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Investigation of the Role of Cytochrome P450 2B4 Active Site Residues in Substrate Metabolism Based on Crystal Structures of the Ligand-bound Enzyme

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Abstract

Based on the x-ray crystal structures of 4-(4-chlorophenyl)imidazole (4-CPI)- and bifenazole (BIF)-bound P450 2B4, eight active site mutants at six positions were created in an N-terminal modified construct termed 2B4dH and characterized for enzyme inhibition and catalysis. I363A showed a > 4-fold decrease in differential inhibition by BIF and 4-CPI ($IC_{50,BIF}/IC_{50,4-CPI}$). F296A, T302A, I363A, V367A, and V477A showed a ≥ 2 -fold decreased k_{cat} for 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation, whereas V367A and V477F showed an altered K_m . T302A, V367L, and V477A showed a > 4-fold decrease in total testosterone hydroxylation, whereas I363A, V367A, and V477F showed altered stereo- and regioselectivity. Interestingly, I363A showed a ≥ 150 -fold enhanced k_{cat}/K_m with testosterone, and yielded a new metabolite. Furthermore, testosterone docking into three-dimensional models of selected mutants based on the 4-CPI-bound structure suggested a re-positioning of residues 363 and 477 to yield products. In conclusion, our results suggest that the 4-CPI-bound 2B4dH/H226Y crystal structure is an appropriate model for predicting enzyme catalysis.

Keywords

cytochrome P450; structure-function relationships; site-directed mutagenesis; enzyme catalysis and inhibition; P450 2B4 crystal structure

Introduction

Cytochromes P450 from the 2B subfamily have been studied extensively with regard to structure-function relationships [1]. Prior to elucidation of mammalian P450 x-ray crystal structures, chimeragenesis, site-directed mutagenesis, and homology modeling based on bacterial structures were the primary tools available to identify key residues responsible for substrate specificity and stereo- and regioselectivity [1]. Most of these residues belong to the substrate recognition sites (SRS) proposed by Gotoh based on analogy with the crystal structure of bacterial P450 101 [2]. The mammalian P450 2C5 x-ray structure renewed interest in modeling other mammalian P450 enzymes and studying structure-function relationships using site-directed mutagenesis [1,3-10]. These studies suggest that in addition to the active site,

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residues that line the substrate access channel are critical determinants of substrate metabolism, specificity, and stereo- and regioselectivity.

More recently, our laboratory has resolved ligand-free, 4-(4-chlorophenyl)imidazole (4-CPI)-, and bifonazole (BIF)-bound x-ray crystal structures of P450 2B4 in an N-terminal modified and C-terminal His-tagged construct termed 2B4dH [11-13]. The open ligand-free structure of 2B4dH was resolved from homodimers, in which residue His-226 from one enzyme fills the active site of the second enzyme [11]. Later, residue His-226 was replaced with a tyrosine, and P450 2B4dH/H226Y was then crystallized with a small ligand 4-CPI [12]. In this structure, helices F through G, helices B' and C, the N-terminus of helix I, and the β_4 region are relocated to surround 4-CPI, yielding a closed conformation. The P450 2B4dH/H226Y-4CPI structure shows ten residues in the active site: Ile-101, Ile-114, Phe-115, Phe-297, Ala-298, Glu-301, Thr-302, Ile-363, Val-367, and Val-477. More recently, P450 2B4dH/H226Y was crystallized with a relatively large ligand, BIF [13], in which the I-helix bends to accommodate the inhibitor in the active site. The BIF-bound structure shows ten residues in the active site: Ser-128, Met-132, Val-292, Leu-295, Phe-296, Ala-298, Gly-299, Glu-301, Thr-302, and Ile-363. In both the 4-CPI and BIF-bound crystal structures an imidazole nitrogen points towards the heme iron to form a coordinate bond. The ligandbound x-ray crystal structures revealed remarkable ligand-induced conformational plasticity. Subsequently, solution studies by isothermal titration calorimetry (ITC) with several nitrogenous inhibitors of different sizes that show distinct thermodynamic signatures further suggested how 2B4 can accommodate a variety of ligands while maintaining its overall fold [14].

The crystal structures provide final snap shots of P450-ligand interactions. Therefore, mutagenesis of selected active site residues is needed to provide insight into their specific roles in ligand binding and substrate specificity, stereo-, and regioselectivity. In addition, active site mutagenesis helps us to examine the reliability of inhibitor-bound P450 2B4dH/H226Y crystal structures in predicting substrate metabolism. In this study, six amino acids residues that interact with either 4-CPI (Val-367 and Val-477), BIF (Val-292 and Phe-296), or both (Thr-302 and Ile-363) were replaced with smaller and/or bulkier amino acids residues. The resulting mutants V292A, F296A, T302A, I363A, V367A, V367L, V477A, and V477F were characterized for inhibition by 4-CPI and BIF and metabolism of 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) and testosterone. Among the mutants I363A showed the most dramatic perturbation in enzyme inhibition and catalysis.

Experimental Procedures

Materials

7-EFC and 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) were purchased from Molecular Probes, Inc. (Eugene, OR). NADPH was from Sigma Chemical Co. (St. Louis, MO). [4- 14 C]-testosterone was obtained from Amersham Biosciences (Piscataway, NJ). Authentic steroid standards were obtained from Steraloids Inc. (Newport, RI). Oligonucleotide primers were obtained from Sigma Genosys (Woodlands, TX). The QuikChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). The GeneClean kit was purchased from BIO 101 (Irvine, CA). Rat NADPH-cytochrome P450 reductase (CPR) and cytochrome b_5 (b_5) were prepared as described previously [15]. All other chemicals were of the highest grade available and were obtained from standard commercial sources.

Mutagenesis, expression, and purification

All of the mutants were generated by polymerase chain reaction (PCR) using the QuickChange site-directed mutagenesis kit. P450 2B4dH/H226Y was used as the template, and the forward and reverse primers are presented in Table 1. As exceptions, construction of F296A and I363A

required 6% and 4% DMSO, respectively, during the PCR. The resulting constructs were sequenced to verify the desired mutation and absence of unintended mutations (Protein Chemistry Laboratory, University of Texas Medical Branch, Galveston, TX). P450 2B4dH/H226Y and mutants were expressed in *Escherichia coli* (*E. coli*) TOPP3 cells (Stratagene, La Jolla, CA) and purified with a Ni-NTA affinity column as described previously [16]. P450 2B4dH/H226Y and most of the mutants had an expression level of 200-450 nmol P450 l⁻¹, except for F296A and V367L, which had low expression (66-120 nmol l⁻¹). The specific contents of P450 2B4dH/H226Y and mutants were between 9 and 20 nmol of P450 per mg protein.

Enzymatic assays

7-EFC *O*-deethylation was measured in a final reaction volume of 100 µl as described earlier [16]. In brief, the reaction mixture contained 150 µM 7-EFC in the standard reconstitution system (P450:CPR:*b*₅, 1:4:2) at 5 pmol P450 in 50 mM Hepes (pH 7.4), 15 mM MgCl₂, and 2% MeOH. Samples were incubated at 37 °C for 10 min before the addition of NADPH (1 mM) to initiate the reaction. After 5 min the reaction was terminated by the addition of 50 µl of 20% trichloroacetic acid. Then, 2 ml of Tris-HCl buffer (pH 9.0) was added to the reaction mixture (50 µl), and the product was quantified by measuring 7-HFC at Λ_{ex} and Λ_{em} of 410 and 530 nm, respectively. The turnover number (nmol/min/nmol P450) was determined using a standard curve of 7-HFC. For steady-state kinetic analysis 5 - 150 µM 7-EFC was used.

Testosterone hydroxylation was measured in a 100-µl reaction volume using a radiometric method as described earlier [7]. In brief, the reaction mixture contained 200 µM testosterone containing [4-¹⁴C] testosterone (20,000 dpm/nmol) and 10 pmol P450 in the reconstitution and buffer systems as described above. The samples were incubated at 37 °C for 10 min prior to addition of 1 mM NADPH to initiate the reaction. After 10 min the reaction was terminated by the addition of 50 µl tetrahydrofuran. Metabolites (from 50 µl of the reaction mixture) were resolved on a TLC plate by two cycles of chromatography in the solvent system dichloromethane : acetone (4:1, v/v), localized by autoradiography, and quantified by scintillation counting. For steady-state kinetic analysis 5 - 200 µM testosterone was used. To confirm the production of 2 α - and 15 α -hydroxytestosterone by I363A, standards and reaction mixture were run in the same lane. After chromatography, the autoradiogram was overlaid with the standards, which were detected with a short wavelength UV lamp (254 nm).

Enzyme Inhibition Assay

Enzyme inhibition was measured using the 7-EFC *O*-deethylation assay in a final reaction volume of 100 µl at 0.005 - 0.1 µM 4-CPI and 0.1 - 2.5 µM BIF concentrations as described previously [17]. BIF inhibition studies for all the mutants and 4-CPI inhibition studies for T302A and V367A utilized 10 pmol P450, whereas 4-CPI inhibition studies for rest of the mutants utilized 2.5 pmol P450 and the same reconstitution system defined above.

Data analysis

The K_m and k_{cat} values were calculated using Michaelis-Menton nonlinear regression parameters with SigmaPlot (Jandel Scientific, San Rafael, CA). Nonlinear regression analysis was performed to fit the data using a four-parameter logistic function to derive the IC_{50} values for 4-CPI and BIF [13]. Each experiment included P450 2B4dH/H226Y and mutants (presented in Tables 2-5) simultaneously for more accurate comparison of the data.

Molecular modeling studies

A molecular model of P450 2B4dH/H226Y was constructed using the InsightII software package (Homology, Discover_3, Biopolymer, Builder and Docking from Molecular

Simulations Inc., San Diego, CA) and 4-CPI-bound P450 2B4dH/H226Y x-ray crystal structure (1SUO) as the template as described previously [13]. For the mutants, the coordinates of the corresponding residues were changed in the P450 2B4dH/H226Y 3D model by Biopolymer, and the resulting mutants were minimized. The parameters for heme and ferryl oxygen were those described by Paulsen and Ornstein [18]. The structure of testosterone was constructed using the Builder module.

Testosterone was docked into the 3D models of P450 2B4dH/H226Y and the mutants in a reactive binding orientation leading to 16 α -, 16 β -, 2 α -, or 15 α -hydroxylation using InsightII as described previously [7]. Since the initial oxidation step involves hydrogen abstraction, the reactive carbon atom was placed 3.7 Å from ferryl oxygen with C-H-ferryl O angle of 180° to promote hydrogen bond formation. The nonbond interaction energies were evaluated with the Docking module of InsightII, and the lowest energy orientation obtained after molecular mechanics minimization of P450 2B4dH/H226Y and the mutants are shown in Figure 3.

Results and Discussion

Inhibition studies with 4-CPI and BIF

To examine the effect of the mutations on inhibitor binding, P450 2B4dH/H226Y and site-directed mutants were analyzed for 4-CPI and BIF inhibition using 7-EFC Odeethylation. The results are presented in Table 2 and Figure 1. Because of the dominance of the strong coordinate bond between the heme iron of P450 and nitrogen of the imidazole ring phenylimidazole compounds show non-competitive inhibition, such that the IC_{50} value is comparable to the K_i value. Furthermore, inhibition studies with P450 2B4 and 2B5 using different substrates and at different concentrations yielded similar IC_{50} values for 4-phenylimidazole [17]. Therefore, IC_{50} values were used to examine the effect of active site mutations on inhibitor binding. P450 2B4dH/H226Y showed a 55-fold lower IC_{50} for 4-CPI than BIF (0.01 μ M vs. 0.55 μ M). Of the eight mutants generated, the most interesting results were observed with I363A. This mutant showed a 1.5-fold lower IC_{50} , BIF and a 2.7-fold higher IC_{50} , 4-CPI than P450 2B4dH/H226Y, leading to > 4-fold decreased differential inhibition (IC_{50} , BIF/ IC_{50} , 4-CPI) (Table 2, Fig. 1). Compared with P450 2B4dH/H226Y, F296A and V477F showed a modest increase in IC_{50} , BIF and IC_{50} , 4-CPI but no appreciable change in IC_{50} , BIF/ IC_{50} , 4-CPI. Other mutants showed similar IC_{50} , BIF and IC_{50} , 4-CPI values to P450 2B4dH/H226Y.

The above results suggested that Ile-363 is the most critical residue in modulating inhibitor sensitivity in P450 2B4dH/H226Y. The modest or lack of perturbation of the sensitivity of the other single mutants to 4-CPI and BIF suggests that the coordinate bond between the imidazole nitrogen of the inhibitor and the heme iron in P450 2B4dH/H226Y plays a dominant role in inhibitor binding. In an earlier study, the Ile³⁶³→Val substitution in full-length P450 2B4 increased the IC_{50} for 4-phenylimidazole by 2-fold [17]. In addition, simultaneous substitution of P450 2B5 residues 114, 294, 363, and 367 with the corresponding residues of P450 2B4 decreased the IC_{50} for 4-phenylimidazole by 12-fold. Similarly, the reciprocal simultaneous substitutions in P450 2B4 increased the IC_{50} by 14-fold. Overall, the results suggest that the inhibitor binding site is strongly influenced by residue-residue interaction in addition to the coordinate bond between the inhibitor and the heme.

7-EFC O-deethylation: steady-state kinetic analysis

All of the mutants retained the ability to metabolize 7-EFC, although F296A, T302A, I363A, V367A, and V477A showed a significant decrease in catalytic activity at 150 μ M 7-EFC (data not shown) compared with P450 2B4dH/H226Y. The results of steady-state kinetic analysis are presented in Table 3. T302A and V367A showed ~5-fold lower k_{cat} , whereas F296A,

I363A, and V477A showed ~2-fold lower k_{cat} than P450 2B4dH/H226Y. V367A showed a > 2-fold higher K_{m} , whereas T302A, V367L, and V477F showed a > 1.6-fold lower K_{m} than P450 2B4dH/H226Y. Compared with P450 2B4dH/H226Y, the $k_{\text{cat}}/K_{\text{m}}$ of T302A and V367A was decreased by ≥ 5 -fold, whereas the $k_{\text{cat}}/K_{\text{m}}$ of V477F was enhanced by > 2-fold. Although, F296A and I363A showed a decreased k_{cat} , their $k_{\text{cat}}/K_{\text{m}}$ was similar to that of P450 2B4dH/H226Y. V292A showed a negligible change in k_{cat} or K_{m} compared with P450 2B4dH/H226Y (Table 3).

The above results suggested that Thr-302 is a critical residue for determining catalytic activity in P450 2B4dH/H226Y, which is consistent with prior site-directed mutagenesis studies on P450 2B and other mammalian P450 enzymes. Thr-302 corresponds to the highly conserved threonine found in the active site of many mammalian and bacterial P450s [1,2], and is thought to be involved in proton delivery to the oxygen species [19]. Consistent with our results, T302A in P450 2B4 shows decreased activity with cyclohexane, 1-phenylethanol, and benzphetamine [20], and T303A in 2E1 shows reduced epoxidation of several substrates [21]. Furthermore, T309A in P450 2D6 and P450 3A4 shows decreased activity with several substrates [22] (unpublished observations). With regard to the other 2B4dH mutants, V477F in P450 2B6dH shows increased 7-EFC *O*-deethylation [23]. V292A and F296A in 2B4dH, which were created primarily based upon BIF-bound P450 2B4dH/H226Y crystal structure, caused minimal effect on 7-EFC turnover. Although, Val-292 and Phe-296 are in the active site in a large open cavity of the BIF-bound structure, V292A and F296A are least likely to perturb substrate metabolism. In an earlier study, however, L292A in P450 2B1 showed decreased activity with several substrates including 7-EFC [9]. Residues 292 and 296 in P450 2B4 are not present in the active site based on bacterial or P450 2C5 crystal structures [1-3].

Stereo- and regioselectivity of testosterone hydroxylation

To examine the effect of mutations on stereo- and regioselectivity P450 2B4dH/H226Y and the mutants were analyzed for testosterone hydroxylation at 200 μM substrate (Table 4 and Figure 2). P450 2B4dH/H226Y showed preferential hydroxylation at the 16 carbon, producing 16 α -hydroxytestosterone (~65% of the total activity) followed by 2 α -hydroxytestosterone (~25%) and 16 β -hydroxytestosterone. T302A, V367L, and V477A showed a > 4-fold lower total testosterone hydroxylase activity than P450 2B4dH/H226Y. Although its testosterone 16 α -hydroxylation was unaffected, F296A showed 2-fold higher 2 α -hydroxylation than P450 2B4dH/H226Y. V367A showed preference for testosterone 16 β -hydroxylation, whereas V477F showed preference for 2 α -hydroxylation with unaltered total testosterone hydroxylase activity. Compared with P450 2B4dH/H226Y, I363A showed a dramatic increase (> 25-fold) in total testosterone hydroxylation, and produced a new metabolite (15 α -hydroxytestosterone), which accounts for ~60% of the total activity. In addition, I363A showed altered regioselectivity for C15 over C16 hydroxylation. Similar to 7-EFC metabolism, V292A showed minimal effects on testosterone metabolism compared with P450 2B4dH/H226Y.

The above results suggested that in addition to its role in differential inhibition, Ile-363 is a critical residue for determining testosterone stereo- and regioselectivity. Residue 363 has been shown to be involved in differential substrate specificity and strict stereo- and regioselectivity in several P450 2B enzymes [1,7,23-27]. For example, a smaller residue at position 363 in P450 2B4 and P450 2B5 prefers androstenedione 15 α -hydroxylation, whereas a larger one prefers androstenedione 16 α -hydroxylation [25]. In addition, several other studies with 2B enzymes such as 2B1 have shown that decreasing the size of residue at 363 favors 15 α -hydroxylation of androstenedione [26]. V367A in P450 2B4 shows altered stereoselectivity of androstenedione hydroxylation [25], and I477F in P450 2B1dH shows altered testosterone hydroxylase stereoselectivity [7].

Steady-state kinetic analysis of testosterone hydroxylation

Subsequently, steady-state kinetic analysis of testosterone hydroxylation by P450 2B4dH/H226Y, F296A, I363A, and V367A was carried out (Table 5). The K_m of P450 2B4dH/H226Y was $\sim 175 \mu\text{M}$; thus, the accuracy of the K_m is limited by the highest testosterone concentration used ($200 \mu\text{M}$). I363A showed the greatest increase in k_{cat} for testosterone 16α -hydroxylation (8-fold) and 16β -hydroxylation (20-fold) compared with P450 2B4dH/H226Y. In addition, I363A showed the highest k_{cat} for testosterone 15α -hydroxylation ($\sim 60\%$ of the total k_{cat}). V363A also showed a > 5 -fold lower K_m than P450 2B4dH/H226Y ($\sim 30 \mu\text{M}$ vs. $175 \mu\text{M}$), leading to a > 150 -fold enhancement in total k_{cat}/K_m for testosterone hydroxylation. F296A showed a very similar k_{cat}/K_m to P450 2B4dH/H226Y. V367A did not show a significant change in k_{cat} or K_m values compared with P450 2B4dH/H226Y but did show altered stereoselectivity (Table 4).

I363A turned out to be the most efficient P450 2B4 enzyme for metabolizing testosterone with a k_{cat}/K_m of $0.644 \text{ min}^{-1} \mu\text{M}^{-1}$. In earlier studies, I363V in 2B4 shows a > 3 -fold enhanced activity with androstenedione and V363L in P450 2B1dH shows a 2-fold enhanced activity with testosterone compared with the respective wild-type [7,25,26]. In contrast, V363A in 2B1 shows a > 4 -fold lower androstenedione hydroxylation than 2B1 [26]. From > 50 site-directed mutants in 2B enzymes, L209A in P450 2B1dH is the most efficient site-directed mutant for testosterone hydroxylation [8]. More recently, directed evolution of L209A yielded a triple mutant, F202L/L209A/S334P, which is ~ 2.5 fold more efficient than L209A [28].

Molecular docking of testosterone

To explain the changes in stereo- and regioselectivity observed testosterone was docked into the 3D models of P450 2B4dH/H226Y, I363A, V367A, and V477F using InsightII. Testosterone docking was carried out by fixing the angle (C-H-ferryl oxygen, 180°) and distance (C and ferryl oxygen, $3.7 \text{ \AA}$). Figure 3 shows testosterone docked into the active site of P450 2B4dH/H226Y (3A), I363A (3B-D), V367A (3E), and V477F (3F). Testosterone fit well in the active site of P450 2B4dH/H226Y, with no van der Waals overlaps when docked in an orientation that yields 16α -hydroxytestosterone (Figure 3A). However, docking of testosterone in other orientations, especially in 2α , was energetically unfavored (data not shown). The estimated angle (C-H-ferryl oxygen) and distance (between C- 16α and ferryl oxygen) in P450 2B4dH/H226Y are 168° and $4.1 \text{ \AA}$, respectively. Similarly, testosterone fit well in the active site of I363A when docked in orientations yielding 15α -OH, 16α -OH, and 16β -OH testosterone. The estimated angle and distance in I363A are 146° and $4.3 \text{ \AA}$ for the 15α -orientation, 172° and $3.4 \text{ \AA}$ for the 16α -orientation, and 153° and $3.7 \text{ \AA}$ for the 16β -orientation, respectively. Testosterone docking into the active site of V367A in an orientation leading to 16β -hydroxylation gave an estimated angle and distance of 116° and $5.7 \text{ \AA}$, respectively. When testosterone was docked in V477F in a 2α -orientation, the estimated angle and distance were 131° and $3.2 \text{ \AA}$, respectively. Although the estimated angle and distance deviated from 180° and $3.7 \text{ \AA}$, respectively, the docking energy was within the acceptable range.

Docking of testosterone into the active site of P450 2B4dH/H226Y I363A suggests a local energetically favored rearrangement of the active site residues such as Ala-363 and Val-477, which appears to provide testosterone more room to access the heme in multiple orientations leading to multiple product formation. Similarly, preferred 16β -hydroxylation in P450 2B4dH/H226Y V367A can also be explained by repositioning of Val-477, which appears to prefer 16β - over 16α -orientation. Testosterone docking into the active site of P450 2B4dH/H226Y V477F in a 2α -orientation shows a dramatic shift of residue 477. Phe-477 stacks on the top of testosterone and appears to stabilize it through hydrophobic interactions.

Conclusions

This is the first study to examine the utility of the ligand-bound P450 2B4dH/H226Y x-ray crystal structures for predicting differential inhibition by BIF and 4-CPI as well as substrate turnover and stereo- and regioselectivity. The study was carried out by substituting amino acid residues that interact with 4-CPI-, BIF-, or both with smaller and/or larger ones followed by characterization of enzyme inhibition and catalysis. Interestingly, T302A, I363A, V367A, and V477A, which were created based upon 4-CPI- or both 4-CPI and BIF-bound crystal structures [12,13], showed altered substrate turnover and/or stereo- and regioselectivity. However, only I363A showed a substantial perturbation in enzyme inhibition. Although Val-292 and Phe-296 are in the active site in a large open cavity of the BIF-bound crystal structure, V292A and F296A caused minimal effect on enzyme inhibition and catalysis. Therefore, we suggest that the 4-CPI-bound crystal structure of 2B4 is superior to the BIF-bound crystal structure for predicting important active site residues. The 4-CPI-bound crystal structure of 2B4 can be used for further analysis, such as modeling and re-engineering of other P450s and predicting substrate oxidation. As future prospects, substrate-bound crystal structures of P450 2B enzymes will enhance further our understanding of the precise role of active site residues in P450 function.

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FIG 1.

A. Inhibition of P450 2B4dH/H226Y (filled circles), V477F (open circles), and I363A (open triangles) by BIF. BIF concentrations included in the assay were 0.1, 0.2, 0.3, 0.4, 0.8, 1.5, 2.5 μM. **B.** Inhibition of P450 2B4dH/H226Y (filled circles), F296A (open circles), and I363A (open triangles) by 4-CPI. 4-CPI concentrations included in the assay were 0.005, 0.01, 0.015, 0.02, 0.03, 0.04, 0.1 μM.

FIG 2.

A representative thin layer chromatography of testosterone metabolites produced by 2B4dH/H226Y and mutants at 200 μ M substrate.

FIG 3. Docking of testosterone into the active site of three-dimensional models of P450 2B4dH/H226Y and mutants. Substrate was docked with **A)** P450 2B4dH/H226Y in an orientation leading to the formation of 16 α -hydroxytestosterone, **B-D)** I363A in an orientation leading to the formation of 16 α -hydroxytestosterone (B), 15 α -hydroxytestosterone (C), and 16 β -hydroxytestosterone (D), **E)** V367A in an orientation leading to the formation of 16 β -

hydroxytestosterone, and **F**) V477F in an orientation leading to the formation of 2 α -hydroxytestosterone. The heme (red sticks), testosterone (pink sticks), hydrogen atoms (grey), replaced residues (green sticks), amino acid residues that were subject of study here (blue sticks), and I helix (blue and green) are shown.

Table 1

Oligonucleotides used in PCR for site-directed mutagenesis of P450 2B4dH/H226Y.

Mutants	Oligonucleotides
V292A	5'-ATCCTCACCGCGCTCTCGTC-3' 5'-GAGCGAGAGCGCGGTGAGGAT-3'
F296A	5'-TGCTCTCGCTCGCCTTCGCCGGCACC-3' 5'-GGTGCCGGCGAAGGCGAGCGAGAGCA-3'
T302A	5'-GCACCGAGGCCACCAGCAC-3' 5'-GTGCTGGTGGCCTCGGTGC-3'
I363A	5'-GGACCTCGCCCCCTTCGG-3' 5'-CCGAAGGGGGCGAGGTCC-3'
V367A	5'-TTCGGGGCGCCCCACACG-3' 5'-CGTGTGGGGCGCCCCGAA-3'
V367L	5'-TTCGGGTGCCCCACACGGTC-3' 5'-GACCGTGTGGGGCAACCCGAA-3'
V477A	5'-GAGAGTGGCGGGCAACGT-3' 5'-ACGTTGCCCCGCGCCACTCTC-3'
V477F	5'-GAGAGTGGCTTCGGCAACGTGC-3' 5'-GCACGTTGCCGAAGCCACTCTC-3'

The nucleotides changed to make the desired mutation are underlined.

Table 2

Inhibition of P450 2B4dH/H226Y and mutants by 4-CPI and BIF: determination of IC₅₀ using a 7-EFC O-deethylation assay.

P450	BIF (μM)	IC ₅₀ 4-CPI (μM)	BIF/4-CPI
2B4dH/H226Y	0.55 ± 0.02	0.010 ± 0.003	55
V292A	0.52 ± 0.02	0.011 ± 0.001	47
F296A	0.88 ± 0.09	0.022 ± 0.008	40
T302A	0.41 ± 0.09	0.008 ± 0.000	51
I363A	0.36 ± 0.01	0.027 ± 0.005	13
V367A	0.47 ± 0.03	0.007 ± 0.001	67
V367L	0.56 ± 0.01	0.012 ± 0.002	47
V477A	0.61 ± 0.10	0.011 ± 0.003	55
V477F	0.85 ± 0.16	0.015 ± 0.002	56

Results are the mean ± standard deviation of at least three independent experiments.

Table 3

Steady state kinetic analysis of 7-EFC O-deethylation by P450 2B4dH/H226Y and mutants.

P450	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
2B4dH/H226Y	9.9 (0.7) ^a	108 (14)	0.09
V292A	11 (0.7)	102 (12)	0.11
F296A	5.0 (0.6)	73.3 (18)	0.07
T302A	1.3 (0.1)	67.8 (10)	0.02
I363A	5.3 (0.6)	81.8 (18)	0.07
V367A	1.8 (0.1)	284 (90)	0.01
V367L	8.3 (0.3)	67.0 (5.6)	0.12
V477A	4.7 (0.3)	85.7 (9.2)	0.05
V477F	9.5 (0.3)	42.6 (3.2)	0.22

Results are the representative of at least two independent determinations. The variation between the experiments is $\leq 20\%$.

^a Standard errors for fit to Michaelis-Menten are shown in parenthesis.

Table 4
Testosterone hydroxylation by 2B4dH/H226Y and mutants at 200 μ M substrate.

	Turn over (nmol/min/nmol P450)			Stereoselectivity	Regioselectivity
	16 α -OH	16 β -OH	2 α -OH		
P450					
2B4dH/H226Y	0.43	0.06	0.17	16 α -OH:16 β -OH	16-OH:2-OH
V292A	0.26	ND	0.13	7.2	2.9
F296A	0.44	ND	0.34	ND	2.0
T302A	0.01	0.03	ND	ND	1.3
I363A ^a	3.7	1.4	0.51	0.3	ND
V367A	0.17	0.31	0.07	2.7	10
V367L	0.07	ND	0.09	0.6	6.9
V477A	ND	ND	ND	ND	0.80
V477F	0.08	0.01	0.46	8.0	ND
					0.19

Results are the mean of at least three determinations. The standard error of mean (SEM) was approximately $\pm$ 25% of the mean values. SEM was not indicated in the Table to enhance the clarity.

^a I363A also catalyzes P450 testosterone 15 α -hydroxylation (12 nmol/min/nmol P450), whereas P450 2B4dH/H226Y showed non-determinable (ND) testosterone 15 α -hydroxylase activity.

Table 5
Steady state kinetic analysis for testosterone hydroxylation by P450 2B4dH/H226Y and mutants.

P450	16 α -OH		16 β -OH		15 α -OH	
	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat} (min ⁻¹)	K_m (μ M)	k_{cat} (min ⁻¹)	K_m (μ M)
2B4dH/H226Y	0.7 (0.1) ^a	175 (38)	--	--	--	--
F296A	1.0 (0.2)	96.0 (35)	--	--	--	--
I363A	6.6 (0.3)	30.4 (4.3)	2.0 (0.1)	32.6 (3.2)	12.0 (0.7)	28.4 (5.5)
V367A	--	--	0.9 (0.2)	180 (67)	--	--

Results are the representative of at least two independent determinations. The variation between the experiments is $\leq 20\%$.

^aStandard errors for fit to Michaelis-Menten are shown in parenthesis