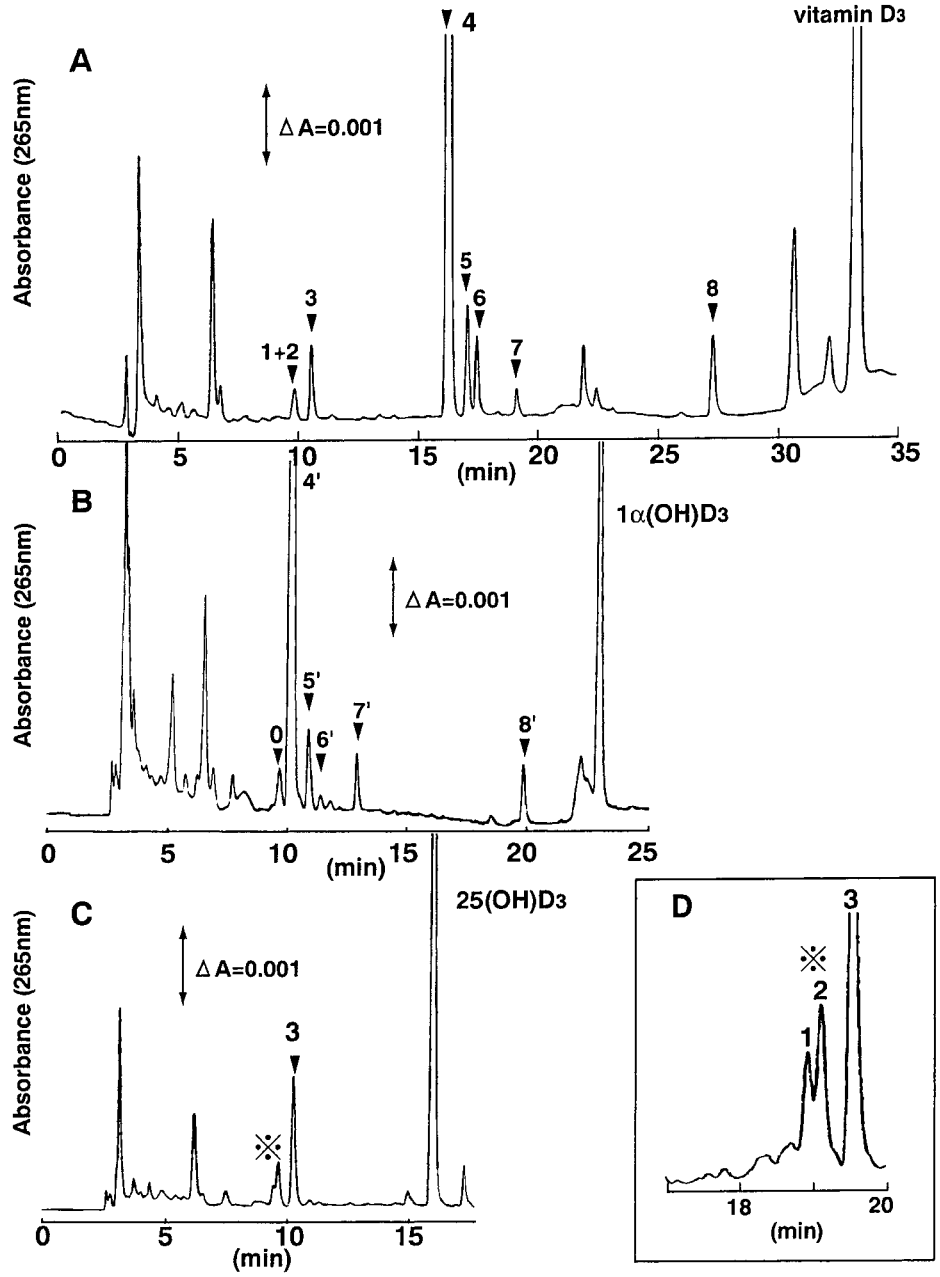


**FIG. 1.** HPLC profiles of vitamin $D_3$ and its metabolites (A), 1$\alpha$ (OH)$D_3$ and its metabolites (B), 25(OH)$D_3$ and its metabolites (C) in the reconstituted system containing the membrane fraction prepared JM109/pKH27A1 cells. Following 30 min of incubation with 5.0 $\mu$M of vitamin $D_3$ (A) and 5.0 $\mu$M of 1$\alpha$ (OH)$D_3$ (B) 5. 0 $\mu$M of 25(OH) $D_3$ (C), the reaction mixture was extracted and analyzed by HPLC as described under Materials and Methods. The metabolites numbered 1 and 2 were also analyzed by another HPLC condition (D) as described under Materials and Methods.

1A) was identical with that of the authentic 25(OH)$D_3$. Another major peak (peak 4) was suggested to be 27(OH)$D_3$ based on the report by Dilworth *et al.* (16). The metabolite designated as peak 1 + 2 in Fig. 1A was clearly separated to two peaks whose retention times were identical with those of 24*R*,25(OH)$_2D_3$and 1$\alpha$,25(OH)$_2D_3$ (Fig. 1D) under another HPLC condition described under Materials and Methods. All the metabolites were not observed in the reconstituted system without ADX. In addition, the membrane fraction prepared from the control JM109/pKSNdl cells showed no metabolites, suggesting that all the metabolites were produced by CYP27A1. On the metabolism of 1$\alpha$(OH)$D_3$, similar metabolism was observed with the major metabolite of 1$\alpha$,25 (OH)$_2D_3$ as shown in Fig. 1B. As in the case of vitamin $D_3$, another major metabolite appeared to be 27-hydroxylated product of 1$\alpha$(OH)$D_3$. Comparison of HPLC profiles between Figs. 1A and 1B

suggests that the metabolites designated as 6', 7' and 8' appeared to correspond to those designated as 6, 7 and 8. Mass spectrometric analysis confirmed these assumptions (data not shown). The metabolite numbered 0 shown in Fig. 1B appeared to be 1$\alpha$,24$(OH)_2D_3$, judging from the results reported by Guo *et al.* (17). On the metabolism of 25$(OH)D_3$, two metabolites with the same retention times of authentic standards of 24*R*,25$(OH)_2D_3$ and 1$\alpha$,25 $(OH)_2D_3$ were detected (Figs. 1C and 1D). The major peak appeared to contain both 25,26$(OH)_2D_3$ and 25,27$(OH)_2D_3$. On the metabolism of 1$\alpha$,25 $(OH)_2D_3$, the metabolite with the same retention time as authentic 1$\alpha$,24*R*,25 $(OH)_3D_3$ and the putative 1$\alpha$,25,26 $(OH)_3D_3$ and 1$\alpha$,25,27$(OH)_3D_3$ were observed (data not shown).

*Mass spectrometric analysis of the metabolites.* The eight metabolites were examined for mass spectrometric analysis. The metabolite numbered 1 and 2 showed similar spectra with those of authentic 24*R*,25$(OH)_2D_3$ and 1$\alpha$,25$(OH)_2D_3$ (Figs. 2A to 2D) respectively. It was noted that a molecular ion at 417 (M+H) was observed in the spectra of authentic 24*R*,25$(OH)_2D_3$ and the metabolite numbered 1. Molecular ion at *m/z* 446 appeared to be a reaction product with methanol in the LC-MS system used in this study as described previously (5). These results together with the HPLC analysis strongly suggest that the metabolites numbered 1 and 2 were 24*R*,25 $(OH)_2D_3$ and 1$\alpha$,25$(OH)_2D_3$, respectively. Mass spectrum of the metabolite numbered 3 indicates that it is also dihydroxylated product of vitamin $D_3$ (Fig. 2E). Based on the report indicating that CYP27A1 secondly prefer the hydroxylation at C27 position of vitamin $D_3$ (18), the metabolite numbered 3 was postulated as 25,26$(OH)_2D_3$. It should be noted that the positions of C26 and C27 were changed by the addition of hydroxy group to C25 as shown in Fig. 4. 25,27$(OH)_2D_3$ would be also produced judging from the fact that a considerable amount of 26$(OH)D_3$ was produced from vitamin $D_3$. Thus, it seems likely that the metabolite numbered 3 contained not only 25,26$(OH)_2D_3$ but also 25,27$(OH)_2D_3$. As shown in Fig. 2G, the metabolite numbered 4 in Fig. 1 showed nearly the same spectrum as that of authentic 25$(OH)D_3$ (Fig. 2F) with molecular ions at *m/z* 401 (M+H), 383 (401-$H_2O$) and 365 (401-2 $H_2O$). Molecular ion at *m/z* 430 appeared to be a reaction product with methanol in the LC-MS system used in this study as described previously (5). The metabolites numbered 5 (Fig. 2I) and 6 (Fig. 2H) also showed mass spectra with molecular ions at *m/z* 430, 401 (M+H), 383 (401-$H_2O$) and 365 (401-2 $H_2O$), suggesting that they were monohydroxylated product of vitamin $D_3$. These results are consistent with the assumption that the metabolite numbered 5 is 27$(OH)D_3$. In addition, it is possible to assume that the metabolite numbered 6 is 26$(OH)D_3$, on the basis of the substrate recognition of CYP27A1 proposed by Dilwarth *et al.* (18). The metabolite numbered 7 (Fig. 2J) showed a mass spectrum with molecular ions at *m/z* 428, 399 (M+H), 381 (399-$H_2O$) and 363 (399-2 $H_2O$), suggesting that the ketone was formed by the addition of another hydroxy group at the same position to form a *gem*-diol intermediate, which spontaneously rearranges with loss of a water (4, 5). Since C25 position cannot form the ketone, the metabolite numbered 7 was suggested to be 27-oxo-$D_3$. Mass spectrum of the metabolite numbered 8 strongly suggests that the metabolite is a dehydrogenated form of vitamin $D_3$. HPLC analysis (Fig. 1A) suggests that the metabolite is not 3-oxo-vitamin $D_3$ because 3-oxo-vitamin $D_3$ should be eluted after vitamin $D_3$. Mass spectrum of the metabolite numbered 8' (Fig. 1B) also suggested that the metabolite was a dehydrogenated form of 1$\alpha$$(OH)D_3$ (data not shown). However, no dehydrogenated form was detected in the metabolites of 25$(OH)D_3$.

*Determination of $K_m$ and $V_{max}$ values for each reaction.* When the substrate concentration was varied, the reaction followed Michaelis-Menten type kinetics on 25-hydroxylation towards vitamin $D_3$ and 1$\alpha$$(OH)D_3$. The $K_m$ and $V_{max}$ values for vitamin $D_3$ and 1$\alpha$$(OH)D_3$ determined with Hanes-Woolf plots were 3.2 $\mu$M and 0.27 (mol/min/mol P450) for vitamin $D_3$ and 6.9 $\mu$M and 0.79 (mol/min/mol P450) for 1$\alpha$$(OH)D_3$, respectively (Table I). In addition, the $K_m$ and $V_{max}$ values for 1$\alpha$-hydroxylation, 24-hydroxylation and 26(27)-hydroxylation toward 25$(OH)D_3$ were estimated. (Table II). Although these hydroxylation reactions showed significantly small $V_{max}$ values as compared with $V_{max}$ value of the 25-hydroxylation of vitamin $D_3$, they showed similar $K_m$ values. It is noted that the determined $K_m$ value for 1$\alpha$-hydroxylation toward 25$(OH)D_3$ was similar to the $K_m$ value for the same reaction catalyzed by CYP27B1 (6, 7).

*Metabolic pathway of vitamin $D_3$.* Figure 3 shows the time courses of the concentrations of the eight metabolites in the reaction sytem. They would be roughly divided into three types. The first type consists of the metabolites numbered 3, 4, 5 and 7 which were assumed to be 25$(OH)D_3$ (Fig. 3A), 27$(OH)D_3$ (Fig. 3B), 26$(OH)D_3$ (Fig. 3B) and dehydrogenated form of vitamin $D_3$ (Fig. 3C). The time course is quite consistent with the fact that they are the products of one-step reaction. The second type consists of the metabolites numbered 1, 2 and 3 which were assumed to be 24*R*,25$(OH)_2D_3$, 1$\alpha$,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ (Fig. 3D). The time course suggests that the three metabolites are produced from 25$(OH)D_3$ once released from substrate-pocket of CYP27A1 as a product of vitamin $D_3$ 25-hydroxylation. The third type is the metabolite numbered 6 which were suggested to be 27-oxo-$D_3$ (Fig. 3B). The time course appeared the intermediate between the first and second types, suggesting that sequential monooxygenation without release of 27$(OH)D_3$

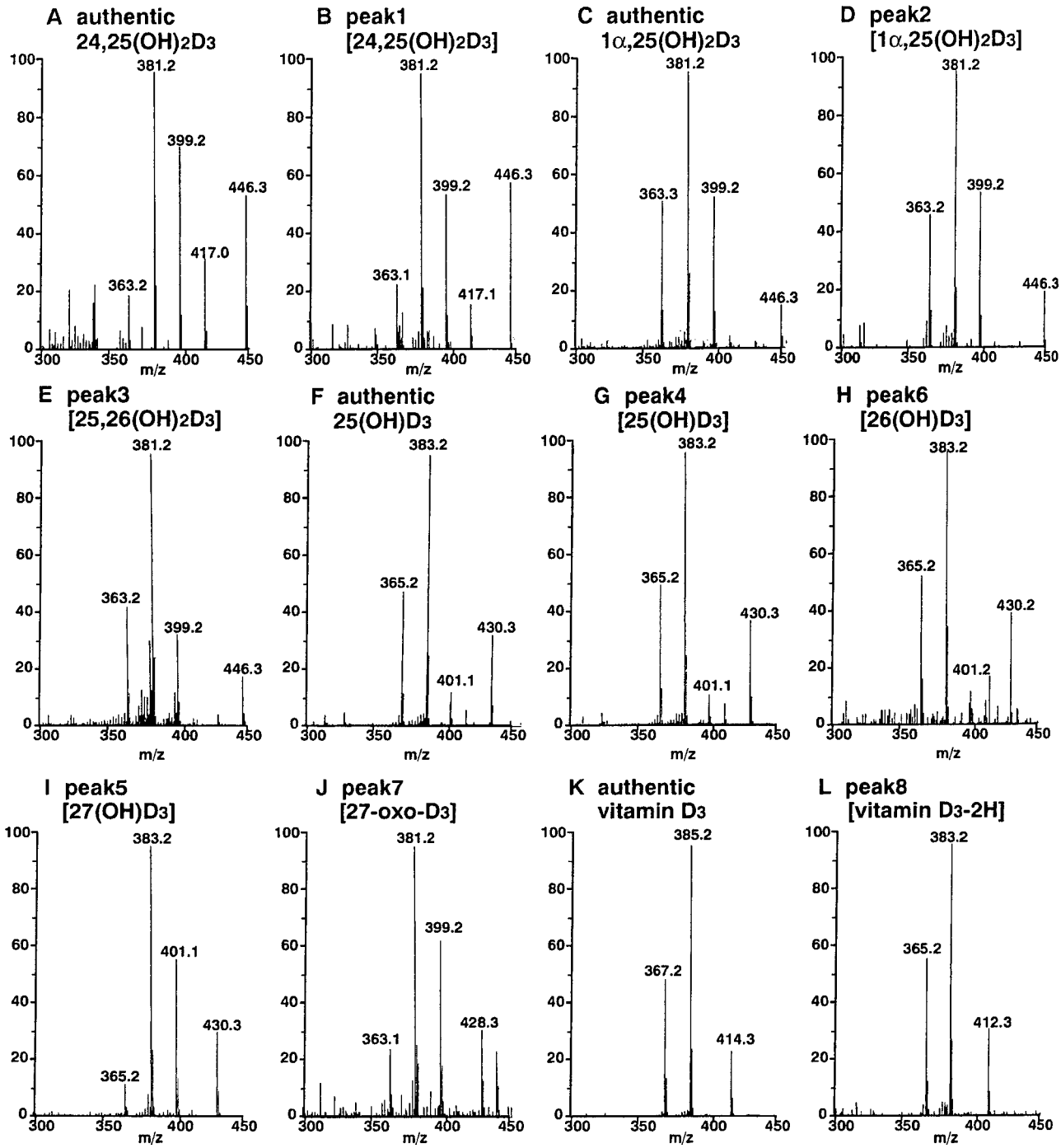


**FIG. 2.** Mass spectra of the metabolites numbered 1–8 in Fig. 1, and authentic standards of 24*R*,25(OH)$_2$D$_3$ (A), 1α,25(OH)$_2$D$_3$ (C), 25(OH)D$_3$ (F), and vitamin D$_3$ (K).

from substrate-pocket of CYP27A1 (12, 18) partially occurs on the conversion from vitamin D$_3$ through 27(OH)D$_3$ to 27-oxo-D$_3$.

## DISCUSSION

Detailed studies have been performed on the CYP27A1-dependent metabolism of cholesterol and 5β-cholestan 3α, 7α, 12α-triol (THC) (11, 18, 19). On the contrary, insufficient analysis has been carried out on the metabolism of vitamin D$_3$, whereas Berginer *et al.* (1) confirmed a physiological significance of human CYP27A1-dependent vitamin D$_3$ 25-hydroxylation. No kinetic studies showing $K_m$ value for vitamin D$_3$ 25-hydroxylation had been published although $K_m$ value for 1 α(OH)D$_3$ 25-hydroxylation catalyzed by rabbit

**TABLE I**

Kinetic Parameters of Human CYP27A1 for 25-Hydroxylation of Vitamin $D_3$ and 1$\alpha$(OH)$D_3$

| Substrate | $K_m$ ($\mu$M) | $V_{max}$ (mol/min/mol P450) |
|---|---|---|
| Vitamin $D_3$ | 3.2 ± 0.5 | 0.27 ± 0.03 |
| 1$\alpha$(OH)$D_3$ | 6.9 ± 1.7 | 0.79 ± 0.09 |

*Note.* Each $K_m$, $V_{max}$ and $V_{max}/K_m$ value represents the mean ± SD from four separate experiments.

CYP27A1 was published by Furster *et al.* (22). In this study, detailed enzymatic properties of human CYP27A1 on the vitamin $D_3$ metabolism have been revealed using *E. coli* expression system. Our previous studies using yeast expression system showed an extremely small activity of rat CYP27A1 toward vitamin $D_3$ (21). Ergosterol in the yeast membrane, which is known to be a substrate of CYP27A1 (21), might inhibit the vitamin $D_3$ metabolism. On the other hand, the recombinant *E. coli* membrane which contains no steroids appears quite appropriate system to reveal the metabolism of vitamin $D_3$ by CYP27A1. The obtained $K_m$ value of 3.2 $\mu$M appears consistent with the hypothesis that CYP27A1 is the physiologically essential enzyme as vitamin $D_3$ 25-hydroxylase. Although the $V_{max}$ value seems quite small, physiologically enough amount of 25(OH)$D_3$ may be retained due to the long half life time of 25(OH)$D_3$ in blood circulation (23). The ratio among 25, 27 and 26-hydroxylation, and the putative dehydrogenation was roughly estimated to be 100:15:3:8. The 27-hydroxylation includes both 27(OH)$D_3$ and 27-oxo-vitamin $D_3$. These results suggest that the reaction specificity of CYP27A1 for vitamin $D_3$ is not so restrict. As described by Pikuleva *et al.* (18), CYP27A1 catalyzes conversion from cholesterol through 27-hydroxycholesterol and 3$\beta$-hydroxy-5-cholestenal (27-oxo-form) to 3$\beta$-hydroxy-5-cholestenoic acid. Thus, the formation of 27-oxo-vitamin $D_3$ appears quite reasonable. However, the formation of the dehydrogenated form of vitamin $D_3$ seems an atypical reaction for P450. Although 24- and 25-dehydro-forms were detected in bile as a metabolite of 1$\alpha$,25(OH)$D_3$ as

**TABLE II**

Kinetic Parameters of Human CYP27A1 for 1$\alpha$-Hydroxylation, 24-Hydroxylation, and 26(27)-Hydroxylation toward 25(OH)$D_3$

| Reaction | $K_m$ ($\mu$M) | $V_{max}$ (mol/min/mol P450) |
|---|---|---|
| 1$\alpha$-hydroxylation | 3.5 ± 0.4 | 0.021 ± 0.002 |
| 24-hydroxylation | 5.5 ± 0.7 | 0.014 ± 0.003 |
| 26(27)-hydroxylation | 2.9 ± 0.7 | 0.054 ± 0.008 |

*Note.* Each $K_m$ and $V_{max}$ value represents the mean ± SD from three separate experiments.

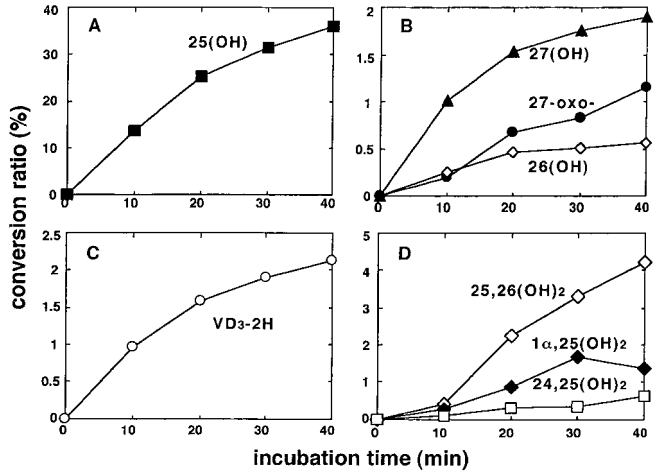


**FIG. 3.** Time courses of the conversion ratios of the metabolites in the reconstituted system containing the membrane fraction prepared from JM109/pKH 27A1 cells.

described by Onisco *et al.* (24), the reaction mechanism seems different because the dehydrogenated forms in this study were not detected in the metabolites of 1$\alpha$,25(OH)$_2D_3$ and 25(OH)$D_3$ but detected in those of vitamin $D_3$ and 1$\alpha$(OH)$D_3$. Further analysis is needed to determine the chemical structures of the dehydrogenated forms and reveal the reaction mechanism. Although physiological meanings of other reactions than 25-hydroxylation have not been confirmed yet, the multiple-step reaction yields physiologically significant compounds such as 1$\alpha$,25(OH)$_2D_3$ and 24*R*,25(OH)$_2D_3$ (25–27). On 1$\alpha$,25(OH)$_2D_3$ production from vitamin $D_3$, the first reaction is the conversion from vitamin $D_3$ to 25(OH)$D_3$, judging from no detection of 1$\alpha$(OH)$D_3$ as a metabolite of vitamin $D_3$. Thus, the 25-hydroxyl group

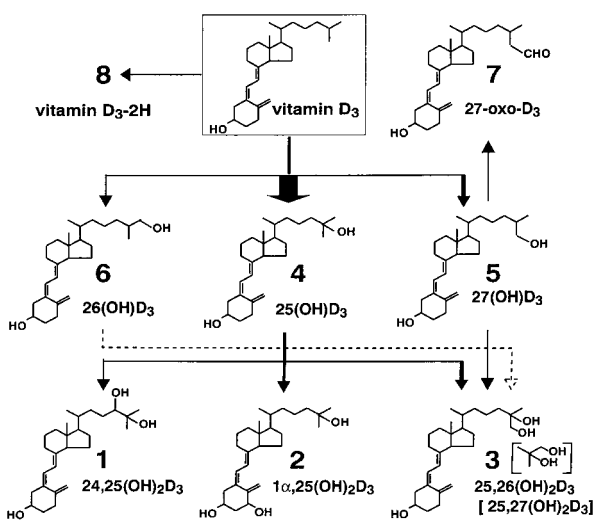


**FIG. 4.** Putative metabolic pathways of vitamin $D_3$ by human CYP27A1. The numbers of the metabolites correspond to those in Fig. 1A.

is essential for the 1 $\alpha$-hydroxylation catalyzed by CYP27A1, suggesting that CYP27A1 has the same substrate specificity as CYP27B1 (6, 7) on the 1 $\alpha$-hydroxylase activity. In addition, the $K_m$ value of CYP27A1 for 25(OH)$D_3$ 1 $\alpha$-hydroxylation is quite similar to that of CYP27B1 (6, 7), suggesting the similar binding mode of 25(OH)$D_3$ in the substrate-binding pockets of CYP27A1 and CYP27B1. Axen *et al.* (9), reported the 1$\alpha$-hydroxylation of 25(OH)$D_3$ by CYP27A1 and indicated the possibility that CYP27A1 was a physiologically essential "25(OH)$D_3$ 1$\alpha$-hydroxylase." However, Pikuleva *et al.* (2) suggested that the activity was too small for the physiological meaning. In addition, the cDNA cloning and functional expression of CYP27B1 (28) confirmed that CYP27B1 was the real 25(OH)$D_3$ 1$\alpha$-hydroxylase. Recent study to indicate the megalin-mediated transport of 25(OH)$D_3$ in proximal of kidney cell also confirmed the mechanism of the production of 1$\alpha$,25$(OH)_2D_3$ by CYP27B1 (29). On the other hand, our previous work (10) demonstrated the presence of a patient with pseudovitamin D-deficient rickets with nearly normal serum 1$\alpha$,25$(OH)_2D_3$ level in spite of the complete defects of CYP27B1 genes. The ratio among the serum levels of vitamin $D_3$, 25(OH)$D_3$ and 1$\alpha$,25$(OH)_2D_3$ is roughly estimated to be 80:800:1 (1). As described above, vitamin $D_3$ 25-hydroxylase activity catalyzed by human CYP27A1 with $K_m$ value of 3.2 $\mu$M and $V_{max}$ value of 0.27 (mol/min/mol P450) is considered to be physiologically important (1). Comparison of each of the serum substrate concentration and the $V_{max}/K_m$ value between CYP27A1-dependent vitamin $D_3$ 25-hydroxylation and 25(OH)$D_3$ 1$\alpha$-hydroxylation (Table II) suggests that 25(OH)$D_3$ 1$\alpha$-hydroxylation activity catalyzed by human CYP27A1 should not be physiologically neglected. Thus, the nearly normal 1$\alpha$,25$(OH)_2D_3$ level in the serum of the patient mentioned above appears to be derived from CYP27A1-dependent 25(OH)$D_3$ 1$\alpha$-hydroxylase activity.

Vitamin D analogs are potentially useful for clinical treatments of type I rickets, osteoporosis, renal osteodystrophy, psoriasis, leukemia and breast cancer (30). In the kidney, CYP24 plays an important role in the metabolism of the vitamin $D_3$ analogs used as drugs. However, CYP27A1 could be responsible for the metabolism in the liver. Thus, *E. coli* expression system for human CYP27A1 in this study appears useful to predict the metabolism of vitamin D analogs before clinical trials.

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