Changes in the Enzymatic Properties of CYP2D6 by the Substitution of Phenylalanine at Position 120 by Alanine

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The functional roles of phenylalanine at position 120 (Phe-120) in the oxidation of bunitrolol (BTL), debrisoquine (DB) and bufuralol (BF) by cytochrome P450 2D6 (CYP2D6) were examined using a yeast cell expression system (*Saccharomyces cerevisiae* AH-22 strain). The substitution of Phe-120 by alanine markedly increased the activities of enantiomeric BTL 4-hydroxylase and DB 4-hydroxylase, whereas it did not remarkably affect BF 1″-hydroxylase activities. Kinetic studies revealed that the substitution of Phe-120 by alanine increased the *Km* and *Vmax* values for enantiomeric BTL 4-hydroxylation, but increased only the *Vmax* value for DB 4-hydroxylation without changing the *Km* value. *Km* and *Vmax* values for BF 1″-hydroxylation were similar between the mutant and the wild-type. The dissociation constants of the mutant calculated from the binding spectra for BTL enantiomers were higher than those of the wild-type, suggesting that the substitution of Phe-120 by alanine decreased the affinity of CYP2D6 for BTL enantiomers. These results indicate that Phe-120 has an important role in the oxidation of substrates by CYP2D6.

Key words —— cytochrome P450 2D6, phenylalanine-120, alanine-120, bunitrolol, debrisoquine

INTRODUCTION

Cytochrome P450 (CYP) is not a single but diverse enzymes responsible for the oxidation of an extremely wide range of compounds including chemically produced, biologically produced and naturally occurring compounds.[1] However, the numbers of mammalian CYP enzymes mainly involved in the oxidation of medicines are limited to isoforms belonging to CYP1A, -1B, -2A, -2B, -2C, -2D, -2E, -3A and -4A.[2] Among these isoforms, CYP2D6 is a unique CYP enzyme because it shows extensive genetic polymorphism especially in Caucasians.[3]

According to the latest data of the International Committee of Human CYP Allele Nomenclature (http://www.imm.ki.se/CYPalleles/), 45 CYP2D6 alleles have been reported. There are various kinds of single nucleotide polymorphism in the gene of *CYP2D6*, and some cause the substitution of an amino acid residue, resulting in changes of the enzymatic properties.

We have been studying the relatedness between the structure and the function of CYP2D enzymes in various mammals, including humans.[4–6] In our previous project, CYP2D2 (P450BTL) was purified employing bunitrolol (BTL) 4-hydroxylase as the index of CYP2D2 functions.[4] BTL is a β-adrenoceptor blocking agent, and rat CYP2D2,[4] as well as human CYP2D6,[7] catalyzes BTL 4-hydroxylation with relatively low *Km* values. Our interest has also been directed to the mechanism(s) responsible for the stereoselectivity in the oxidation of various chiral substrates by CYP2D enzymes.[8] For example, rat CYP2D2 shows substrate enantioselectivity of [(+)-BTL < (–)-BTL],[4] whereas human CYP2D6 exhibits the reverse selectivity of [(+)-BTL > (–)-BTL].[7]

As part of our overall studies, the present study was conducted to determine what amino acid residues in the active site of human CYP2D6 are involved in the enantioselective oxidation of BTL enantiomers by the enzyme. In the search of the active site, it seems likely that the phenyl ring of phe-

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nylalanine at position 120 (Phe-120) may affect the positioning of the aromatic ring of the substrate in the active site of CYP2D6, giving the idea that the substitution of Phe-120 by another amino acid residue may change the activity or substrate enantioselectivity in BTL 4-hydroxylation by CYP2D6. The results revealed that phenylalanine at position 120 strongly affects the oxidation capacity of CYP2D6 towards some but not all typical substrates. This report describes the results and proposes a possible mechanism.

MATERIALS AND METHODS

Computer-Assisted Molecular Modeling —— The conformation of CYP2D6 was constructed by Swiss Plots using the crystallographic data of CYP2C5 as a template at the web site (http://www1.embl-heidelberg.de/predictprotein/predictprotein.html). The conformation of BTL was obtained by using the Insight II/Search-Compare module on SGI Indigo 2. The initial insertion of BTL into the active site of the protein was carried out by hand on a personal computer screen using Swiss PDB Viewer and a home-made modeling program CG. Energy optimizations and molecular dynamics of the model were performed using Insight II/Discover. Because of the limited capability of our computer, only the active site was subjected to calculation by eliminating the amino acid residues other than Pro-103 to Leu-121, Leu-208 to Leu-224, Leu-236 to Leu-248, Ile-297 to Ser-311, Ile-369 to Thr-375, Phe-481 to Val-485. Movements of the alpha carbons in those peptides were suppressed by the tethering technique to keep the original backbone structure of CYP2D6 unchanged: otherwise, gradual drifting of the fragments should have taken place as the computation progressed. The effect of water was taken into account by adding water molecules into the model system directly with the cutoff distance of 10 angstroms rather than by assuming an effective dielectric constant.

Materials —— Debrisoquine (DB) and 4-hydroxydebrisoquine (4-OH-DB) as hemisulfates were obtained from Hoffmann La-Loche (Basel, Switzerland); BTL and 4-hydroxybunitrolol (4-OH-BTL) as hydrochlorides were from Nippon Boehringer Ingelheim Co. (Hyogo, Japan); bufuralol (BF) and 1″-hydroxybufuralol (1″-OH-BF) as hydrochlorides were from Daiichi Pure Chemical Co. (Tokyo, Japan); glucose 6-phosphate (G-6-P), G-6-P dehydrogenase and NADPH were from Oriental Yeast Co., Ltd. (Tokyo, Japan); procainamide, cytochrome c, and phenylmethane sulfonylfluoride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Site-Directed Mutagenesis and Expression of Mutated cDNA in Yeast Cells —— A cDNA encoding CYP2D6 (F120A) was constructed from the wild-type *CYP2D6* in pBluescript II KS (+) as a template with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. The forward and reverse primers for the introduction of mutation were 5′-cgcgttcccaaggggtggccctggcgcgctatgggc-3′ and 5′-gcccatagcgcgccagggccacccctttgggaacgcg-3′, respectively. The nucleotide positions to be mutated are underlined. To confirm successful mutagenesis, the plasmid insert was sequenced in both directions with an ALF express DNA sequencer (Amersham Biosciences, Piscataway, NJ, U.S.A.) using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and Cy5-labeled primers designed based on the CYP2D6 nucleotide sequence.

Expression in yeast cells was carried out as previously described.[7] Briefly, the mutation-bearing plasmid was digested with *Hin*dIII, and the resultant fragment was inserted into *Hin*dIII-digested dephosphorylated pGYRI and transformed into *Escherichia coli* (*E. coli*) HB101. The yeast expression vector pGYRI has a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter and includes the yeast NADPH-CYP reductase gene. The recombinat vector containing a mutated CYP2D6 cDNA in the proper orientation with respect to GAPDH was selected by DNA sequencing using a 5′-Cy5-labeled oligonucleotide primer (5′-ctcaagggaggatgtgtgggtg tgg-3′) designed on the basis of the nucleotide sequence directly upstream of the GAPDH promoter. Finally, *Saccharomyces cerevisiae* AH 22 strain was transfected with the pGYRI vector containing a mutated CYP2D6 cDNA to express the corresponding CYP2D6 mutant protein.[7] Cultivation of the yeast cells was performed as described previously.[9] The CYP2D6 wild-type was expressed in yeast cells according to a method previously reported.[7]

Assays of CYP2D6 Holo- and Apoproteins —— Microsomal fractions were prepared from yeast cells by the method previously published.[9] The fractions were diluted to a protein concentration of 2 mg/ml with 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, and the total holo-CYP content was spectrophotometrically measured

from reduced carbon monoxide (CO) spectra according to the method of Omura and Sato[10] using 91 $mM^{-1}cm^{-1}$ as an absorption coefficient. Appropriate portions of yeast cell microsomal fractions and whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% slab gels. Following the electrophoresis, proteins on the gel were electroblotted to a polyvinylidene fluoride (PVDF) membrane, and were analyzed by Western blotting according to the method of Guengerich *et al.*[11] using monoclonal antibodies raised against CYP2D6 (Daiichi Pure Chemicals, Co., Tokyo, Japan). Relative degrees of staining of CYP2D6 protein bands on the membrane were assessed with NIH Image (version 1.2) run on a Macintosh G4 computer with an Epson CC-550L scanner.

Assay of Drug Oxidation Activities —— DB 4-hydroxylase activity was measured by the HPLC method reported previously.[6] Briefly, a 500-μl incubation mixture in an Eppendorf-type tube (1.5 ml) contained 10 pmol of CYP, 5 mM G-6-P, 1 IU of G-6-P dehydrogenase, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH and 100 μM DB in 100 mM potassium phosphate buffer (pH 7.4). After a 5-min preincubation at 37°C, the reaction was started by adding NADPH and continued for 5 min. After the reaction was stopped by adding 50 μl of 60% $HClO_4$ aqueous solution and vortex mixing, the tube containing the reaction mixture was centrifuged at 5000 $\times$ *g* for 10 min at room temperature. An aliquot (10 μl) of the supernatant was subjected to HPLC under the conditions described below.

BTL 4-hydroxylase activity was assayed according to the HPLC method previously published.[12] Briefly, a 500-μl incubation mixture in a brown glass conical tube (10 ml) with a stopper contained 10 pmol of CYP, 5 mM G-6-P, 1 IU of G-6-P dehydrogenase, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH, and 10 μM (+)-BTL or (–)-BTL in 100 mM potassium phosphate buffer (pH 7.4). After a 5-min preincubation at 37°C, the reaction was started by adding NADPH and was performed at 37°C for 5 min. After the reaction was stopped by adding 1 ml of 1 N NaOH aqueous solution and vortex mixing, 1 ml of 1 M sodium carbonate buffer (pH 9.6) and 20 ng of procainamide (internal standard) were added, and 4-OH-BTL was extracted into 5 ml of ethyl acetate by vigorous shaking. After centrifugation, 4 ml of the organic layer was taken, and evaporated to dryness under N_2 stream. The residue was dissolved in 100 μl of an HPLC mobile phase described below, and an aliquot (10 μl) was subjected to HPLC under the conditions described below.

BF 1″-hydroxylase activity was also measured by the HPLC method as described before.[6] Briefly, a 500 μl incubation mixture in a brown glass conical tube (10 ml) with a glass stopper contained 10 pmol CYP, 5 mM G-6-P, 1 IU of G-6-P dehydrogenase, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH and 100 μM BF racemate in 100 mM potassium phosphate buffer (pH 7.4). Manipulations hereafter were the same as those employed in the assay of BTL 4-hydroxylation except for using propranolol racemate (100 nmol) instead of procainamide as an internal standard. In preliminary experiments, linearity of product formation as a function of time was confirmed for each substrate.

HPLC Conditions —— A Hitachi 655-12A liquid chromatograph equipped with a Hitachi L-7480 fluorescence detector, a Rheodyne Model 7125 injector and a Shimadzu C-R3A Chromatopac data processor; column, Inertsil ODS (4.6 mm $\times$ 250 mm, GL Science, Tokyo, Japan) for all of the enzyme assays; mobile phase-A, acetonitrile/20 mM perchloric acid (pH 2.5) (15 : 85, by volume) at a flow rate of 1 ml/min for DB 4-hydroxylation; mobile phase-B, acetonitrile/methanol/water/acetic acid (20 : 20 : 60 : 1, by volume) at a flow rate of 1 ml/min for BTL 4-hydroxylation; mobile phase-C, acetonitrile/20 mM perchloric acid (pH 2.5) (35 : 65, by volume) at flow rate of 1 ml/min for BF 1″-hydroxylation; detection, fluorescence 219/286 nm (excitation/emission) for DB 4-hydroxylation, 310/380 nm for BTL 4-hydroxylation and 252/302 nm for BF 1″-hydroxylation. Kinetic analyses for DB 4-hydroxylation, BTL 4-hydroxylation and BF 1″-hydroxylation by yeast cell microsomes were performed using DB, BTL and BF concentration ranges from 2.5 to 150 μM, from 0.1 to 5 μM, and from 0.5 to 100 μM, respectively. Apparent Michaelis-Menten constants (*Km*) and maximal velocities (*Vmax*) were calculated using the computer program Prism version 3.0 (GraphPad Software, San Diego, CA, U.S.A.) designed for non-linear regression analysis of a hyperbolic Michaelis-Menten equation.

Study of Substrate Binding to Yeast-Expressed CYP2D6 and the Mutant —— Microsomal fractions from yeast cells expressing wild-type CYP2D6 or CYP2D6 (F120A) were diluted to 2 mg protein/ml with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol. One milliliter each of the diluted microsomal fractions was put into each of two 1-ml cuvettes, and a baseline was re-

corded from 380 to 520 nm. Varying amounts of BTL enantiomer dissolved in distilled water were added at final concentrations from 1 to 50 μM to the sample cuvette, the same volume of distilled water being added to the reference cuvette. The difference spectra were recorded and dissociation constants (*Kd*) were calculated according to the published method.[13]

Others —— Protein concentrations were measured by the method of Lowry *et al.*[14] using bovine plasma albumin as a standard.

RESULTS

The active site of CYP2D6 is shown in Fig. 1, where Phe-120 is depicted in ball and stick configuration. In this situation, we hypothesized that the phenyl ring of Phe-120 may be an obstacle for the approach of BTL to an activated oxygen coordinated to the heme iron or cause a possible hydrophobic interaction (π-π interaction) with the aromatic rings of the substrates. In order to evaluate this hypothesis, we examined the effect of the substitution of Phe-120 by alanine, another hydrophobic amino acid residue without a phenyl ring, on the enzymatic functions of CYP2D6.

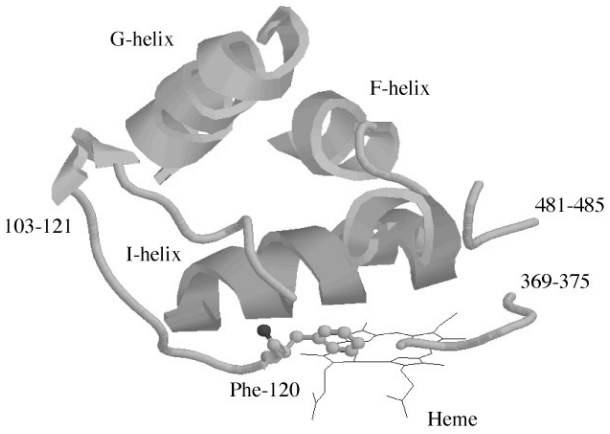

Fig. 1. The Active Site of CYP2D6

The active site was constructed with a heme molecule and six parts of CYP2D6 protein; from Pro-103 to Leu-121, from Leu-208 to Leu-224 (a part of F-helix), from Pro-234 to Leu-248 (a part of G-helix), from Ile-297 to Ser-311 (a part of I-helix), from Ile-369 to Thr-375, from Phe-481 to Val-485.

In the next step, we prepared a cDNA having thymine-358 and thymine-359 instead of guanine-358 and cytosine-359 of the nucleotide, resulting in the introduction of alanine instead of phenylalanine at position 120 of CYP2D6. Yeast cells were then transfected with the plasmid vector pGYR1 into which the cDNA was inserted, followed by cultivation. The contents of CYP2D6 wild-type and its F120A mutant were 62.1 and 76.5 pmol/mg microsomal protein, respectively (the mean value of two independent expression samples).

Enantiomeric BTL 4-hydroxylase activities of CYP2D6 (F120A) are compared with those of the wild-type in Fig. 2A. BTL 4-hydroxylase activities of the mutant were much higher than those of the wild-type at 10 μM BTL enantiomer concentration. Substrate enantioselectivity [(+)-BTL > (–)-BTL] was higher for the mutant than for the wild-type. Table 1 summarizes the parameters obtained from the kinetics performed using a substrate concentration range from 1 to 50 μM. Interestingly, apparent *Km* values as well as *Vmax* values were found to be markedly increased for the mutant as compared with those of the wild-type.

A similar tendency was observed for DB 4-hydroxylation (Fig. 2B). The DB 4-hydroxylase activity of CYP2D6 (F120A) was about 8-fold that of the wild-type at a substrate concentration of 100 μM.

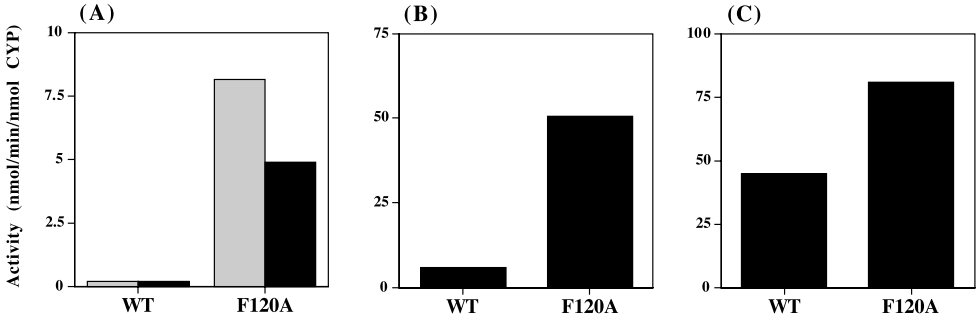

Fig. 2. Comparison of BTL 4-Hydroxylation (A), DB 4-Hydroxylation (B) and BF 1″-Hydroxylation (C) by CYP2D6 and its Mutant

Substrate concentrations used were 10 μM for BTL enantiomers and 100 μM for DB and BF. (A) hatched columns, from (+)-BTL; dotted columns, from (–)-BTL. Each value represents the mean of two independent determinations.

Table 1. Kinetic Parameters for BTL 4-Hydroxylation by Recombinant Wild-Type CYP2D6 and CYP2D6 (F120A)

		Km (μM)	*Vmax* (nmol/min/nmol CYP)	*Vmax/Km* (ml/min)
WT	(+)-BTL	0.10 ± 0.01	0.54 ± 0.02	5.4
	(−)-BTL	0.09 ± 0.02	0.46 ± 0.02	5.1
	(+)/(−) ratio	1.1	1.2	1.1
F120A	(+)-BTL	35.9 ± 3.5 (386)	40.0 ± 2.6 (74)	1.1
	(−)-BTL	2.5 ± 0.5 (22)	7.5 ± 0.5 (16)	3.0
	(+)/(−) ratio	14.4	5.3	0.4

Values in parentheses are relative to those of the wild-type (WT) taken as 1. Each value represents the mean ± S.D. of two independent determinations.

Table 2. Kinetic Parameters for DB 4-Hydroxylation And BF 1″-Hydroxylation by Recombinant Wild-Type CYP2D6 and CYP2D6 (F120A)

	Km (μM)	*Vmax* (nmol/min/nmol CYP)	*Vmax/Km* (ml/min)
DB 4-hydroxylation			
WT	12.6 ± 0.9	5.4 ± 0.2	0.43
F120A	13.4 ± 2.6 (1.1)	59.2 ± 4.8 (11.0)	4.42 (10.3)
BF 1″-hydroxylation			
WT	3.1 ± 0.6	51.4 ± 2.3	16.6
F120A	3.8 ± 1.1 (1.2)	96.8 ± 7.6 (1.9)	25.5 (1.5)

Values in parentheses are relative to those of the wild-type (WT) taken as 1. Each value represents the mean ± S.D. of two independent determinations.

The kinetics showed that in contrast to the results of BTL 4-hydroxylation (Table 1), the apparent *Km* value for DB 4-hydroxylation was similar between the mutant and the wild-type (Table 2), while the *Vmax* value of the mutant was 11-fold that of the wild-type.

On the other hand, BF 1″-hydroxylase activities were not changed so much as compared with the results of BTL and DB (Fig. 2C). The activity of the mutant was only 1.8-fold that of the wild-type at a substrate concentration of 100 μM. In kinetic parameters listed in Table 2, *Km* values were almost the same between the mutant and the wild-type, and the *Vmax* value of the mutant was 1.9-fold that of the wild-type, reflecting well the results of Fig. 2C.

We then measured the substrate binding spectrum between BTL enantiomers and wild-type CYP2D6 or the mutant. Addition of varying amounts of BTL enantiomers to the microsomal fractions from yeast cells expressing wild-type CYP2D6 or CYP2D6 (F120A) yielded a typical type I spectrum having a peak at 390 nm and a trough at 420 nm. Dissociation constants (*Kd*) for the complexes of BTL enantiomer with wild-type and mutant CYP2D6 are listed in Table 3. *Kd* values for the complexes of BTL enantiomer with wild-type CYP2D6 were 1 to 2 μM, whereas *Kd* values for the complexes of BTL enantiomer with the CYP2D6 mutant increased to 2- to 6-fold those of the wild-type. These results indicate that the affinity of the mutant CYP2D6 for BTL enantiomers, especially for (+)-BTL, was remarkably decreased compared with that of the wild-type.

Table 3. Dissociation Constants for Interaction of BTL Enantiomers with Recombinant Wild-Type CYP2D6 and CYP2D6 (F120A)

		Kd (μM)
WT	(+)-BTL	1.16 ± 0.89
	(−)-BTL	1.77 ± 0.91
	(+)/(−) Ratio	0.66
F120A	(+)-BTL	7.21 ± 3.1 (6.2)
	(−)-BTL	4.03 ± 2.5 (2.3)
	(+)/(−) Ratio	1.79

Values in parentheses are relative to those of the wild-type (WT) taken as 1. Each value represents the mean ± S.D. of two independent determinations.

DISCUSSION

BTL is a β-blocker having a relatively simple chemical structure compared to numerous substrates of CYP2D6. BTL also has a simple oxidation pathway, *i.e.*, aromatic 4-hydroxylation by CYP2D6.[8] In addition, BTL 4-hydroxylation by CYP2D6 is known to have a clear substrate enantioselectivity [(+)-BTL > (–)-BTL].[8] Hence, BTL is thought to be an appropriate substrate to study mechanism(s) causing the stereoselectivity in the oxidation of chiral substrates by CYP2D6.

In the present study, the conformation of CYP2D6 was automatically constructed using Swiss Plots on the web site (http://www1.embl-heidelberg.de/predictprotein/predictprotein.html) using the PDB file of CYP2C5 as a template. The active site structure of CYP2D6 was cut off from the constructed CYP2D6 model. To the active site model, each of the BTL enantiomers was inserted. From the predicted configuration of the active site where BTL enantiomer is positioned close to an activated oxygen molecule coordinated to a heme iron, it seems likely that the phenyl ring of Phe-120 may affect the positioning of the aromatic ring of the substrate in the active site of CYP2D6. These considerations suggested to us the idea that the substitution of Phe-120 by another amino acid residue may change the activity or substrate enantioselectivity in BTL 4-hydroxylation by CYP2D6.

As expected, the substitution of Phe-120 by alanine markedly increased the *Vmax* values of 4-hydroxylation of BTL enantiomers, particularly of (+)-BTL, resulting in an increase in the ratio of *Vmax* values from 1.2 for the wild-type to 5.3 for the mutant (Table 1). Interestingly, the substitution of phenylalanine by alanine at position 120 also caused remarkable increases in the *Km* values. When DB or BF, other typical substrates for CYP2D6 were used, different profiles were observed. In DB 4-hydroxylation, the *Vmax* value was markedly increased by the substitution, whereas the *Km* value was unchanged. In BF 1″-hydroxylation, *Vmax* values were not so different (1.9-fold at highest) between the mutant and the wild-type, and *Km* values were very similar among them.

Since striking effects of the substitution were observed for enantiomeric BTL 4-hydroxylation, possible changes in the affinities of BTL enantiomers for wild-type and mutant CYP2D6 were assessed by measuring the substrate binding spectra. *Kd* values for the mutant-BTL enantiomer complexes were found to be higher than those for the wild-type CYP2D6-BTL enantiomer complexes, indicating that the affinities of BTL enantiomers were decreased by the substitution of phenylalanine with alanine at position 120.

It is noteworthy that remarkable increases in *Km* values as well as *Vmax* values were observed for enantiomeric BTL 4-hydroxylation by CYP2D6 (F120A) compared to that by the wild-type. Is the increased *Km* values wholly due to decreased affinities of the mutant for the substrates? The Michaelis-Menten type reaction can be expressed as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} E + P$$

$$Km = \frac{k_2 + k_3}{k_1}$$

where E, S, ES, P and *Km* are enzyme, substrate, enzyme-substrate complex, product and Michaelis-Menten constant, respectively. When k_1 and k_2 are much higher than k_3, *Km* can be taken as the association-dissociation constant of E and S.

The dissociation constants of CYP2D6 (F120A) calculated from the binding spectra for BTL enantiomer were found to be higher than those of wild-type CYP2D6. Furthermore, the velocities of the mutant in enantiomeric BTL 4-hydroxylation were much higher than those of the wild-type. Therefore, it is reasonable to think that both of the decreased affinities (increased k_2) and increased velocities (increased k_3) caused the remarkably increased *Km* values of enantiomeric BTL 4-hydroxylation by the mutant.

The apparent *Km* value (about 0.1 μM) of BTL 4-hydroxylation by CYP2D6 was much lower than those for DB 4-hydroxylation (about 13 μM) and BF 1″-hydroxylation (about 3 μM) examined in this study. In addition, the *Vmax* value of BTL 4-hydroxylation by wild-type CYP2D6 was one-tenth and one-hundredth of those for DB 4-hydroxylation and BF 1″-hydroxylation, respectively. These results suggest that BTL is more useful as an inhibitor of CYP2D6 than as a substrate.

Why did the substitution of phenylalanine by alanine at position 120 markedly increase the velocities of the oxidations of BTL? Various models have been proposed for the active site of CYP2D6,[15–18] and most of the substrates are thought to be trapped in the active site *via* hydrophobic interaction between the aromatic ring of the substrates and the phenyl ring of phenylalanine at positions 481

or 483. In this context, such hydrophobic interactions reported to date have been thought to help the oxidation of the substrates by the enzyme.

In contrast, the phenyl ring of Phe-120 may restrict the access of the substrate to an activated oxygen molecule coordinated to the heme iron *via* its hydrophobic interaction with and/or steric hindrance against the aromatic rings of the substrates, particularly BTL. The substitution of Phe-120 by alanine may remove such obstacles against the substrate, resulting in the marked increase in BTL 4-hydroxylation. There is another possibility that the phenyl ring of Phe-120 captures the substrate and even its metabolite *via* hydrophobic interaction (π-π interaction). The substitution of Phe-120 by alanine without the phenyl ring may help the release of the metabolite from the active site, resulting in the increased k_3 value of the equation describe above.

We already presented the results of the CYP2D6 mutant having alanine instead of Phe-120 for oxidations of BTL and DB except for the data of *Kd* values of BTL enantiomers in the 41st Meeting of Chugoku-Shikoku Blanch of the Pharmaceutical Society of Japan in November, 2002.[19] Very recently, Flanagan *et al*.[20] reported the possibility of Phe-120 to contribute to the regiosspecificity of CYP2D6. They also paid attention to the role of Phe-120 and revealed that the substitution of Phe-120 by alanine yielded 7-hydroxydextromethorphan, a new metabolite of dextromethorphan, while BF 1″-hydroxylase activities remained unchanged.[20] Therefore, our present results and their reported ones are agreed in the point that BF 1″-hydroxylase activities are similar between the CYP2D6 wild-type and the mutant (F120A).

Recently, Williams *et al*.[21] have reported the crystal structure of CYP2C9 and proposed that CYP2C9 may have a substrate pocket in the active site. Similarly to the case of CYP2C9, there is a possibility that CYP2D6 also has such a pocket in the active site, which traps BTL *via* hydrophobic interaction with the phenyl ring of Phe-120, and trapping of the substrate by the pocket may conversely cause low efficiency of the oxidation of a substrate newly coming into the active site. Further studies will be necessary for understanding the mechanism(s) involved in the present results.

In summary, the substitution of Phe-120 by alanine remarkably increased the enantiomeric BTL 4-hydroxylase activities and DB 4-hydroxylase activities, but did not markedly affect BF 1″-hydroxylase activities. Kinetic studies revealed that the substitution of Phe-120 by alanine increased the *Km* and *Vmax* values for enantiomeric BTL 4-hydroxylation and increased only the *Vmax* values for DB 4-hydroxylation without changing the *Km* value, while the substitution did not strikingly affect the kinetic parameters for BF 1″-hydroxylation. The dissociation constants of the mutant calculated from the binding spectra for BTL enantiomers were higher than those of the wild type. These results indicate that phenylalanine has an important role in the oxidation of the substrates by CYP2D6.

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