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Role of Gln 85 of human CYP27A1 in 25-hydroxyvitamin D_3-binding and protein folding

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Abstract

CYP27A1 catalyzes vitamin D_3 25-hydroxylation and further hydroxylation at C-1α, C-24 or C-26(27). Molecular modeling of human CYP27A1 and docking with 25-hydroxyvitamin D_3 predicted that Gln 85 might be important for 1α-hydroxylation activity of CYP27A1 by forming a hydrogen bond with the 25-OH group of 25-hydroxyvitamin D_3. Expectedly, the mutant Q85H expressed in *Escherichia coli* showed no detectable 1α-hydroxylation activity toward 25-hydroxyvitamin D_3. In addition, Q85H prefers 24-hydroxylation toward 25-hydroxyvitamin D_3 whereas the wild-type prefers 26(27)-hydroxylation. A molecular modeling study also suggests that Gln 85 of CYP27A1 simultaneously interacts with Asn 107 and the hydroxyl group of the substrate. The fact that Q85L did not contain a heme molecule suggests that the hydrogen bond between Gln 85 and Asn 107 is important for protein folding of CYP27A1. Based on these results, it is possible that Gln 85 plays essential roles in both substrate-binding and protein folding.

Keywords: CYP27A1; Cytochrome P450; Vitamin D hydroxylase; Molecular modeling; Site-directed mutagenesis

In humans, a hormonally active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3, is produced by two-step hydroxylation at the 25-position in the liver by CYP27A1 and CYP2R1, and then at the 1α-position by CYP27B1 in the kidney. 1α,25$(OH)_2D_3$ directs cellular processes associated with calcium transport, cellular differentiation and growth, and the immune system [1]. On the other hand, CYP24A1 plays a central role in the metabolism of 1α,25$(OH)_2D_3$ in target tissues such as kidneys, intestine, and bones [2]. Ubiquitously expressed CYP27A1 catalyzes multiple oxidation reaction at the C-27 of bile acid intermediates in liver and cholesterol in extra hepatic tissue. Thus, CYP27A1 is a physiologically essential P450 in vertebrates. Defect in CYP27A1 causes cerebrotendinous xanthomatosis (CTX) [3], which is an autosomal recessive, lipid storage disease. Large amounts of cholestanol, cholesterol, and bile alcohol are produced and accumulated in many tissues [4]. The clinical characteristics of CTX are premature bilateral cataracts, formation of tendon xanthomatosis, and neurological and neuropsychiatric abnormalities. Berginer et al. [5] reported significantly low levels of 25-dihydroxyvitamin D_3 in serum, extensive osteoporosis, and increased risk of bone fractures in patients with CTX.

So far, the crystal structures of more than 10 prokaryotic CYPs and six mammalian microsomal CYPs have been solved. The overall folding of those CYPs is quite similar, although their sequence identity is less than 20%.

Abbreviations: P450, cytochrome P450; CYP, cytochrome P450; ADR, NADPH-adrenodoxin reductase; ADX, adrenodoxin; 25$(OH)D_3$, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDDR-I, vitamin D-dependent rickets type I; CTX, cerebrotendinous xanthomatosis.

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Recently, we constructed a three-dimensional structure of CYP27B1 by a homology modeling technique using the structure of CYP2C5 as a template [6–8]. Using the three-dimensional model we studied the docking of 25-hydroxyvitamin D_3 into the substrate-binding pocket of CYP27B1, and predicted that Thr409 of human CYP27B1 is responsible for substrate-binding. Mutation studies clearly demonstrated that the prediction is basically correct. In a similar manner, we have identified the amino acid residue of CYP27B1 essential for interaction with aderenodoxin [9], and amino acid residues of CYP24A1 essential for substrate-binding [10]. Thus, a homology modeling technique together with mutation studies provides useful information on the structure–function analysis of CYPs.

In this study, we constructed a three-dimensional model of human CYP27A1, and predicted the amino acid residue responsible for the 1α-hydroxylation of vitamin D_3.

Materials and methods

Materials. DNA modifying enzymes and restriction enzymes were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Linker and primer DNAs were purchased from Japan Bio-Service (Tokyo, Japan). *Escherichia coli* DH5α (Takara Shuzo, Kyoto, Japan) was used a host strain. Bovine NADPH-adrenodoxin reductase (ADR) and adrenodoxin (ADX) were kindly given by Dr. Y. Nonaka (Koshien University). Vitamin D_3, 25(OH)D_3, 1α,25(OH)$_2D_3$, and 24*R*,25(OH)$_2D_3$ were purchased from Funakoshi (Tokyo, Japan). Other chemicals used were of the highest quality commercially available.

Sequence alignment and homology modeling. Multiple alignments of human CYP27A1, CYP27B1, and rabbit microsomal CYP2C5 were performed by using ClustalW interfaced with Clustal X (ver. 1.81) for Windows. Since sequence identity of CYP27A1 and CYP27B1 is 41%, we constructed CYP27A1 by the MUTATE MONOMER method (SYBYL 6.9) using the model structure of CYP27B1 [8] as a template. There are some deletion and insertion parts in which four amino acid residues to be deleted (G174, G248, S355, and S356 of CYP27B1) and five residues to be inserted (from P455 to R459 of CYP27A1) are located only in loop regions of the template CYP. Five residues (P455–R459) were inserted by a loop search method. Then, the model structure was optimized on Tripos force field. Energy minimization was carried out 1000 iterations. The model structure was evaluated by PROCHECK Program.

25(OH)D_3 for 1α-hydroxylation was manually docked into the substrate-binding pocket by a similar manner to that of the docking of 25(OH)D_3 into CYP27B1 [8]. Vitamin D_3 for 25-hydroxylation was docked into the pocket as the side-chain and the A-ring was accommodated in the pocket with the opposite direction for 1α-hydroxylation. Optimization of the resultant docking models was performed on the Tripos force field, in which the protein part was fixed.

Recombinant DNA procedures. Recombinant DNA procedures and transformation of *E. coli* was performed as described previously [11]. Polymerase chain reaction (PCR) was carried out with a PC2000 apparatus (Perkin-Elmer, Foster City, CA, USA). DNA sequencing was performed by using a Shimadzu dye terminator cycle sequencing ready reaction kit.

Construction of expression plasmids. The expression plasmid for human CYP27A1, pKH27A1, was constructed as described previously [12]. The expression plasmids for mutant CYP27A1 were generated by Quick Change™ Site-directed Mutagenesis kit (Stratagene) according to the instruction manual. The oligonucleotide primers for mutagenesis are shown in Table 1.

Cultivation of the recombinant E. coli cells. Recombinant *E. coli* cells were grown in TB broth containing 50 μg/ml ampicillin at 29 °C under good aeration. The induction of transcription of CYP27A1 cDNA under the promoter was initiated by addition of isopropyl-thio-β-D-galactopyranoside (IPTG) at a final concentration of 1 mM. δ-Aminolevulinic acid and chloramphenicol were also added at a final concentration of 0.5 mM and 1 μg/ml, respectively [11].

Table 1
Oligonucleotides used to generate CYP27A1 mutants

Mutation	Oligonucleotides
Q85H	5′-CTGCACCAGTTACA<u>C</u>GTGCTTTACAAGGCC-3′
	5′-GGCCTTGTAAAGCAC<u>G</u>TGTAACTGGTGCAG-3′
Q85E	5′-ACTGCACCAGTTA<u>G</u>AGGTGCTTTACAAGGC-3′
	5′-GCCTTGTAAAGCACCT<u>C</u>TAACTGGTGCAGT-3′
Q85L	5′-CTGCACCAGTTA<u>TT</u>GGTGCTTTACAAGGCC-3′
	5′-GGCCTTGTAAAGCACC<u>AA</u>TAACTGGTGCAG-3′

Western blot analysis. The membrane fractions prepared from the recombinant *E. coli* cells cultured for 48 h were subjected to electrophoresis on 4–20% linear gradient polyacrylamide/SDS gels and transferred electrophoretically from the gel to poly (vinylidene difluoride) membrane. The membrane was probed with anti-rat CYP27A1 anti-serum [13], and then followed by Enhanced Chemiluminescence immunodetection method (Amersham Pharmacia Biotech, Buckinghamshire, England). The expression levels of the proteins were estimated from the band intensities on the X-ray film by NIH Image (National Institute of Health, Bethesda, MD, USA), as described previously [14].

Measurement of reduced-CO difference spectra. Reduced-CO difference spectra were measured by a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously [12]. The concentration of CYP27A1 was determined from the reduced-CO difference spectrum using a difference of extinction coefficient at 446 and 490 nm of 91 mM^{-1} cm^{-1} [15].

Measurement of hydroxylation activity of CYP27A1 and its mutant Q85H toward vitamin D_3 and 25(OH)D_3. The hydroxylation activity was measured in the recombinant system containing the membrane fraction containing 0.2 μM CYP27A1 or Q85H, 0.5–8.0 μM of a substrate, 0.2 μM NADPH-adrenodoxin reductase, 2 μM adrenodoxin, 0.5 mM NADPH, 100 mM Tris–HCl, pH 7.4 and 1 mM EDTA at 37 °C as described previously [12]. The reaction mixture was extracted with four volumes of chloroform–methanol (3:1, v/v). The organic phase was recovered and dried up, and analyzed by HPLC using a YMC-Pack ODS-AM column (YMC Co., Kyoto, Japan) (4.6 × 300 mm) with a linear gradient of 50–100% acetonitrile aqueous solution [12], peak area of known amounts of the authentic compounds subjected to the identical HPLC.

LC-MS 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, positive mode. The conditions of LC were: column, reverse-phase ODS column (μBondapak C18, 5 μm, Waters) (6 × 150 mm); mobile-phase, 80% methanol aqueous solution per 25 min; flow-rate, 1.0 mL min^{-1}; UV detection, 265 nm.

Other method. The concentrations of vitamin D_3, 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24*R*,25(OH)$_2D_3$ were estimated by their molar extinction coefficient of 1.8×10^4 M^{-1} cm^{-1} at 264 nm [16].

Results

Three-dimensional structure of human CYP27A1

The overall folding of human CYP27A1 was quite similar to those of CYP2C5 [6] and the three-dimensional model of CYP27B1 [7,8] (Fig. 1). We evaluated the model structures by using the PROCHEK program. Ramachandran plots of human CYP27A1 showed 98.8% of residues either in the most favored or allowed region. Fig. 1A shows a stereo view of the whole structure model of human CYP27A1 complexed with vitamin D_3 which is a natural

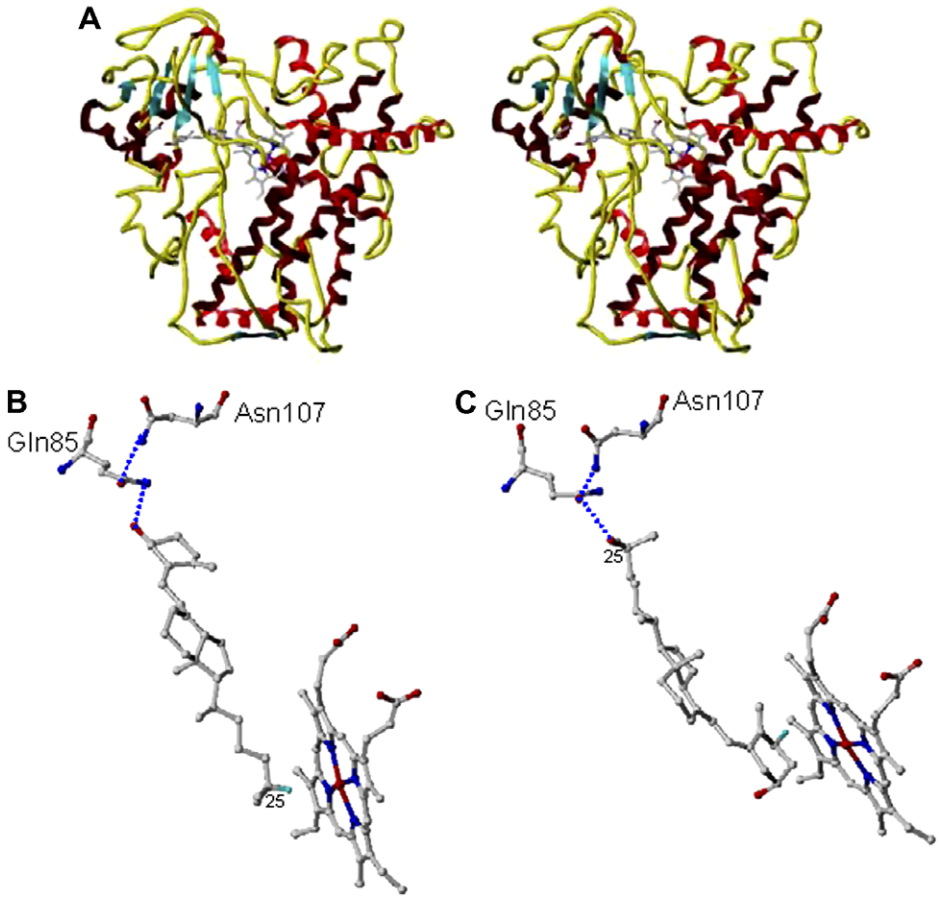

Fig. 1. Three-dimensional structure model of human CYP27A1 (A) and its docking model with vitamin D_3 for 25-hydroxylation (B) and 25(OH)D_3 for 1α-hydroxylation (C). Amino acid residues, heme, and substrate are represented as ball and stick model. Hydrogen bonds are drawn by a blue dotted line. (A) Stereo view of the complex model of human CYP27A1 and vitamin D_3. Overall folding of CYP27A1 is represented by ribbon-loop drawing. Helix, β-sheet, and loop are represented as red, cyan, and yellow color, respectively. (B) 3β-Hydroxyl group of vitamin D_3 forms hydrogen bond with the side-chain of Gln85 which also forms hydrogen bond with Asn107. Hydrogen at C(25) is positioned above Fe of the heme suggesting that 25-hydrogen could be oxidized. (C) 25-Hydroxyl group of 25(OH)D_3 forms hydrogen bond with Gln85 which forms hydrogen bond with Asn107.

ligand of this CYP. Vitamin D_3 is accommodated in CYP27A1 with stable conformation in which the A-ring adopts α-form and 3β-hydroxyl group forms hydrogen bond with the side-chain of Gln85 (Fig. 1B). The distance between oxygen of the 3-hydroxyl group and the nitrogen of the side-chain of Gln85 is 2.8 Å. Hydrogen at C(25) is positioned above Fe of the heme and distances between Fe atom and 25-hydrogen and C(25) are 3.8 and 4.6 Å, respectively suggesting that 25-hydrogen could be oxidized. Fig. 1C shows the docking model of 25(OH)D_3 into CYP27A1 for 1α-hydroxylation. 25-Hydroxyl group forms a hydrogen bond with Gln85. The distance between oxygen of the 25-hydroxyl group and the oxygen of the side-chain of Gln85 is 2.8 Å. Distances between the Fe atom and 1α-hydrogen and C(1) are 4.0 and 4.8 Å, respectively, suggesting that 1α-hydrogen could be oxidized.

Expression of human CYP27A1 and its mutant Q85H in E. coli cells

Fig. 2 shows Western blot analysis of membrane fractions prepared from the recombinant *E. coli* cells cultivated for 48 h. Western blot analysis showed that the expression levels of Q85E, Q85H, and Q85L were approximately 50%, 50%, and 25% of the wild-type, respectively. The mutant Q85H showed a reduced CO-difference spectrum with a maximum at 446 nm similar to the wild-type (data not shown). However, the mutants, Q85E and Q85L showed the spectra similar to the control (data not shown),

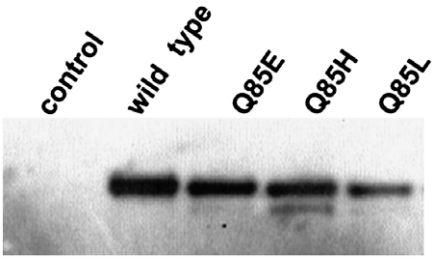

Fig. 2. Western blot analyses of CYP27A1 and its mutants. After 48 h of cultivation, the membrane fractions containing 1.0 μg protein prepared from the recombinant *E. coli* cells producing wild-type of CYP27A1 and its mutants, Q85E, Q85H, and Q85L were analyzed as described in Materials and methods.

suggesting no expression of the mutant hemoproteins. In contrast, the expression level of Q85H hemoprotein was 30–40% of the wild-type based on the specific content of the membrane fraction. In addition, Q85H showed substrate-induced Type I spectra by the addition of vitamin D_3 or 5β-cholestane-3α,7α,12α-triol (THC) similar to the wild-type (data not shown). These results indicate that this mutant retains a heme molecule, and can bind the substrate.

Metabolism of 25(OH)D_3 by the wild-type of CYP27A1 and the mutant Q85H

Fig. 3 shows HPLC profiles of 25(OH)D_3 and their metabolites by the wild-type of CYP27A1 and the mutant Q85H. Based on our previous study on the metabolism of 25(OH)D_3 by CYP27A1, the metabolites were considered to be 24*R*,25(OH)$_2$$D_3$, 1α,25(OH)$_2$$D_3$, and 25,26(27)(OH)$_2$$D_3$ [12]. Under the HPLC conditions used in this study, 25,26(OH)$_2$$D_3$ and 25,27(OH)$_2$$D_3$ cannot be separated. Thus, the third peak in Fig. 3 appears to contain both 25,26(OH)$_2$$D_3$ and 25,27(OH)$_2$$D_3$. On the other hand, HPLC profile of Q85H was quite different from that of the wild type. Although the first peak at 19 min was observed, the second peak corresponding to 1α,25(OH)$_2$$D_3$ was not observed. In addition, the peak corresponding to 25,26(27)(OH)$_2$$D_3$ was also remarkably reduced. These results strongly suggest that the conversion of Gln to His at 85-position dramatically changed enzymatic properties of human CYP27A1. To confirm that a major metabolite of 25(OH)D_3 by Q85H is 24,25(OH)$_2$$D_3$, the metabolite was analyzed by LC-MS. Fig. 4 shows mass spectra of the metabolite and authentic standards of 1α,25(OH)$_2$$D_3$ and 24*R*,25(OH)$_2$$D_3$. The fragmentation pattern of the metabolite was very similar to 24*R*,25(OH)$_2$$D_3$. Although the retention time of 23,25(OH)$_2$$D_3$ is nearly the same as 24*R*,25(OH)$_2$$D_3$ under the HPLC conditions used in this study, 23,25(OH)$_2$$D_3$ should have a major fragment ion at *m*/*z* 325 and 307 [17]. These results strongly suggest that the major metabolite by Q85H is 24*R*,25(OH)$_2$$D_3$.

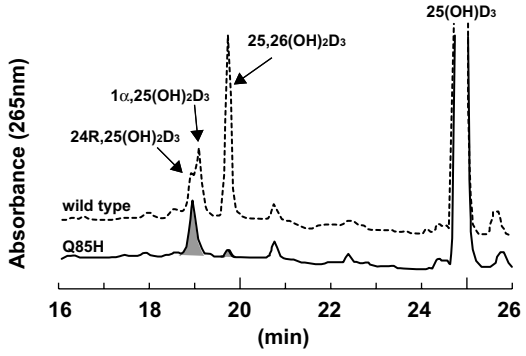

Fig. 3. HPLC profiles of 25(OH)D_3 and its metabolites in the reconstituted system containing membrane fraction prepared from DH5a/pKH27A1 (wild-type) and DH5a/pKHQ85H (Q86H). The peak designated as 25,26(OH)$_2$$D_3$ appears to contain both 25,26(OH)$_2$$D_3$ and 25,27(OH)$_2$$D_3$.

Kinetic analysis of vitamin D hydroxylation activity by the wild-type of CYP27A1 and the mutant Q85H

Table 2 shows kinetic parameters of wild-type and Q85H for 25-hydroxylation toward vitamin D, and 1α,24, and 26(27)-hydroxylation activity toward 25(OH)D_3. In vitamin D 25-hydroxylation, Q85H showed somewhat smaller k_{cat} and larger K_m than the wild-type. Thus, k_{cat} /K_m of Q85 was 27% of the wild-type. Since Q85H showed no detectable 1α-hydroxylation activity, the kinetic parameters were not determined. The fact that Q85H abolishes 1α-hydroxylation activity toward 25(OH)D_3 appears to be closely related to the fact that Q65H of CYP27B1 corresponding to Q85H of CYP27A1 abolishes the 1α-hydroxylase activity to cause VDDR-I. In 25(OH)D_3 24-hydroxylation, K_m value of Q85H was significantly increased, and k_{cat}/K_m value of Q85H reduced to 32% of the wild-type. On the other hand, a significant

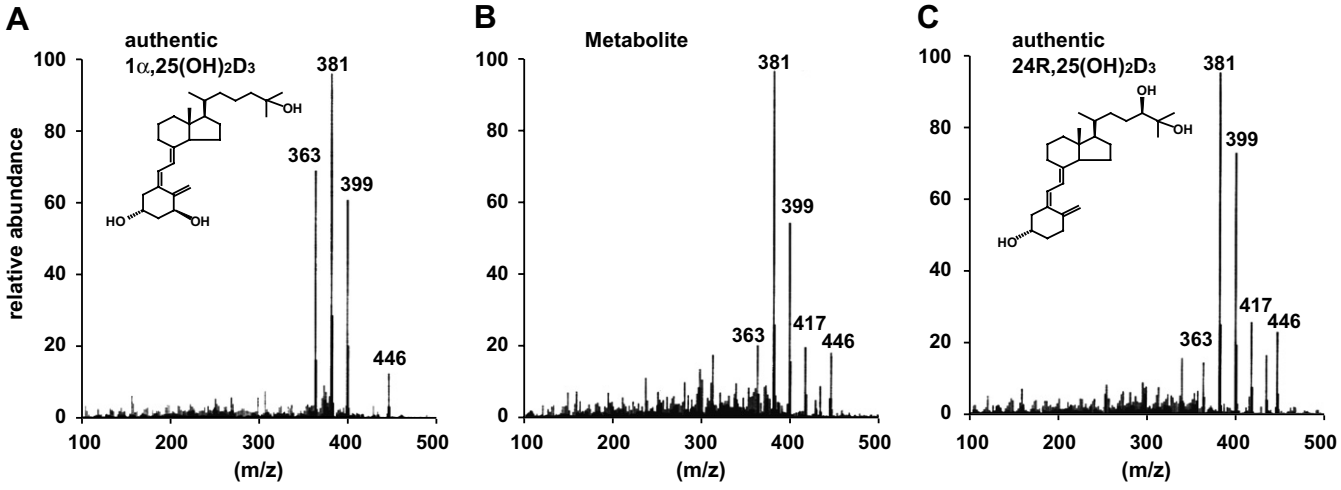

Fig. 4. Mass spectra of authentic standard of 1α,25(OH)$_2$$D_3$ (A), the major metabolite by Q85H (B), and authentic standard of 24*R*,25(OH)$_2$$D_3$ (C).

Table 2
Kinetic parameters of the wild-type of human CYP27A1 and its mutant Q85H for hydroxylation of vitamin D_3 and 25(OH)D_3

CYP27A1	Substrate	Position	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
Wild	VD_3	25	0.27 ± 0.01	3.2 ± 0.5	0.084
Q85H	VD_3	25	0.15 ± 0.03	6.5 ± 2.5	0.023
Wild	25(OH)D_3	1α	0.021 ± 0.002	3.5 ± 0.4	0.006
Q85H	25(OH)D_3	1α	[a]	[a]	[a]
Wild	25(OH)D_3	24	0.014 ± 0.003	5.5 ± 0.7	0.0025
Q85H	25(OH)D_3	24	0.054 ± 0.006	69 ± 15	0.0008
Wild	25(OH)D_3	26(27)	0.054 ± 0.008	2.9 ± 0.7	0.0186
Q85H	25(OH)D_3	26(27)	0.012 ± 0.001	55 ± 11	0.0002

[a] 1α-Hydroxylase activity of Q85H was not detected. Each value of k_{cat} and K_m represents the mean ± SD for three separate experiments.

reduction of k_{cat} value and a significant increase of K_m value made an extreme reduction of k_{cat}/K_m in 25(OH)D_3 26(27)-hydroxylation.

Discussion

CYP27A1 is a multi-function enzyme responsible for the production of bile acids and the hydroxylation of cholesterol and vitamin D. CYP27A1 plays an important role as vitamin D 25-hydroxylase as does CYP2R1 whose deficiency causes VDDR-I, as reported by Cheng et al. [18]. In addition, we suppose that detectable 1α,25$(OH)_2D_3$ in the serum of the patients with VDDR-I is derived from the 1α-hydroxylation activity of CYP27A1 [12]. We revealed the roles of the amino acid residues of CYP27B1 and CYP27A1 whose mutation causes VDDR-I and CTX, respectively [8,11]. Pikuleva et al. [19,20], and Murtazina et al. [21] revealed amino acid residues of CYP27A1 involved in electron transfer, substrate-binding, or membrane–protein interaction by site-directed mutagenesis. Recently, Proccer et al. [22] identified amino acid residues, Phe248, Ser404, and Val515, which moderately affect the 25- and 27-hydroxylation of 1α(OH)D_3, by modeling of human CYP27A1 and site-directed mutational analyses. Thus, the combination of molecular modeling and site-directed mutagenesis has provided much information on the structure–function relationships of these CYPs. In this study, we found that Gln85 greatly affected substrate-recognition of CYP27A1.

As reported in our previous study, molecular modeling studies have suggested that Gln65 and/or Thr409 of human CYP27B1 might play an essential role in substrate binding. Site-directed mutagenesis studies using mouse CYP27B1 clearly demonstrated that Ser408 of mouse CYP27B1 corresponding to Thr409 of human CYP27B1 is responsible for the binding of substrate 25(OH)D_3 [8]. The substitution of Ser408 to Ala, Val or Ile greatly reduced the 1α-hydroxylation activity of CYP27B1, suggesting that the OH-group of the side-chain of the residue was found to be essential. Based on the fact that Val 425 of human CYP27A1corresponds to Thr409 of human CYP27B1, we assumed that substitution of Val 425 to Thr might increase the 1α-hydroxylation activity. However, no increase of 1α-hydroxylation activity was observed while the expression level was significantly reduced (data not shown). On the other hand, amino acid substitution of Gln65 of mouse CYP27B1 likely affected the conformation of CYP27B1 based on site-directed mutagenesis studies. The three-dimensional model suggested that Gln65 of CYP27B1 can either interact with Y87 on the β-sheet or with the 25-hydroxyl group of the substrate, but it cannot simultaneously interact with them [8]. Thus, when Gln65 of CYP27B1 interacts with Tyr87, it does not form a hydrogen bond with the 25-hydroxyl group of 25(OHD_3. Note that Gln65 and Tyr87 of CYP27B1 correspond to Gln85 and Asn107 of CYP27A1, respectively. Unexpectedly, the current molecular modeling study suggests that Gln 85 of CYP27A1 simultaneously interacts with Asn107 and the hydroxyl group of the substrate as shown in Fig. 1. A hydrogen bond between Gln85 and Asn107 might be important for protein folding because Q86L did not show the structure containing heme. Although we cannot explain no expression of Q85E hemoprotein, a negative charge might affect protein folding. Despite the unsuccessful expression of Q85L and Q85E hemoproteins, we successfully expressed Q85H hemoprotein in *E. coli* cells. A molecular modeling study suggests that His 85 of Q85H can interact with Asn 107. Judging from HPLC profiles shown in Fig. 3, Q85H appears to be a novel enzyme distinct from the wild type.

Docking with 25(OH)D_3 suggests that Gln 85 is important for 1α-hydroxylation activity of CYP27A1 by forming a hydrogen bond with the 25-OH group of 25(OH)D_3. Thus, no 1α-hydroxylation activity of the Q85H satisfies our expectations. In contrast, unexpected results were obtained in 24/25/26(27)-hydroxylation by Q85H. Note that substrate 25(OH)D_3 is inserted into the substrate-binding pocket of CYP27A1 in opposite directions for 1α-hydroxylation and 24/25/26(27)-hydroxylation. In 24- and 26(27)-hydroxylation toward 25(OH)D_3, Q85H preferentially hydroxylates at the C-24 position whereas the wild-type prefers 26(27)-hydroxylation. These results suggest that Gln 85 affects the orientation of 25(OH)D_3 by an interacting A-ring of 25(OH)D_3. In vitamin D_3 25-hydroxylation, Q85H showed no significant discrepancy from the wild-type. The k_{cat}/K_m value of Q85H for vitamin

D_3 25-hydroxylation is much higher than 25(OH)D_3 24-hydroxylation. Thus, Q85H appears to prefer the hydroxylation positions in the turn of C-25 > C-24 > C-26(27), whereas the wild-type prefers the hydroxylation positions in the turn of C-25 > C-26(27) > C-24. Based on these results, it is possible that Gln85 plays essential roles in both substrate-binding and protein folding.

In this study, we generated a C-24-specific hydroxylase for 25OHD_3 with one point mutation of CYP27A1. Although CYP24A1 also catalyzes 24-hydroxylation of 25(OH)D_3 to produce 24,25$(OH)_2D_3$, CYP24A1 catalyzes further oxidation as described previously [2]. Thus, Q85H is a novel enzyme to produce 24,25$(OH)_2D_3$ as a major metabolite from vitamin D_3 by both vitamin D 25-hydroxylation and 24-hydroxylation activities. So far, many reports have shown that 24,25$(OH)_2D_3$ has a physiological function different from that of 1α,25$(OH)_2D_3$. Thus, *E. coli* cells expressing Q85H might be practically useful to produce 24,25$(OH)_2D_3$ from vitamin D_3 although further studies are needed.

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