Identification of the Amino Acid Residue of CYP27B1 Responsible for Binding of 25-Hydroxyvitamin D₃ Whose Mutation Causes Vitamin D-dependent Rickets Type 1*

Received for publication, May 12, 2005, and in revised form, June 22, 2005
Published, JBC Papers in Press, June 22, 2005, DOI 10.1074/jbc.M505244200

We previously reported the three-dimensional structure of human CYP27B1 (25-hydroxyvitamin D₃ 1α-hydroxylase) constructed by homology modeling. Using the three-dimensional model we studied the docking of the substrate, 25-hydroxyvitamin D₃, into the substrate binding pocket of CYP27B1. In this study, we focused on the amino acid residues whose point mutations cause vitamin D-dependent rickets type 1, especially unconserved residues among mitochondrial CYPs such as Gln₆⁵ and Thr₄⁰⁹. Recently, we successfully overexpressed mouse CYP27B1 by using a GroEL/ES co-expression system. In a mutation study of mouse CYP27B1 that included spectroscopic analysis, we concluded that in a 1α-hydroxylation process, Ser₄⁰⁸ of mouse CYP27B1 corresponding to Thr₄⁰⁹ of human CYP27B1 forms a hydrogen bond with the 25-hydroxyl group of 25-hydroxyvitamin D₃. This is the first report that shows a critical amino acid residue recognizing the 25-hydroxyl group of the vitamin D₃.

The hormonally active form of vitamin D₃, 1,25-(OH)₂D₃, plays essential roles in calcium homeostasis, immunology, and cell differentiation (1). 1,25-(OH)₂D₃ is produced by two-step hydroxylations at the 25-position in the liver by mitochondrial CYP27A1 and then at the 1α-position in the kidney by CYP27B1. The cDNA for CYP27B1 was first cloned in 1997 (2–5), and the sequence analysis of the CYP27B1 gene confirmed that defects in CYP27B1 cause vitamin D-dependent rickets type 1 (VDDR1). To date, 16 one-point mutants and several frameshift mutants have been reported (6–9). The mutated amino acid residues seemed to play important roles in the function of 1α-hydroxylase, such as substrate binding, activation of molecular oxygen, interaction with adrenodoxin, and folding of the P450 structure (10, 11).

To investigate the mutations in depth, spectral analyses including reduced CO-difference spectra and substrate-induced difference spectra are indispensable. However, the expression levels of wild type and CYP27B1 mutants were too low to carry out spectral analyses (11, 12). Thus, enhancement of the expression level of CYP27B1 is essential for structure-function analysis of CYP27B1. Recently, we successfully overexpressed mouse CYP27B1 by using a GroEL/ES co-expression system (13). The expression level of CYP27B1 is sufficient for the preparation of large amounts of wild type and CYP27B1 mutants for structural analyses. In addition, we successfully constructed a three-dimensional structure of CYP27B1 by the homology modeling technique using the structure of rabbit microsomal CYP2C5 as a template, which is the first solved x-ray structure of a eukaryotic CYP (14, 15). The three-dimensional model of CYP27B1 provided much information about the roles of amino acid residues at the mutated positions seen in VDDR1 patients. In this study, we focused on the mutants from VDDR1 whose mutated amino acids are not conserved among six mitochondrial P450s (Fig. 1). We demonstrate which amino acid residue is responsible for substrate binding by mutation studies that include spectral analysis of CYP27B1.

EXPERIMENTAL PROCEDURES

Materials—DNA modifying enzymes, restriction enzymes, and the DNA sequencing kit were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Primer DNAs for mutation were purchased from GENSET KK (Kyoto, Japan). Table I). Escherichia coli DHB5 (Takara Shuzo Co.) was used as a host strain. The pKSDdl was constructed from pk223-3 as described previously (13). The GroEL/ES expression plasmid, pGro12, was kindly given by the HSP research laboratory (Kyoto, Japan). CHAPS was purchased from Dojindo (Kumamoto, Japan). NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). Bovine adrenodoxin and NADPH-adrenodoxin reductase were kindly given by Dr. Y. Nonaka of Koshien University. 25-(OH)₂D₃ was purchased from
Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of the highest quality commercially available.

**Molecular Modeling and Substrate Docking**—Molecular modeling and graphical manipulations were performed using SYBYL 6.9 (Tripos, St. Louis, MO). 25-Hydroxyvitamin D_3 was docked into the substrate binding pocket manually.

**Construction of Expression Plasmids**—The expression plasmid for mouse CYP27B1 with the His tag at the C terminus, pKCHis-m1, was constructed as described (6). The expression plasmids for CYP27B1 mutants (S408I, S408T, S408V, S408A, S408H, Q65H, Q65E, Q65A, Q65L, Q65S, S408A, S408H, Q65H, Q65E, Q65A, Q65L, Q65S, Q65N) were generated by the QuikChange® Site-directed Mutagenesis kit from Stratagene (Amsterdam, the Netherlands) according to the instruction manual. The oligonucleotide primers for mutagenesis are shown in Table I. Corrected generation of the desired mutations was confirmed by DNA sequencing.

**Cultivation of the Recombinant E. coli Cells**—The E. coli DH5α harboring pGro12 was transformed with the expression plasmid for wild type CYP27B1 (pKCHis-m1α) or its mutants. Recombinant E. coli cells were grown in TB media (pH 7.0) containing 50 μg/ml ampicillin and 25 μg/ml kanamycin at 26 °C under good aeration for 24 h. The induction of transcription of CYP27B1 cDNA and the GroEL/ES gene was initiated by addition of isopropyl-1-thio-μ-D-galactopyranoside and arabinose at a final concentration of 1 mM and 4 mg/ml, respectively.

**Solubilization of Wild Type and CYP27B1 Mutants by CHAPS**—The recombinant E. coli cells were suspended in 100 mM Tris-HCl buffer (pH 7.4) containing 1% CHAPS, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 20% glycerol, and disrupted by sonication for 15 min at 4 °C. Cell debris was removed at 1,200 × g for 10 min. Then the supernatant was ultracentrifuged at 100,000 × g for 1 h at 4 °C. The resultant supernatant was used for spectral and enzymatic analyses.

**Measurement of Reduced CO-difference Spectra**—Reduced CO-difference spectra were measured by a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously (18). The concentration of CYP27B1 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 by Omura and Sato (17).

**Measurement of Substrate-induced Difference Spectra**—Substrate-induced difference spectra of wild type and CYP27B1 mutants were measured in the presence of 1.0 μM 25-(OH)D_3 by a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan).

**Western Blot Analysis of Gln^{50} Mutants**—Anti-CYP27B1 antiserum was prepared using a purified sample of mouse CYP27B1 as antigen (13). The purified CYP27B1 (0.1 mg) was mixed with an equal volume of Freund's complete adjuvant and injected intradermally into a young male Japan White rabbit. At 2, 4, and 6 weeks after the first injection, the rabbit was boosted with additional injections of 0.1 or 0.2 mg each of the antigen mixed with Freund's incomplete adjuvant.

**Docking of 25-(OH)D_3 into CYP27B1**—The three-dimensional structure of CYP27B1, which was constructed and reported in our previous paper (14), gave abundant insights in regards to the function of each residue. We are greatly interested in how the CYP27B1 recognizes 25-(OH)D_3 and binds it as a substrate. We noted amino acid residues whose point mutant cause VDDR1 (Fig. 1). Fig. 1 shows comparison of the residues of CYP27B1 with other human mitochondrial CYPs at the mutated positions seen in patients with VDDR1. The amino acid residues that are identical and homologous in all the CYPs are shaded dark and light, respectively. The amino acid residues at the non-conserved position are surrounded by open boxes.

**RESULTS**

The three-dimensional structure of CYP27B1 demonstrated that P143L, E189L, S323Y, T409I, and R429P are not conserved and are thought to be involved in specific roles of each enzyme such as substrate binding. Thus, we focused on mutants Q65H, P143L, E189L, S323Y, T409I, and R429P. The three-dimensional structure of CYP27B1 with the mutated positions seen in patient with VDDR1. The amino acid residues that are identical and homologous in all the CYPs are shaded dark and light, respectively. The three-dimensional structure of CYP27B1 with the mutated positions seen in patients with VDDR1. The amino acid residues that are identical and homologous in all the CYPs are shaded dark and light, respectively.

**Docking of 25-(OH)D_3 into CYP27B1**—The three-dimensional structure of CYP27B1, which was constructed and reported in our previous paper (14), gave abundant insights in regards to the function of each residue. We are greatly interested in how the CYP27B1 recognizes 25-(OH)D_3 and binds it as a substrate. We noted amino acid residues whose point mutant cause VDDR1 (Fig. 1). Fig. 1 shows comparison of the residues of CYP27B1 with other human mitochondrial CYPs at the mutated positions seen in patients with VDDR1. Residues conserved in these CYPs are thought to be responsible for common roles among mitochondrial CYPs, whereas residues not conserved are thought to be involved in specific roles of each enzyme such as substrate binding. Thus, we focused on mutants Q65H, P143L, E189L, S323Y, T409I, and R429P. The three-dimensional structure of CYP27B1 demonstrated that P143L, E189L, S323Y, T409I, and R429P are responsible for protein folding as previously reported (14) (Fig. 2a). On the other hand, Gln^{50} and Thr^{109} are lining the end of broad cavity above heme in the three-dimensional model of CYP27B1 (Fig. 2a).

In the docking study of 25-(OH)D_3 into the pocket, it is important to determine the substrate binding site and the conformation of 25-(OH)D_3. Considering the importance of...
Gln\textsuperscript{65} and Thr\textsuperscript{409} whose mutations cause VDDR1, we selected the substrate binding site where \(25-(OH)D_3\) can form the hydrogen bond between the \(25-OH\) group and Gln\textsuperscript{65} and/or Thr\textsuperscript{409}. We docked \(25-(OH)D_3\) as follows: 1) the A-ring of \(25-(OH)D_3\) was superimposed on 1R-camphor accommodated in the substrate binding pocket of the P450cam protein (Protein Data Bank code 1DZ4) (19), because, structurally and biologically, the best characterized P450 is P450cam and we analyzed the docking modes of camphor into P450cam and found the modes being approximately conserved. In addition, P450cam belongs to the class 1 enzyme in the P450 superfamily as well as mitochondrial CYPs. 2) The side chain of \(25-(OH)D_3\) was positioned near Gln\textsuperscript{65} and Thr\textsuperscript{409}. 3) Spatial location of \(25-(OH)D_3\) was manually adjusted so as to minimize the van der Waals bump between the substrate vitamin and the amino acid residues lining the substrate binding pocket. The resulting structure of CYP27B1 and \(25-(OH)D_3\) complex is shown in Fig. 2\textit{a}. The distances between the \(25-(OH)D_3\) and Gln\textsuperscript{65} and the \(25\)-hydroxyl group and Thr\textsuperscript{409} are 2.83 and 2.82 Å, respectively. This suggests that the \(25\)-hydroxyl group forms pincer-type hydrogen bonds with Gln\textsuperscript{65} and Thr\textsuperscript{409}. The \(1a\)-hydrogen of \(25-(OH)D_3\), which will be subjected to hydroxylation, orients to the iron atom of the heme. The distance between C(1) and iron is 4.3 Å and that between hydrogen at the \(1a\)-position and iron is 3.3 Å (Fig. 2\textit{b}), which are consistent with the distances observed in the crystal structures: C(5)-iron (4.2 Å) and H(5)-iron (3.2 Å) in the camphor-P450cam complex (1DZ4) (19); C(5)-iron (3.9 Å) and H(5)-iron (3.3 Å) in the camphor-P450cam complex (1AKD) (20); C(1)-iron (3.8 Å) and H(1)-iron (2.9 Å) in the androstenedione-P450eryF complex (1EUP) (21). If the A-ring of \(25-(OH)D_3\) adopts the \(\alpha\)-form, distances of C(1)-iron and C(2)-iron are 3.9 and 3.8 Å, respectively, resulting in the absence of a rational explanation for the selective hydroxylation that occurred at the \(1a\)-position but not the \(2\)-position. Thus, our docking model well explains the stereospecific hydroxylation at the \(1a\)-position of \(25-(OH)D_3\).

Expression of Wild Type and CYP27B1 Mutants with GroEL/ES Co-expression System—Molecular modeling study of CYP27B1 strongly suggests that Ser\textsuperscript{408} of mouse CYP27B1 corresponding to Thr\textsuperscript{409} of human CYP27B1, and/or Gln\textsuperscript{65} are involved in substrate binding (Fig. 3). To reveal each function of the mutated amino acid residues, we generated multiple forms of CYP27B1 mutants at positions 408 (409 in human CYP27B1) and 65. As shown in Fig. 4, mutant S408I showed the reduced CO-difference spectra similar to wild type. S408T and S408A, and S408V, also showed similar spectra (data not shown). The expression level of the wild type of CYP27B1 was 200–300 nmol/liter of culture, as described previously (13). The
expression levels of Ser408 mutants, S408I and S408A, were nearly the same as the wild type, whereas those of S408T and S408V were higher (400–450 nmol/liter of culture). On the other hand, the expression levels of Gln65 mutants (Q65H, Q65E, Q65A, Q65L, Q65N) were too low to be determined by the reduced CO-difference spectra. However, Q65E showed a substrate-induced difference spectrum. Thus, the expression level of the Q65E hemoprotein was estimated to be 10 nmol/liter based on the assumption that Q65E shows a substrate-induced difference spectrum similar to wild type. In contrast, Western blot analysis showed that the distinct bands reacted with anti-CYP27B1 antiserum in the Gln65 mutants. The expression levels of the Gln65 mutants were not so different from that of the wild type (Fig. 5). These results suggest that most Gln65 mutants are expressed as apoproteins without a heme molecule in E. coli cells.

Analysis of Substrate Binding of Wild Type and Ser408 Mutants of CYP27B1 with 25-(OH)D₃—As shown in Fig. 6, substrate-induced difference spectra of wild type and CYP27B1 mutants with 25-(OH)D₃ showed Type I spectra, indicating the change of spin state of heme iron of CYP27B1 from low spin to high spin. The magnitude of ΔA900–420 in S408T was slightly larger than the wild type of CYP27B1. In contrast, the magnitude of ΔA900–420 in S408V and S408A was quite small, but S408I showed no detectable spectral change. These results suggest that the substrate, 25-(OH)D₃, can remove the H₂O molecule as the sixth axial ligand of the heme iron of wild type CYP27B1 and mutant S408T. In addition, the hydroxyl group at the side chain of the amino acid at position 408 appears to be essential for removal of the H₂O molecule. It should be noted that S408I corresponding to T409I from patients with VDDR1 cannot remove the H₂O molecule by 25-(OH)D₃.

Analysis of 1α-Hydroxylation Activity of Wild Type and CYP27B1 Mutants toward 25-(OH)D₃—The 1α-hydroxylation activity toward 25-(OH)D₃ was examined using solubilized CYP27B1 by CHAPS as described under “Experimental Procedures.”

---

**TABLE II**

| CYP27B1 | kcat | Km | kcat/Km |
|---|---|---|---|
| Wild type | 23.1 ± 0.9 | 0.28 ± 0.06 | 84 ± 19 |
| S408T | 5.9 ± 1.6 | 0.13 ± 0.03 | 46 ± 11 |
| S408A | 0.34 ± 0.06 | 0.24 ± 0.04 | 1.5 ± 0.3 |
| S408V | 0.78 ± 0.40 | 0.54 ± 0.18 | 1.5 ± 0.7 |
| S408I | 0.050 ± 0.020 | 0.18 ± 0.06 | 0.31 ± 0.10 |

**TABLE III**

| CYP27B1 | kcat | Km | kcat/Km |
|---|---|---|---|
| Wild type | 0.60 ± 0.12 | 0.52 ± 0.05 | 1.1 ± 0.1 |
| S408V | 0.022 ± 0.010 | 0.66 ± 0.09 | 0.033 ± 0.009 |

---

*Analysis of Substrate Binding of Wild Type and Ser408 Mutants of CYP27B1 with 25-(OH)D₃—* As shown in Fig. 6, substrate-induced difference spectra of wild type and CYP27B1 mutants with 25-(OH)D₃ showed Type I spectra, indicating the change of spin state of heme iron of CYP27B1 from low spin to high spin. The magnitude of ΔA900–420 in S408T was slightly larger than the wild type of CYP27B1. In contrast, the magnitude of ΔA900–420 in S408V and S408A was quite small, but S408I showed no detectable spectral change. These results suggest that the substrate, 25-(OH)D₃, can remove the H₂O molecule as the sixth axial ligand of the heme iron of wild type CYP27B1 and mutant S408T. In addition, the hydroxyl group at the side chain of the amino acid at position 408 appears to be essential for removal of the H₂O molecule. It should be noted that S408I corresponding to T409I from patients with VDDR1 cannot remove the H₂O molecule by 25-(OH)D₃.

*Analysis of 1α-Hydroxylation Activity of Wild Type and CYP27B1 Mutants toward 25-(OH)D₃—* The 1α-hydroxylation activity toward 25-(OH)D₃ was examined using solubilized CYP27B1 by CHAPS as described under “Experimental Procedures.”
dures." As shown in Table II, kinetic parameters, \( K_m \) and \( k_{cat} \), of the wild type CYP27B1 were estimated to be 0.28 \( \mu \)M and 23.1 min\(^{-1} \), respectively. The \( k_{cat} \) and \( K_m \) values of S408T were significantly lower than those of the wild type CYP27B1. However, S408T appeared to have enough activity as a 1\( \alpha \)-hydroxylase for 25-(OH)D\(_3\) based on its \( k_{cat}/K_m \) value. On the other hand, S408A and S408V showed much smaller \( k_{cat} \) values than the wild type. As expected by spectral analysis, S408I showed the smallest activity among the mutants. Note that S408A, S408V, and S408I showed \( K_m \) values not so different from the wild type.

In the same way, 1\( \alpha \)-hydroxylation activity toward 25-(OH)D\(_3\) of Gln65 mutants was measured. Mutant Q65E showed only a small activity, although other Gln65 mutants showed no detectable activity. The \( K_m \) value of Q65E was estimated to be 0.80 \( \mu \)M, which is considerably higher than those of Ser460 mutants (Table II). Because the concentration of Q65E hemoprotein was not determined from reduced CO-difference spectrum probably because of its unstability, the \( k_{cat} \) value was not determined. On the assumption that Q65E shows a substrate-induced difference spectrum similar to wild type, \( k_{cat} \) was estimated to be 0.55 min\(^{-1} \).

**Analysis of 1\( \alpha \)-(OH)D\(_3\) 25-Hydroxylation Activity of the Wild Type and S408V—** We consider that the mutant T409I of human CYP27B1 corresponds to S408I of mouse CYP27B1. However, it might be possible that the conversion of Thr to Ile corresponds to the conversion of Ser to Val, judging from their chemical structure. Thus, enzymatic properties of S408V were compared with those of the wild type. As shown in Table III, the kinetic parameters, \( K_m \) and \( k_{cat} \), of the wild type for 1\( \alpha \)-(OH)D\(_3\) 25-hydroxylation was estimated to be 0.52 \( \mu \)M and 0.60 min\(^{-1} \), respectively. Thus, the \( k_{cat}/K_m \) value was only 1.3% of 1\( \alpha \)-(OH)D\(_3\) 25-hydroxylation. Ser460 showed a similar \( K_m \) value but a much smaller \( k_{cat} \) value than wild type. These results are quite similar to those for 25-(OH)D\(_3\) 1\( \alpha \)-hydroxylation, suggesting that Ser460 is involved in the binding of not only 25-(OH)D\(_3\) but also 1\( \alpha \)-(OH)D\(_3\).

**Vitamin D\(_3\) Metabolism by the Wild Type and S408V—** Fig. 7 shows the HPLC profiles of vitamin D\(_3\) by wild type CYP27B1 and S408V. Both 1\( \alpha \)-(OH)D\(_3\) and 1\( \alpha \),25-(OH)D\(_2\) were detected in the metabolism by the wild type. However, 25-(OH)D\(_3\) was not detected as previously described (13). On the other hand, S408V showed a clear peak of 25-(OH)D\(_3\) in addition to those of 1\( \alpha \)-(OH)D\(_3\) and 1\( \alpha \),25-(OH)D\(_2\). LC-MS analysis confirmed that this metabolite is 25-(OH)D\(_3\) (data not shown). It is possible to assume that 25-(OH)D\(_3\) is not detected as an intermediate because of its rapid conversion to 1\( \alpha \),25-(OH)D\(_2\) by the wild type CYP27B1, but 25-(OH)D\(_3\) is detected because of its slow conversion by S408V. Fig. 8 shows the time courses of vitamin D\(_3\) metabolism. In the metabolism of wild type CYP27B1, 1\( \alpha \)-(OH)D\(_3\) increased up to 10 min and thereafter reached plateau, whereas 1\( \alpha \),25-(OH)D\(_2\) continued increasing. On the other hand, 25-(OH)D\(_3\) was not detected as described previously (13).

In contrast, 25-(OH)D\(_3\) was detected as a metabolite of vitamin D\(_3\) by S408V. As shown in Fig. 7, 25-(OH)D\(_3\) increased up to 10 min and thereafter reached plateau, whereas 1\( \alpha \)-(OH)D\(_3\) continued increasing. On the other hand, 1\( \alpha \),25-(OH)D\(_2\) appeared at 10 min, and then the rate of 1\( \alpha \),25-(OH)D\(_2\) formation increased with increasing time. Vitamin D\(_3\) metabolism together with 25-(OH)D\(_3\) 1\( \alpha \)-hydroxylation and 1\( \alpha \)-(OH)D\(_3\) 25-hydroxylation by S408V strongly indicated that S408V has a dual pathway to produce 1\( \alpha \),25-(OH)D\(_2\) from vitamin D\(_3\) as shown in Fig. 9. Although 25-(OH)D\(_3\) was not detected in the wild type-dependent metabolism of vitamin D\(_3\), it is possible that the wild type has a dual pathway as well as S408V.

**DISCUSSION**

Kitanaka et al. (6, 7) cloned eight types of missense mutations and one nonsense mutation from Japanese VDDR1 patients, and other groups identified nine missense mutations from patients (8, 9). None of the CYP27B1 mutants expressed in mammalian cells (6) and E. coli cells (11, 12) showed 1\( \alpha \)-hydroxylase activity toward 25-(OH)D\(_3\). Thus, the mutated amino acid residues seemed to play important roles in the function of 1\( \alpha \)-hydroxylase. Our previous study (11) suggested that Arg\(^{286}\), Gly\(^{125}\), and Pro\(^{297}\) destroyed the tertiary structure of the substrate-heme pocket. It was also suggested that Arg\(^{286}\) and Arg\(^{253}\) of CYP27B1 were involved in heme-propionate binding and that Asp\(^{164}\) stabilized the 4-helix bundle consisting of D, E, I, and J helices, possibly by forming a salt bridge. Thr\(^{321}\) was found to be responsible for the activation of molecular oxygen.

As shown in Fig. 1, amino acid residues at positions 65, 143,
Fig. 9. Metabolic pathway of vitamin D₃ by CYP27B1 and the mutant S408V.

189, 323, 409, and 429 of human CYP27B1 were not conserved among mitochondrial P450s. Of these mutations, P143L, E189G(K,L), and R429P are assumed to disrupt protein folding because Pro and Gly residues are known to be helix breakers. In addition, S323Y in the I helix appears to play an important role in protein folding because the side chain of the amino acid residue at position 323 is oriented to the opposite side of a heme molecule, buried inside the protein structure (14). The three-dimensional structure model of CYP27B1 implied that Gln⁶⁵ and/or Thr⁴⁰⁹ interacts with 25-(OH)D₃, probably by forming a hydrogen bond with the 25-hydroxyl group of the substrate. We have not successfully overexpressed human CYP27B1 yet, but we have successfully overexpressed mouse CYP27B1 by using a GroEL/ES co-expression system. Thus, we generated mouse CYP27B1 mutants for Gln⁶⁵ and Ser⁴⁰⁸, corresponding to Thr⁴⁰⁹ of human CYP27B1. The substitution of Ser⁴⁰⁸ to Thr did not cause significant alterations in substrate binding and 1α-hydroxylation activity toward 25-(OH)D₃. However, the substitutions to Ala, Val, and Ile dramatically decreased 1α-hydroxylation activity and changed the substrate binding manner. Judging from the Kₘ values of S408A, S408V, and S408I, these mutants have significant affinity for 25-(OH)D₃. However, based on their substrate-induced manner, their binding mode of the substrate appears unsuitable for displacement of H₂O as the distal ligand. Because the displacement of the H₂O molecule by the substrate is essential for P450 reactions, all our data, including the determination of the Kₘ values of Q65E, is consistent with an involvement of Gln⁶⁵ in binding of the substrate. It is noteworthy that the predictions derived from the three-dimensional model showed good agreement with the experimental data. Thus, this three-dimensional model gives essential information on the structure-function relationship of CYP27B1.

REFERENCES

1. Feldman, D., Glorieux, F. H., and Pike, J. W. (eds) (1997) Vitamin D, Academic Press, New York.
2. Takeyama, K., Kitazaka, S., Sato, T., Kobori, M., Yanagisawa, J., and Kato, S. (1997) Science 277, 1827–1830.
3. Uchida, E., Kagawa, N., Sakaki, T., Urushino, N., Sawada, N., Kamakura, M., and Tanaka, T. (1998) J. Biol. Chem. 273, 257, 381–389.
4. Yoshida, T., Monkawa, T., Tenenhouse, H. S., Goodyer, P., Shinki, T., Kato, S., and Inouye, K. (1999) Eur. J. Biochem. 265, 950–956.
5. Okumura, K., Nogami, M., Hasegawa, Y., Nishi, H., Yanagisawa, J., Tanaka, T., and Kato, S. (1999) J. Biochem. 125, 6607–6615.
6. Sawada, N., Sakaki, T., Kitazaka, S., Kato, S., and Inouye, K. (2000) Biochem. Biophys. Res. Commun. 277, 595–599.
7. Williams, P. A., Comse, J., Ringe, D., Petsko, G. A., and Sligar, S. G. (2000) Science 287, 1615–1622.
8. Hiwatashi, A., Nishi, Y., and Ichikawa, Y. (1982) FEBS Lett. 167–171.
9. Hiwatashi, A., Nishi, Y., and Ichikawa, Y. (1982) FEBS Lett. 1615–1622.
10. Schlichting, I., Jungen, C., and Schulze, H. (1997) FEBS Lett. 415, 253–257.
11. Sawada, N., Sakaki, T., Kitanaka, S., Kato, S., and Inouye, K. (2001) Eur. J. Biochem. 265, 6607–6615.
12. Sawada, N., Sakaki, T., Kitazaka, S., Kato, S., and Inouye, K. (2001) J. Clin. Endocrinol. Metab. 86, 511–515.
13. Kondo, S., Sakaki, T., Ohkawa, H., and Inouye, K. (1999) Biochem. Biophys. Res. Commun. 257, 273–278.
14. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 397–404.
15. Hiwatashi, A., Nishi, Y., and Ichikawa, Y. (1982) Biochim. Biophys. Res. Commun. 125, 595–599.
16. Williams, P. A., Comse, J., Ringe, D., Petsko, G. A., and Sligar, S. G. (2000) Science 287, 1615–1622.
17. Schlichting, I., Jungen, C., and Schulze, H. (1997) FEBS Lett. 415, 253–257.
18. Hiwatashi, A., Nishi, Y., and Ichikawa, Y. (1982) FEBS Lett. 167–171.
19. Hiwatashi, A., Nishi, Y., and Ichikawa, Y. (1982) FEBS Lett. 1615–1622.
20. Schlichting, I., Jung, C., and Schulze, H. (1997) FEBS Lett. 415, 253–257.
21. Cupp Vickery, J., Anderson, R., and Hatzizis, Z. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3050–3055.