Determinants of the substrate specificity of human cytochrome *P*-450 CYP2D6: design and construction of a mutant with testosterone hydroxylase activity

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Cytochrome *P*-450 CYP2D6, human debrisoquine hydroxylase, metabolizes more than 30 prescribed drugs, the vast majority of which are small molecules containing a basic nitrogen atom. In contrast, the similar mouse protein Cyp2d-9 was first characterized as a testosterone 16 α -hydroxylase. No common substrates have been reported for the two enzymes. Here we investigate the structural basis of this difference in substrate specificity. We have earlier used a combination of NMR data and homology modelling to generate a three-dimensional model of CYP2D6 [Modi, Paine, Sutcliffe, Lian, Primrose, Wolf, C. R. and Roberts (1996) *Biochemistry* **35**, 4541–4550]. We have now generated a homology model of Cyp2d-9 and compared the two models to identify specific amino acid residues that we believe form the substrate-binding site in each protein and therefore influence catalytic selectivity. Although there are many similarities in active site structure, the most notable difference is a phenylalanine residue (Phe-483) in CYP2D6, which in the model is located such that the bulky phenyl ring is positioned across the channel mouth, thus limiting the size of substrate that can access the active site. In Cyp2d-9, the corresponding position is occupied by an isoleucine residue, which imposes fewer steric restraints on the

size of substrate that can access the active site. To investigate whether the amino acid residue at this position does indeed influence the catalytic selectivity of these enzymes, site-directed mutagenesis was used to change Phe-483 in CYP2D6 to isoleucine and also to tryptophan. CYP2D6, Cyp2d-9 and both mutant CYP2D6 proteins were co-expressed with NADPH cytochrome *P*-450 reductase as a functional mono-oxygenase system in *Escherichia coli* and their relative catalytic activities towards bufuralol and testosterone were determined. All four proteins exhibited catalytic activity towards bufuralol but only Cyp2d-9 catalysed the formation of 16 α -hydroxytestosterone. Uniquely, the CYP2D6_{F483I} mutant acquired the ability to metabolize testosterone to a novel product, which was identified by MS and proton NMR spectroscopy as 15 α -hydroxytestosterone. NMR spin relaxation experiments were used to measure distances between the haem iron and protons of testosterone bound to the CYP2D6_{F483I} mutant. These experiments demonstrate that very minor modifications to the active site structure of CYP2D6 can have a profound influence on the substrate specificity of the enzyme.

INTRODUCTION

The cytochrome *P*-450 superfamily of haem-containing proteins is responsible for the phase I oxidative metabolism of a wide variety of structurally diverse substrates [1,2]. Cytochrome *P*-450 CYP2D6, the human debrisoquine hydroxylase, is responsible for the metabolism of more than 30 prescribed drugs [3]. CYP2D6 expression is subject to genetic polymorphism, as a consequence of which CYP2D6 protein levels have been shown to vary widely between individuals. The molecular basis for this variation is now reasonably well understood; many alleles at the CYP2D6 gene locus have been reported [4,5]. Inheritance of CYP2D6 alleles containing one or more gene-inactivating mutations leads to the 'poor metabolizer' (PM) phenotype which affects approx. 7% of the Caucasian population [6,7]. Inheritance of the PM phenotype has been associated with altered susceptibility to various diseases, including cancer and Parkinson's disease [5,8]. It is therefore of central importance that we understand the structural requirements both of CYP2D6 itself and the com-

pounds that it metabolizes, so that we can predict *a priori* whether a compound will be metabolized by CYP2D6 and understand at a molecular level any potential metabolic implications of genetic polymorphism involving amino acid substitutions.

Attempts to rationalize the substrate specificity of the *P*-450 mono-oxygenase superfamily have been limited by the lack of available structural information for mammalian *P*-450 enzymes. In the absence of defined crystal structures, molecular modelling is now commonly used to predict the three-dimensional structure of these enzymes, based on comparisons with the primary amino acid sequences of those bacterial *P*-450 proteins for which a crystal structure has been solved.

Until relatively recently, the only *P*-450 for which a crystal structure was available was *P*-450_{cam} (CYP101), a camphor-metabolizing bacterial protein isolated from *Pseudomonas putida* [9]. More recently, however, after the determination of crystal structures for *P*-450_{terp} (CYP108) [10], the *P*-450 domain of *P*-450_{BM3} (CYP102) [11] and *P*-450_{eryf} [12], a consensus sequence

Abbreviations used: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PM, poor metabolizer.

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alignment was generated [13] in which local structural idiosyncrasies were averaged out and a more accurate estimate of mammalian *P*-450 structure could be constructed. We and others have used such an approach to predict the three-dimensional structure of CYP2D6 [14–16] and to identify specific amino acid residues that seem to define the substrate-binding site of the enzyme. The models of human *P*-450 species obtained by homology modelling can be improved by the inclusion of interatomic distances between the haem iron and atoms of the bound substrate determined from NMR spin relaxation experiments [16].

Although CYP2D6 substrates are structurally diverse, most contain a basic nitrogen atom and binding is thought to involve an electrostatic interaction between this basic nitrogen and a negatively charged amino acid in the substrate-binding site of the enzyme [17]. By making mutants designed on the basis of our models of the active site structure of CYP2D6, we have demonstrated that aspartic residue 301 is a critical determinant of enzyme function; a negatively charged residue at this position was shown to be a prerequisite for catalytic activity towards substrates containing a basic nitrogen [18].

To further characterize the regions of CYP2D6 important for defining substrate specificity, we have compared the three-dimensional structures of two members of the CYP2D gene family that exhibit marked differences in substrate specificity, i.e. human CYP2D6 and the analogous mouse protein Cyp2d-9. Mouse Cyp2d-9 was first isolated as a regiospecific and stereospecific male-specific 16 α -hydroxylase of testosterone, progesterone and oestradiol [19]. No other substrates have been reported for the enzyme [20,21]. CYP2D6 and Cyp2d-9 are highly similar, with more than 70 % amino acid identity. We have now generated a three-dimensional model of the Cyp2d-9 substrate binding site to identify amino acids involved both in maintaining the architecture of the active site of the protein and in interaction with substrate. The overall structure and many of the proposed active-site amino acid residues are seen to be conserved between the two proteins. The most notable exception is a phenylalanine residue (Phe-483) in CYP2D6, which is replaced with an isoleucine (Ile-486) at the corresponding position in Cyp2d-9. This residue is located in the substrate access channel of both proteins and might therefore have an important role in defining substrate specificity. To investigate whether this is so, we have used site-directed mutagenesis to generate a series of mutants that have been expressed at high levels as a functional mono-oxygenase system in *Escherichia coli*. We have determined the catalytic activities of the mutant CYP2D6 enzymes and have used the combination of molecular modelling and NMR to rationalize the observed differences in substrate specificity between the wild-type and mutant forms of CYP2D6 and Cyp2d-9.

EXPERIMENTAL

Materials

E. coli strains CJ236 and JM109 were obtained from Stratagene. The cDNA encoding full-length CYP2D6 was generated by PCR amplification of the product of reverse transcription of human liver mRNA [22]. The cDNA encoding the homologous mouse protein Cyp2d-9 was a gift from Dr. M. Negishi (National Institute for Environmental Health Sciences, Research Triangle Park, NC, U.S.A.) Bufuralol/HCl and the corresponding 1-hydroxybufuralol standard were a gift from SmithKline Beecham; testosterone standards were a gift from Glaxo-Wellcome. His-Bind resin was obtained from Novagen; PD10 and Mono Q (5/5) FPLC columns were obtained from Pharmacia. Testosterone and thrombin were obtained from Sigma. All other

chemicals used were obtained commercially and were of at least analytical grade.

Site-directed mutagenesis

Site-directed mutagenesis was performed on the wild-type CYP2D6 cDNA, cloned into the *Eco*RI site of the bacterial expression plasmid pTZ19R, by the method of Kunkel et al. [23] with the *dut⁻ ung⁻* *E. coli* strain CJ236. Two separate point mutations were introduced at codon 483: a TC $\rightarrow$ AT change leading to a Phe $\rightarrow$ Ile substitution (the CYP2D6_{F483I} mutant) and a TC $\rightarrow$ GG change which resulted in a Phe $\rightarrow$ Trp substitution (the CYP2D6_{F483W} mutant). In each case the presence of the desired mutations and the absence of any undesired mutations were confirmed by sequencing of each double-stranded plasmid by the dideoxy chain termination method of Sanger et al. [24] with a Sequenase Kit Version 2.0 (Amersham, Little Chalfont, Bucks., U.K.).

E. coli expression constructs

cDNA clones encoding full-length wild-type human CYP2D6, the CYP2D6_{F483I} and CYP2D6_{F483W} mutants and the homologous mouse protein Cyp2d-9 were excised from the *Eco*RI site of the expression plasmid pMP201 and modified to allow insertion between the *Nde*I and *Xba*I sites of the ampicillin-selectable expression plasmid pCW, which contains two tandemly arranged copies of the isopropyl β -D-thiogalactoside-inducible *P*_{tac} promoter and has previously been used to express *P*-450 proteins in *E. coli* [22,25]. The expression plasmid had been modified to contain a sequence encoding the bacterial *ompA* leader sequence immediately 5' of the *P*-450 initiation codon. To facilitate purification of the expressed proteins, a sequence coding for five histidine residues preceded by a thrombin cleavage site was inserted at the 3' end of each *P*-450 cDNA as reported previously for CYP2D6 expression in baculovirus [16]. To create a catalytically competent *E. coli* expression system, a second expression plasmid was constructed (pACYC184), which encoded human NADPH cytochrome *P*-450 reductase preceded by a bacterial *pelB* leader sequence [22,26], and was selectable by chloramphenicol.

Recombinant protein expression

E. coli strain JM109 was transformed with each expression plasmid, or plasmids as appropriate. A single fresh colony was picked and grown overnight at 37 °C in the presence of 50 μ g/ml ampicillin, supplemented with 25 μ g/ml chloramphenicol as required. After growth overnight, a 1:100 dilution of each culture was made into modified Terrific Broth, supplemented with 1 mM thiamine, 0.5 mM δ -aminolaevulinic acid and trace elements. Cultures were incubated for 4 h at 30 °C before induction of expression by the addition of isopropyl β -D-thiogalactoside (final concentration 1 mM). Cells were then grown for a further 24 h at 30 °C before harvest. Cells for protein purification were collected by centrifugation (2800 *g*, 12 min, 4 °C), resuspended in binding buffer [20 mM Tris/HCl (pH 7.9)/5 mM imidazole/2 mM CHAPS/0.5 M NaCl/60 μ g/ml PMSF/20 % (v/v) glycerol] and stored frozen at –70 °C until required.

Preparation of membrane fractions

Cells were collected by centrifugation as described previously and resuspended in one-tenth the original culture volume of TSE buffer [50 mM Tris acetate (pH 7.6)/250 mM sucrose/250 mM EDTA]; lysozyme was then added to 0.25 mg/ml and the

resulting suspension left shaking at 4 °C for 60 min. Spheroplasts were then pelleted by centrifugation (2800 g, 12 min, 4 °C), the supernatant was discarded and the pellet was resuspended in spheroplast resuspension buffer [100 mM potassium phosphate (pH 7.6)/6 mM magnesium acetate/20 % (v/v) glycerol/100 mM dithiothreitol]. After the addition of protease inhibitors, cells were sonicated (three 30 s bursts) on ice and spun in a Microfuge (12000 g, 12 min, 4 °C) to pellet insoluble material. The resulting supernatant was transferred to ultracentrifuge tubes and spun for 60 min at 180000 g and 4 °C to pellet cell membranes. Membrane fractions were stored at -70 °C in TSE buffer.

Protein purification

All purification steps were performed at 4 °C as described previously [16]. The P-450 concentration was measured by the method of Omura and Sato [27] with an ϵ_{448} of 91 mM⁻¹·cm⁻¹ for the reduced CO complex.

Protein concentration and immunoblotting

Protein concentrations were determined by the method of Lowry [28], with serial dilutions of BSA as standard. Immunoblotting (Western blotting) was performed essentially by the method of Towbin et al. [29], with modifications as described by Lewis et al. [30]. Proteins were separated by SDS/PAGE [9 % (w/v)] gel and transferred to nitrocellulose filters, which were probed with polyclonal antisera to CYP2D6 as described previously [16]. Immunoreactive peptides were revealed with 4-chloro-1-naphthol.

Measurement of catalytic activity

Bufuralol 1-hydroxylase activity was determined by an HPLC-based method, essentially as described by Kronbach *et al.* [31]. Briefly, 125 µg of membrane protein was mixed with 10 µM bufuralol/HCl in 50 mM potassium phosphate buffer, pH 7.4, in a total reaction volume of 150 µl. The solution was preincubated for 5 min at 37 °C and then the reaction was initiated by the addition of cofactor (an NADPH-regenerating system comprising 2.5 mM glucose 6-phosphate, 0.5 mM NADP⁺ and 2 units/ml glucose-6-phosphate dehydrogenase) and incubated in a shaking water bath at 37 °C for 30 min before the reaction was stopped by the addition of 3 % (v/v, final concentration) HClO₄. Samples were spun for 5 min in a Microfuge to remove any precipitate and the resulting supernatant was analysed by HPLC. Metabolites were separated by HPLC (Hewlett Packard) on an ODS-2 5 µm (Spherisorb) column with mobile phases of 0.1 M ammonium acetate, pH 5.0, and acetonitrile at 50 °C. The fluorescent 1-hydroxybufuralol product was detected by fluorescent detection (excitation 252 nm, emission 280 nm) and quantified against a known standard.

The hydroxylation of testosterone by the purified P-450 species was examined by the addition of cumene hydroperoxide (900 µM) to a solution containing enzyme (3 µM) and testosterone (1.5 mM) in 0.1 M phosphate buffer, pH 8.0, containing 10 % (v/v) DMSO. Cumene hydroperoxide was omitted when membrane fractions co-expressing P-450 and reductase were used. The reaction mixture (15 ml) was incubated in an open beaker for 3 h before the reaction was stopped by the addition of 10 ml of 1 M HCl and the products were extracted with ethyl acetate (twice, 10 ml each). The combined organic extracts were then washed with water and evaporated to dryness under a stream of nitrogen gas. The resulting residue was redissolved in 2 ml of 80 % (v/v) methanol, of which 100 µl was then analysed by HPLC (Gilson) with a C₁₈ reverse-phase column (S5 ODS2,

0.46 cm × 25 cm; Phase Separations, Deeside, Clwyd, U.K.) eluted by a 40 ml gradient from 60 % to 90 % (v/v) methanol, at 1 ml/min, with detection at 254 nm. Compounds eluted from the column were identified by ¹H NMR (Bruker AMX600) and electrospray MS (Kratos Concept).

Optical spectroscopy

UV-visible spectra were obtained as described previously [16,32]. Equilibrium constants for substrate binding were estimated by fitting the following equation (see for example, [33]) to the changes in absorbance at 418 nm:

$$\Delta A = \frac{\Delta A_{\infty}}{2E} [E + S + K_d - \{(E + S + K_d)^2 - 4E \cdot S\}^{1/2}] \quad (1)$$

where E and S represent the concentrations of CYP2D6 and testosterone respectively, ΔA and ΔA_{∞} are the changes in absorption at the substrate concentration S and at saturating substrate concentration, respectively, and K_d is the equilibrium dissociation constant of the enzyme-substrate complex.

NMR spectroscopy

Proton NMR measurements were predominantly made at 250 MHz with a Bruker AMX250 spectrometer; studies of the frequency dependence of relaxation rates additionally involved measurements at 600 and 500 MHz. Experiments were performed, and the data analysed, essentially as described previously [16,32]. The relaxation rates of testosterone protons in the presence of the reduced CO complex were found to be identical with those in buffer (0.1 M phosphate buffer, pH 8.0, in 100 % ²H₂O containing 10 % ²H₆-DMSO), indicating that the diamagnetic contribution to relaxation was negligible. The paramagnetic contribution, $R_{1,p}$, to the relaxation rate of the protons of bound substrate arising from the unpaired electrons on the haem iron, was estimated from the observed relaxation rates by using the following equation [16]:

$$R_{1,obs} - R_{1,f} = \frac{E_0}{K_d + S_0} (R_{1,p} - R_{1,f}) \quad (2)$$

where $R_{1,obs}$ and $R_{1,f}$ are respectively the observed spin-lattice relaxation rate and the relaxation rate of the free substrate. Fast exchange of testosterone between the bound and free states was confirmed by the observation of a linear increase in $R_{1,obs}$ as a function of reciprocal temperature in the range 283–310 K (results not shown). $R_{1,p}$ is related to the iron-proton distance by the Solomon-Bloembergen equation [33–35]:

$$R_{1,p} = \frac{1}{T_{1,m}} = \frac{2}{15} \frac{\gamma_I^2 g^2 S(S+1) \beta^2}{r^6} \left(\frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2 \tau_c^2} \right) \quad (3)$$

where r is the distance of the proton from the haem iron, ω_I and ω_S are the nuclear and electronic Larmor frequencies respectively and τ_c is the effective correlation time of the dipolar interaction. The correlation time was estimated by measuring $R_{1,p}$ at three frequencies (250, 500 and 600 MHz) and fitting the data to eqn. (3) for example, (see [17]); the value obtained, 3.0×10^{-10} s, is comparable to that for the wild-type enzyme [16].

Modelling of the complex of testosterone with the CYP2D6_{F483I} mutant

A set of models of the complex was produced by a combination of homology modelling and distance restraints obtained from paramagnetic relaxation data. The approach adopted was similar

to that used previously for modelling the CYP2D6–codeine [16] and CYP2D6–1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [36] complexes, based on a structure-based multiple sequence alignment (Figure 1) together with the structures of the four *P*-450 species available from the Brookhaven Protein Data Bank [37,38]: *P*-450_{cam} (PDB accession number 2CPP) [39], *P*-450_{terp} (1CPT) [10], *P*-450_{BM3} (2HPD) [11] and *P*-450_{eryF} (1OXA) [12]. A set of 20 models of the CYP2D6_{F483I} mutant–testosterone complex was produced and refined on the basis of constraints from homology and from the NMR-derived iron–testosterone distances, by using the program MODELLER [40]. A set of 20 NMR-derived restraints, defining 10 upper and 10 lower distance bounds, were used; these were defined in terms of the carbon atom to which the respective protein is bonded. Lower bounds were then defined as the measured iron–proton distance minus 10% (a liberal estimate of the error) minus 1 Å (the C–H bond length), and upper bounds as the measured distance plus 10% plus 1 Å. The position of the testosterone was defined partly by non-bonded intermolecular interactions between itself and both CYP2D6 and the haem, and partly by the distance restraints derived from the relaxation data. In addition, a set of 20 models of the wild-type CYP2D6–testosterone complex was produced by using the set of iron–testosterone distances obtained from the CYP2D6_{F483I} mutant. Conformational clustering of the final models, and identification of structures representative of the ensemble of models, were performed with the program NMRCLUST [41].

RESULTS

Identification of candidate amino acids that might influence the catalytic selectivity of CYP2D6 and Cyp2d-9

To identify candidate amino acid residues at the active site of the enzyme that might influence the substrate specificity of CYP2D6, a structure-based multiple sequence alignment was performed in which the primary sequences of CYP2D6 and the homologous mouse protein Cyp2d-9 were compared with those of *P*-450_{cam}, *P*-450_{terp}, *P*-450_{eryF} and the *P*-450 domain of *P*-450_{BM-3}, bacterial enzymes for which the crystal structure has been solved [13] (Figure 1). From this alignment the approximate positions of the major structural elements in CYP2D6 and Cyp2d-9 can be estimated and regions of homology identified. We have previously used this approach, together with inter-atomic distances calculated from NMR relaxation experiments [16], to generate a three-dimensional model for CYP2D6. Many of the structural predictions based on these models have now been verified experimentally [16,18,36].

Comparison of active site amino acid residues in our model of CYP2D6 with those in the homology-based model of Cyp2d-9 (Table 1) shows that the active sites of the two proteins are similar. In each case, all of the proposed active site amino acid residues lie within one of six substrate recognition sequences, regions of mammalian Family 2 *P*-450 proteins that are thought to be important for substrate interaction [42]. The most notable difference between CYP2D6 and Cyp2d-9 is the presence of a phenylalanine residue (Phe-483) in CYP2D6, which is replaced by an isoleucine (Ile-486) in Cyp2d-9. The model indicates that these amino acids could line the substrate access channel, and in our model of CYP2D6 the bulky, rigid phenyl ring of Phe-483 is oriented directly across the channel mouth, restricting the size of substrate able to enter the active site. This could help to explain the catalytic differences between CYP2D6 and Cyp2d-9. To test this hypothesis, Phe-483 in CYP2D6 was mutated to isoleucine (CYP2D6_{F483I}). To investigate further the effect of steric bulk at

this position, a second mutation from phenylalanine to tryptophan was also made (CYP2D6_{F483W}).

Expression and purification of recombinant CYP2D6 proteins

To generate a functional mono-oxygenase system, *E. coli* cells were co-transformed with plasmids encoding the *P*-450 enzyme of interest and NADPH cytochrome *P*-450 reductase. In each case, similar levels of immunodetectable membrane protein were obtained for CYP2D6 and the CYP2D6_{F483I} and CYP2D6_{F483W} mutants (Figure 2, lanes 2–4).

To facilitate purification of the recombinant CYP2D6 proteins for NMR and ligand-binding studies, a histidine tag was incorporated at the C-terminus, together with a thrombin cleavage site to allow its removal after purification. Each of the recombinant CYP2D6 proteins was purified by nickel–agarose chromatography, as described previously [16]. The proteins were then treated with thrombin and the histidine peptide was removed by a second nickel–agarose column. A final purification by MonoQ FPLC lead to preparations of wild-type and mutant CYP2D6 proteins that were more than 90% homogeneous.

Purified CYP2D6 and the CYP2D6_{F483I} and CYP2D6_{F483W} mutants show typical *P*-450 optical spectra: in the oxidized state a spectrum was obtained with well-resolved α and β bands at 570 and 535-nm and a strong Soret band at 418 nm, whereas the spectrum of the CO complex of the reduced enzyme showed peaks at 547 and 448 nm. Figure 3 illustrates the spectrum obtained for the CYP2D6_{F483I} mutant. The absence of any absorption band at 420 nm in the CO complex of the reduced proteins shows that there was no inactive *P*-450 in the preparations [43]. The spectra also demonstrate that the introduced mutations had not affected the ability of the recombinant proteins to bind haem. All of the spectra obtained were similar to those previously reported for the wild-type CYP2D6 enzyme expressed in baculovirus [16].

Catalytic activities of CYP2D6, CYP2D6_{F483I}, CYP2D6_{F483W} and Cyp2d-9

Membrane fractions expressing each *P*-450 enzyme, along with NADPH:cytochrome *P*-450 reductase, were used in reactions with bufuralol, a well-characterized substrate for CYP2D6 [44], and testosterone, known to be hydroxylated at the 16 α position by Cyp2d-9 [19]. All four proteins were able to hydroxylate bufuralol with comparable efficiency (Table 2), wild-type CYP2D6 having the lowest K_m and highest V_{max} values and, in all cases, the reaction was completely inhibited by 1 μ M quinidine, an inhibitor of CYP2D6. All these proteins contain an aspartic residue at position 301, which has previously been demonstrated to be important for binding bufuralol [18].

The ability of Cyp2d-9 to hydroxylate testosterone at the 16 α position was confirmed (results not shown); the rate of formation of 16 α -hydroxytestosterone was found to be 0.54 pmol of product/min per mg of protein, in agreement with the previously reported value of 0.3 pmol of product/min per mg of protein, obtained from transient expression of Cyp2d-9 in mammalian cells [45]. Incubation of either CYP2D6 or the CYP2D6_{F483W} mutant with testosterone did not lead to the formation of any detectable product. With the CYP2D6_{F483I} mutant, however, a product was formed that was eluted later in the HPLC gradient (16.2 min) than authentic 16 α -hydroxytestosterone (13.6 min). This demonstrated that the F483I substitution had conferred on CYP2D6 the ability to metabolize testosterone, and that the product

Figure 1 Multiple sequence alignment used for modelling

The top four sequences are those of the four bacterial *P*-450 proteins of known structure. Residues in these *P*-450 sequences that are identical in CYP2D6 are shown on a black background; those residues that are identical in CYP2D6 and Cyp2d-9 are boxed. The position of Phe-483 in CYP2D6 is shown in italics, and the corresponding Ile-486 in Cyp2d-9 is shown in italics on a grey background. The extent of the secondary structure shown at the top of the alignment is taken from [13]. Note that (1) residues 191–207 of *P*-450_{terp} are missing from the crystal structure and are shown in lower-case letters, and (2) a further 12 *P*-450 sequences were used in obtaining this alignment.

Table 1 Amino acid residues identified from modelling as forming the active sites of CYP2D6 and Cyp2d-9

The CYP2D6 residues listed are those identified as contacting the substrate in the model of the CYP2D6-codeine complex described previously [16].

CYP2D6	Cyp2d-9
Pro-103	Conserved
Ile-106	Glu-110
Thr-107	Phe-111
Leu-10	Val-115
Pro-114	Conserved
Ser-116	Conserved
Ala-122	Conserved
Asp-301	Conserved
Ser-304	Gly-307
Ala-305	Conserved
Thr-309	Conserved
Val-370	Conserved
Gly-373	Asn-376
Val-374	Leu-377
Phe-483	Ile-486

formed was a novel one. The rate of formation of the novel testosterone metabolite was found to be 0.51 pmol of product/min per μ g of protein.

Figure 2 Expression of CYP2D6 and NADPH:cytochrome *P*-450 reductase in *E. coli*

Lane 1, human liver microsomal protein; lanes 2–4, membrane fractions prepared from *E. coli* cells expressing CYP2D6, CYP2D6_{F483I} and CYP2D6_{F483W} respectively, co-expressed with NADPH:cytochrome *P*-450 reductase; lanes 5 and 6, membrane fractions prepared from *E. coli* expressing only CYP2D6 or NADPH:cytochrome *P*-450 reductase respectively; lanes 7 and 8, membrane fractions from uninduced cells. Each lane contained 40 μ g of membrane protein, detected by immunoblotting.

To identify the testosterone metabolite produced by CYP-2D6_{F483I}, we studied the metabolism of testosterone by the purified enzyme, by using cumene hydroperoxide as a source of oxygen and electrons. Under these conditions the same metabolite was formed as with *E. coli* membranes containing co-expressed CYP2D6_{F483I} and NADPH:cytochrome *P*-450 reductase, and no product was observed with the wild-type enzyme. The formation of the new metabolite during incubation of testosterone with CYP2D6_{F483I} (measured by HPLC) was linear with time over at

Figure 3 Visible absorption spectra of the CYP2D6_{F483I} mutant

Spectra of the oxidized protein (2.7 μ M) (solid line) and its reduced CO complex (broken line) were run in 0.1 M phosphate buffer, pH 8.0, containing 1 mM CHAPS.

Table 2 Kinetic parameters for the hydroxylation of bufuralol by wild-type and mutant CYP2D6 and Cyp2d-9

All values are means $\pm$ S.D. for at least three independent estimates with *E. coli* membranes containing the appropriate *P*-450 enzyme co-expressed with human NADPH:cytochrome *P*-450 reductase.

Enzyme	K_m (μ M)	V_{max} (pmol/min per mg of protein)
CYP2D6 wild-type	10.8 ± 0.1	86 ± 5
CYP2D6 _{F483I}	16.0 ± 0.3	66 ± 4
CYP2D6 _{F483W}	14.2 ± 0.2	74 ± 7
Cyp2d-9	16.9 ± 0.4	61 ± 5

least 35 min (results not shown), giving an estimate for k_{cat} of 0.5 min^{-1} .

MS of the novel metabolite after purification by reverse-phase HPLC gave a molecular mass of 304 Da compared with 288 Da for testosterone, consistent with the hydroxylation of testosterone at a single position. The site of hydroxylation was determined by ^1H NMR; the spectrum of the novel metabolite is compared with that of testosterone in Figure 4. The testosterone spectrum was assigned by two-dimensional COSY and NOESY experiments, and the results were in agreement with published assignments [46]. The ^1H NMR spectrum of the new metabolite produced by the CYP2D6_{F483I} mutant showed two resonances in the range 3.5–4.5 p.p.m. that arose from protons geminal to hydroxy groups, one from the 17-proton and one from the proton geminal to the new hydroxy group. Double resonance experiments (Figure 5) allowed us to assign these two protons. Irradiation at 3.79 p.p.m. (H-17) clearly affects the signals centred at 2.09 p.p.m. (H-16 α , H-16 β) but not that at 4.015 p.p.m. (Figure 5, trace B). This clearly demonstrates that the new hydroxy group is not in the 16 position, otherwise the two low-field resonances would arise from H-17 and H-16 and would be coupled to one another. Irradiation at 4.015 p.p.m. (Figure 5, trace C) clearly affects not only the resonances of the 16 α - and 16 β -protons at 2.09 p.p.m., but also that of H-14 at 0.995 p.p.m., strongly suggesting that the

hydroxy group is attached to the 15 position. These conclusions were further supported by the reciprocal experiments irradiating at 0.995 and 2.90 p.p.m. (Figure 5, traces D and E). Final confirmation that the product of the action of the CYP2D6_{F483I} mutant on testosterone was 15 α -hydroxytestosterone was obtained by comparison of the spectra in Figures 4 and 5 with the spectra and resonance assignments of 15 α -hydroxytestosterone published by Kirk et al. [47].

Binding of testosterone to CYP2D6, CYP2D6_{F483I} and CYP2D6_{F483W}

On addition of 1.5 mM testosterone to purified wild-type CYP2D6 or to the CYP2D6_{F483W} mutant, there was no change in the UV/visible spectra (results not shown), suggesting that testosterone does not bind to these proteins. With the CYP2D6_{F483I} mutant, however, the addition of increasing concentrations of testosterone led to a decrease in the intensity of the Soret band of the oxidized enzyme at 418 nm and an increase in the intensity of bands at 390 and 650 nm (Figure 6A). These changes are typical of those seen on substrate binding to *P*-450 species; the band at 650 nm is characteristic of high-spin ferric haem proteins [48–50]. The dependence of these changes on the concentration of testosterone (Figure 6B) allowed us to determine the equilibrium constant for testosterone binding by using eqn (1), given in the Experimental section. The K_d for testosterone binding to the CYP2D6_{F483I} mutant was calculated to be $375 \pm 43 \mu\text{M}$ (mean $\pm$ S.D.).

Structural studies of testosterone binding to CYP2D6_{F483I}

Measurements of the paramagnetic relaxation effects of the haem iron on the protons of the testosterone have been used to obtain estimates of distances between individual protons of the bound testosterone and the haem iron of the CYP2D6_{F483I} mutant by methods described previously [16,32]. These require (1) demonstration that the exchange of testosterone between the bound and free states is fast on the relaxation time scale, and (2) determination of the correlation time of the dipolar interaction between the unpaired electrons of the iron and the protons of the substrate. This was achieved by measurement of the temperature and frequency dependence respectively of relaxation (see the Experimental section).

Spin–lattice relaxation rates were measured at testosterone concentrations between 0.3 and 1.0 mM, with the enzyme concentration held constant (1 μ M). After each set of experiments, optical spectra of the reduced CO complex were measured to ensure that the enzyme had not been converted into inactive *P*-420 during their course. The average K_d obtained from the relaxation data, $488 \pm 95 \mu\text{M}$, was in reasonable agreement with that obtained from optical spectroscopy (see above). The spin–lattice relaxation times of the bound substrate, $T_{1,p}$ ($= 1/R_{1,p}$), and the estimated iron–proton distances for testosterone bound to CYP2D6_{F483I} are summarized in Table 3. These measurements give a clear indication of the orientation of the testosterone molecule in the active site of the enzyme, the 15 β and 16 β protons being only 3.60 Å from the haem iron, whereas the 6 β proton is 5.65 Å away.

Models of the complex of testosterone with the CYP2D6_{F483I} mutant were constructed by a combination of homology modelling with the distance restraints derived from the relaxation data. A total of 20 models of the complex were generated then evaluated on the basis of their bonded and non-bonded interactions, and their agreement with NMR-derived distance restraints; eleven of the models were discarded, leaving nine models that were taken as acceptable representations of the

Figure 4 NMR spectra of testosterone (A) and the product of its metabolism by CYP2D6_{F483I} (B)

Spectra were obtained at 600 MHz at a sample temperature of 300 K, in 0.1 M phosphate buffer, pH 8.0, in $^2\text{H}_2\text{O}$ with 10% $^2\text{H}_6$ -DMSO (A), or in $^2\text{H}_4$ -methanol (B). The assignment of the resonances of testosterone is indicated.

Figure 5 NMR double-resonance experiments on the product of metabolism of testosterone by CYP2D6_{F483I}

Trace A, Control spectrum; traces B–E, Effect of low-power irradiation at the frequencies indicated by the arrows: trace B, 3.79 p.p.m. (H-17); trace C, 4.015 p.p.m. (H-15); trace D, 0.995 p.p.m. (H-14); trace E, 2.09 p.p.m. (H-16α, H-16β). The effects of interest, discussed in the text, are indicated by asterisks. Spectra were obtained at 600 MHz at a sample temperature of 300 K in $^2\text{H}_4$ -methanol.

three-dimensional structure of the complex (Figure 7). Testosterone lies across the A ring of the haem and extends closer to both Val-370 and the B ring than do either codeine [16] or MPTP [36] bound to wild-type CYP2D6. The testosterone-binding site (defined as consisting of residues having side-chain atoms closer than 5.0 Å to testosterone in the majority of models) is pre-

Figure 6 Effects of testosterone on the visible spectrum of CYP2D6_{F483I}

(A) Spectrum of the enzyme alone (solid line) and in the presence of saturating concentrations of testosterone (broken line). (B) Changes in absorbance at 418 nm for CYP2D6_{F483I} as a function of testosterone concentration. The enzyme concentration was 6.37 μM in 0.1 M phosphate buffer, pH 8.0, containing 13% (v/v) DMSO. The broken line is the least-squares fit of eqn. (1) to the data.

Table 3 Paramagnetic relaxation times and iron–proton distances for testosterone bound to CYP2D6_{F483I}Values are means $\pm$ S.D. for a minimum of three independent determinations.

Proton	$T_{1\rho}$ (ms)	r (Å)
2 α	2.7 $\pm$ 0.4	9.2 $\pm$ 0.3
2 β	2.7 $\pm$ 0.3	9.2 $\pm$ 0.2
4	1.0 $\pm$ 0.04	7.7 $\pm$ 0.1
6 α	0.056 $\pm$ 0.002	4.82 $\pm$ 0.03
6 β	0.14 $\pm$ 0.004	5.63 $\pm$ 0.02
12 α	0.31 $\pm$ 0.1	6.42 $\pm$ 0.04
15 β	0.01 $\pm$ 0.0005	3.62 $\pm$ 0.02
16 β	0.012 $\pm$ 0.004	3.72 $\pm$ 0.02
17	0.15 $\pm$ 0.005	5.71 $\pm$ 0.02
18-CH ₃	0.25 $\pm$ 0.01	6.17 $\pm$ 0.02
19-CH ₃	0.37 $\pm$ 0.01	6.62 $\pm$ 0.02

dominantly hydrophobic (Figure 7). It comprises residues Leu-110 (in eight out of nine models), Leu-121 (9/9) in 'substrate recognition site 1' (SRS-1) [20]; Ser-304 (8/9), Ala-305 (9/9) and Thr-309 (6/9) in SRS-4; Val-370 (8/9) in SRS-5; and Ile-483 (9/9) in SRS-6.

The observation that CYP2D6_{F483I} hydroxylates testosterone at the 15 α rather than, like Cyp2d-9, at the 16 α position presumably reflects a slight difference in the orientation of testosterone in the active site. The models reported here are not of sufficient precision to throw much light on the origin of this difference. The C-15 atom of the 'most representative' testosterone bound to CYP2D6_{F483I} is 3.3 Å from the haem iron (range 3.1–4.7 Å in the set of nine models), compared with 3.5 Å (range 3.0–5.1 Å) for the C-16 atom.

However, the models do suggest that the two hydroxy-containing residues in the binding site (Ser-304 and Thr-309) could form hydrogen bonds to testosterone (Figure 7, bottom panel). A hydrogen bond between the C-3 carbonyl group of testosterone and Ser-304 hydroxy group is possible in six of the nine models, and one between the C-17 hydroxy group of testosterone and Thr-309 hydroxy group in three of the nine; both hydrogen bonds could exist simultaneously in two of the nine models. Interestingly, the residue in Cyp2d-9 corresponding to Ser-304 in CYP2D6 is Gly-307. Thus, if the orientation of testosterone in the binding site of CYP2D6_{F483I} is partly determined by hydrogen bonding to Ser-304, it might bind in a slightly different orientation to that with Cyp2d-9 owing to the absence of this hydrogen bond. This would be consistent with the experimentally observed difference in regiospecificity of hydroxylation.

DISCUSSION

An understanding of the structural basis of the substrate specificity of human cytochrome *P*-450 proteins is of fundamental importance. Many substrates for CYP2D6 are commonly prescribed therapeutic drugs, several of which have a low therapeutic index [3]. Inter-individual variation in CYP2D6 expression, as a result of the well-characterized genetic polymorphism at the CYP2D6 gene locus, might therefore have significant toxicological consequences. The ability to use structural information to identify a compound as a CYP2D6 substrate is therefore an important goal, in both the design and the prescription of therapeutic drugs.

Attempts to address this issue have been limited by the lack of crystal structure information for the mammalian *P*-450 iso-

enzymes. In the absence of a crystal structure for CYP2D6, we and others have made structural predictions by comparing the primary amino acid sequence of CYP2D6 with those of other *P*450 proteins for which a crystal structure is available. In addition, we have combined this homology modelling with experimental distance constraints derived from NMR to generate improved models of CYP2D6–substrate complexes [16,36]. It is obviously important that the validity of models generated in this manner is substantiated experimentally. This can be done, for example, by using the models to design mutants with altered specificity or to explain the differences in specificity between closely related *P*-450 proteins.

The minimum structural features that identify a compound as a likely substrate for CYP2D6 are now relatively well understood [14,51,52]. In contrast, there is very little information available about the substrate specificity of the homologous mouse protein Cyp2d-9, which was first purified on the basis of its ability to hydroxylate testosterone at the 16 α position [19]. Subsequently, progesterone and oestradiol have also been identified as Cyp2d-9 substrates [53], but extensive characterization of the substrate specificity of the protein has hitherto been limited by the lack of a suitable heterologous expression system.

The comparison described here between models of the active sites of CYP2D6 and Cyp2d-9 showed that, although there were some significant differences in the amino acids making up the active site, many key structural features were retained. These included the conservation of an aspartic residue (Asp-301 in CYP2D6 and Asp-304 in Cyp2d-9), which has been shown previously to be involved in substrate binding [18]. This similarity was confirmed by the finding that Cyp2d-9 also metabolized bufuralol with similar K_m and V_{max} values to those of CYP2D6.

The considerable similarity in predicted active site structure between CYP2D6 and Cyp2d-9 indicated that only very minor modifications to the active site of CYP2D6 would be required to alter its specificity to that of Cyp2d-9 and to convert it into a testosterone hydroxylase. Indeed, comparison of the models of CYP2D6 and Cyp2d-9 revealed a single residue, Phe-483, that seemed to hinder substrate access sterically in CYP2D6 and thus limit the size of molecule that will act as a substrate. In agreement with this prediction, we show here that a mutant of CYP2D6 with only a single amino acid substitution, F483I, has gained the ability to hydroxylate the steroid nucleus regiospecifically.

Testosterone binding to wild-type CYP2D6 could not be detected by optical spectroscopy. To understand the structural basis of this observation, we generated a series of 20 models of the complex of testosterone with wild-type CYP2D6 by using homology modelling combined with the distance restraints obtained from NMR experiments with the CYP2D6_{F483I} mutant. Nine of the 20 models were energetically unfavourable and were discarded, leaving eleven acceptable models. Of these, nine contained a consistent steric problem (a significant deviation from the expected value of a bond angle) that apparently arises from the interaction of testosterone with the side chain of Val-370 (a residue that is conserved in Cyp2d-9). This suggests that the effect of the F483I substitution in permitting testosterone binding to CYP2D6 arises, at least in part, because it allows testosterone to adopt a binding mode that minimizes this steric clash with Val-370.

It has long been considered that a prerequisite for a molecule to be a substrate for CYP2D6 was the presence of a basic nitrogen atom. This was subsequently rationalized by demonstrating that the nitrogen atom forms a hydrogen bond with Asp-301 [16,18], a residue conserved in Cyp2d-9. We and others have recently shown, however, that such an interaction is not needed for some CYP2D6 substrates such as MPTP [36,54,55],

Figure 7 Stereo views of testosterone in its binding site on CYP2D6_{F483I}

Structural models were obtained (as described in the Experimental section) by a combination of homology modelling with distance constraints derived from NMR relaxation experiments. The ten final models obtained are displayed after the superposition of their haem moieties. Top panel: a single testosterone molecule (chosen as the 'most representative' testosterone orientation by using NMRCLUST [37]) is shown, denoted by thick lines, with the residues lining the binding site shown in each of the ten final models. Middle panel: the same view as in the top panel, with testosterone shown for each of the ten final models and residues lining the testosterone-binding site in the 'most representative' protein. Bottom panel: a single model of the complex, illustrating the possible hydrogen bonds between Ser-304 OH-1 and the C-3 carbonyl group of testosterone, and between Thr-309 OH-1 and the C-17 hydroxy group of testosterone.

and testosterone clearly provides another exception to the 'rule' that a basic nitrogen is essential for productive binding to CYP2D6.

Inter-individual variation in CYP2D6 expression, and inheritance of the PM phenotype in particular, has been associated with altered susceptibility to several diseases, including Parkinson's disease and various types of cancer [5,8,56]. Although any plausible mechanistic rationale has yet to be provided for these observations, it seems increasingly likely that CYP2D6, in addition to its ability to metabolize drugs and foreign chemicals, might have a more fundamental metabolic role. Multiple allelic variants have been reported at the CYP2D6 locus [4,5]. Although many of these alleles contain gene-inactivating mutations, several have been reported for which the phenotypic consequences are less well defined. A number of these alleles contain single amino acid substitutions or deletions. In light of the results we report here, it will be of obvious interest to characterize the substrate specificities of these alternative forms of CYP2D6 more fully.

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