part I

USING SITE-DIRECTED MUTAGENESIS FOR DETERMINATION OF SUBSTRATE INTERACTION RESIDUES IN CYTOCHROME P450 2D6
Influence of phenylalanine 120 on Cytochrome P450 2D6 catalytic selectivity and regiospecificity: crucial role in 7-methoxy-4-(aminomethyl)-coumarin metabolism

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Abstract
The polymorphic human debrisoquine hydroxylase, Cytochrome P450 2D6 (P450 2D6), is one of the most important phase I drug metabolising enzymes. It is responsible for metabolising a large number of compounds that mostly share similarity in having a basic N-atom and an aromatic moiety. In homology modeling studies it has been suggested that in fixation of this aromatic moiety there may be an important role for phenylalanine 120 (F120). In this study the role of F120 in ligand binding and catalysis was experimentally examined by mutating it into an alanine. Strikingly, this substitution led to a completely abolished 7-methoxy-4-(aminomethyl)-coumarin (MAMC) O-demethylating activity of P450 2D6. On the other hand, bufuralol metabolism was hardly affected (\(K_m\) 1-hydroxylation mutant: 1.2 \(\mu\)M, wild-type: 2.9 \(\mu\)M, 4-hydroxylation mutant: 1.5 \(\mu\)M, wild-type: 3.2 \(\mu\)M) and neither was dextromethorphan O-demethylation (\(K_m\) mutant: 1.2 \(\mu\)M, wild-type: 2 \(\mu\)M, \(V_{max}\) mutant: 4.5 min\(^{-1}\), wild-type: 3.3 min\(^{-1}\)). However, the F120A mutant also formed 3-hydroxyxymorphan, the double demethylated form of dextromethorphan, which was not detected using wild-type P450 2D6. 3,4-methylenedioxyamphetamine (MDMA) was demethylated by both mutant and wild-type P450 2D6 to 3,4-dihydroxymethamphetamine (3,4-OH-MA, \(K_m\) mutant: 55 \(\mu\)M, wild-type: 2 \(\mu\)M). In addition the mutant formed two additional metabolites; 3,4-methylenedioxyamphetamine (MDA) and N-hydroxy-3,4-methylenedioxymethamphetamine (N-OH-MDMA). Inhibition experiments of dextromethorphan O-demethylation showed a decreased affinity of the F120A mutant for quinidine (IC\(_{50}\) mutant: 240 nM, wild-type: 40 nM), while IC\(_{50}\)'s for quinine were equal (1 \(\mu\)M). These data indicate the importance of F120 in the selectivity and regiospecificity in substrate binding and catalysis by P450 2D6.
INTRODUCTION

Cytochromes P450 (P450s) are heme containing enzymes capable of oxidizing and reducing a large variety of endogenous and exogenous substrates in virtually all living organisms [1, 2]. In humans one of the most important hepatic phase I drug metabolising enzymes is P450 2D6. It is involved in the metabolism of about 30% of the currently marketed drugs, including neuroleptics, antidepressants, β-blockers, opioids and antiarrhythmics [3, 4]. The enzyme is known for its genetic polymorphisms, even increasing its clinical relevance [5, 6]. Although some crystal structures of mammalian P450s have become available in recent years [7, 8], so far no crystal structure of P450 2D6 has been resolved. Structural information on this enzyme still depends on homology modeling and mutagenesis studies.

Recently we have developed a new homology model of P450 2D6 based on the crystal structure of rabbit P450 2C5 [9]. This was the first model of P450 2D6 based on a mammalian P450 template and is therefore considered an improvement over the existing models. This was indicated by the good correlation between experimental data and the modelled protein-substrate interactions. The model identified some active site key residues. First of all E216 has been shown to be a key ligand binding residue involved in hydrogen bonding with a variety of substrates. This residue has been subjected to several mutagenesis studies [10, 11], that indicated involvement of this residue in fixation of basic nitrogen atoms present in many P450 2D6 substrates. A second important active site residue in this and other homology models is F483 [12], interacting via van der Waals forces with substrates like codeine. Earlier mutagenesis of this residue to an isoleucine showed effects on testosterone metabolism [13]. However, the anchoring of aromatic moieties present in most P450 2D6 substrates, as predicted in pharmacophore models [14-16], cannot be completely ascribed to this residue. Therefore other phenylalanine residues contributing to aromatic interactions with substrates have to be present in the active site. Mutation of F481 to other non-aromatic residues led to decreased activities towards model substrates [17], although in the model of Venhorst et al. this residue is not considered to be in the active site of P450 2D6.

Even more interesting is the position of a third phenylalanine at position 120 in the active site of P450 2D6 homology models [9, 11, 12]. In these P450 2C5-based models F120 appears to be positioned directly above the porphyrin ring. From docking and molecular dynamics studies this residue seems to be the anchoring residue for the aromatic moiety of ligands like quinidine and sparteine via π-stacking [9]. In the present study the role of F120 in ligand binding and metabolism by P450 2D6 is studied experimentally by mutating this residue into an alanine. According to computational simulations this mutation will create not only...
more space in the active site, but also eliminates a potentially important aromatic anchoring point. By mutating F120 into an alanine we are aiming at the elucidation of the role of this residue in substrate binding and turnover by P450 2D6.

**Materials and Methods**

**Materials**

The pSP19T7LT_2D6 plasmid containing human P450 2D6 with a C- terminal His6-tag bicistronically co-expressed with human Cytochrome P450 NADPH reductase was kindly provided by Prof. Dr. Ingelman-Sundberg. 7-methoxy-4-(aminomethyl)-coumarin (MAMC), 7-hydroxy-4-(aminomethyl)-coumarin (HAMC), 3,4-methylenedioxy-methyl-amphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) were synthesised as described [18, 19]. Bufuralol hydrochloride was obtained from Gentest. N-methyldioxynamphetamine hydrochloride, dextromethorphan hydrobromide and dextrorphan tartrate were obtained from Sigma. All other chemicals were of analytical grade and obtained from standard suppliers.

**Synthesis of N-hydroxy-3,4-methylenedioxyamphetamine**

N-hydroxy-3,4-methylenedioxyamphetamine (1-(3,4-methylenedioxyphenyl)-N-methyl,2-hydroxylaminopropane) was prepared from piperonyl methyl ketone and N-methyldioxynamphetamine by reductive amination with sodium cyanoborohydride [20]. A two-fold excess of N-methylhydroxylamine hydrochloride was stirred with piperonyl methyl ketone in methanol at room temperature and then sodium cyanoborohydride was added and stirred for additional 48 h. After adding water, neutralising the cyanoborohydride with 6 M HCl and neutralising the acid with 6 M NaOH, the product was extracted in dichloromethane.

\[
\begin{align*}
\text{H NMR (400 Hz, CDCl3):} & \delta 1.00 (d, 3H, CCH3), 2.38 (dd, 1H, H-1), 2.68 (s, 3H, NCH3), 2.91 (m, 1H, CHN), 3.10 (dd, 1H, H-1), 5.90 (s, 2H, OCH2O), 6.63 (dd, 1H, H-6'), 6.70 (d, 1H, H-2'), 6.72 (d, 1H, H-5'). \\
\text{C NMR (400 Hz, CDCl3):} & \delta 14.20 (CH3), 38.82 (CH2), 43.95 (CH3N), 65.35 (CH), 72.82, 100.83 (OCH2O), 108.17 (C-5'), 109.70 (C-2'), 122.13 (C-6'), 133.29 (C-1'), 145.87 (C-4'), 147.59 (C-3'). \\
\text{MS direct injection:} & m/z, M+ 210, MS/MS: 179 (3), 163 (100), 135 (3).
\end{align*}
\]

**Site-directed mutagenesis**

The phenylalanine 120 to alanine (F120A) mutation was introduced into pSP19T7LT_2D6 using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene). The sequences of the forward- and reverse oligonucleotides, respectively, with the mutated residue in **bold italic**, were as follows: 5'-CGT TCC CAA GGG GTG GCC CTG GCG CGC TAT-3' and 5'-ATA GCG CGC CAG GGC GGC CAC CCC TTC GGA ACG-3'. After mutagenesis, the presence of the desired F120A mutation was confirmed by DNA sequencing.

**Expression and membrane isolation**

Both the P450 2D6[F120A] and the wild-type pSP19T7LT_2D6 plasmids were transformed into *Escherichia coli* strain JM109. Expression was carried out in 3L flasks containing 300 ml TB.
(Terrific Broth) medium with additives (1 mM δ-aminolevulinic acid, 400 µL/L trace elements [21], 1 µg/mL thiamine, 100 µg/mL ampicillin). Cultures were inoculated with 3 ml frozen E. coli cells containing the desired plasmid, and induction was initiated by the addition of 2 mM isopropyl β-D-thiogalactoside. Cultures were grown for 48 h at 28 °C and 125 rpm, before they were harvested. P450 contents were determined by CO difference spectra [22].

Harvested cells were pelleted by centrifugation (15 min, 4000 g, 4 °C) and the resulting pellet was resuspended in TSE buffer (50 mM Tris-acetate pH 7.6, 250 mM sucrose, 0.25 mM EDTA), 35 mg wet weight cells/mL. Spheroplasts were prepared by adding 0.1 mg/mL lysozyme and gently shaking the cells for 30 min on ice. Spheroplasts were pelleted by centrifugation (15 min, 4000 g, 4 °C), and resuspended in KPi-glycerol buffer (100 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM DTT), 0.5 g/mL. The spheroplasts were lysed by passage through a French press followed by sonication (Branson Sonifier 250, 10 x 20 sec, 70% full power), and the membrane fraction was isolated by ultracentrifugation (45 min, 120,000 g, 4 °C). Membranes were resuspended in 0.4% of the original culture volume of TSE buffer and P450 contents were determined with CO difference spectra.

**Metabolism and Inhibition Assays**

**MAMC O-demethylation [23]:** Reactions were carried out in duplicate in a 96 wells plate, in a total volume of 200 µL. The reaction mixture consisted of 50 mM potassium phosphate buffer (KPi) pH 7.4, 5 mM MgCl2, 0-500 µM MAMC (10 concentrations) and E. coli membranes (40 nM wild-type 2D6 or 2D6[F120A]). The reactions were initiated by addition of an NADPH regenerating system, resulting in final concentrations of 0.1 mM NADPH, 0.3 mM glucose-6-phosphate, and 0.4 units/mL glucose-6-phosphate dehydrogenase. The reaction was monitored for 40 minutes at 37 °C on a Victor2 1420 multilabel counter (Wallac) (λex= 405 nm, λem= 460 nm). The metabolite of MAMC, i.e. HAMC, was identified and quantified using the synthetic reference compound. HPLC analysis of MAMC metabolism was performed as described [18], using a C18 column (Chrompack Chromspher 5, 250 x 4.6 mm) with a flow rate of 0.4 mL/minute.

**Dextromethorphan O-demethylation [10]:** Reactions were carried out in 300 µL 50 mM KPi pH 7.4, 5 mM MgCl2, 0-40 µM dextromethorphan (10 concentrations) and E. coli membranes (50 nM wild-type 2D6 or 25 nM 2D6[F120A]). After 5 min of pre-incubation at 37 °C, the reactions were initiated with an NADPH regenerating system as described above. The reaction was allowed to proceed for 5 minutes before it was stopped by the addition of 15 µL 70% HClO4. After centrifugation (15 min, 6800 g), 30 µL aliquots of the supernatant were analysed by HPLC. Metabolites were separated using a C18 column (Chrompack Chromspher 5, 250 x 4.6 mm) with a flow rate of 1 mL/minute. The mobile phase consisted of 30% acetonitril and 1% triethylamine, set to pH 3 with 70% HClO4. Metabolites were detected by fluorescence (λex= 280 nm, λem= 311 nm) and identified by LC-MS. Inhibition of O-demethylation by various concentrations of quinidine and quinine was measured under the same conditions using a concentration of 8 µM dextromethorphan.

**Bufuralol hydroxylation [10, 24]:** Reactions were carried out as described above with 0-100 µM bufuralol (9 concentrations) and E. coli membranes (50 nM wild-type 2D6 or 25 nM 2D6[F120A]). Metabolites were separated using a C18 column (Chrompack Chromspher 5, 250 x 4.6 mm) with a flow rate of 0.4 mL/minute.
4.6 mm) with a flow rate of 0.6 ml/minute. The mobile phase consisted of 30% acetonitril and 0.1% triethylamine, set to pH 3 with 70% HClO₄. Metabolites were detected by fluorescence ($\lambda_{ex}=252$ nm, $\lambda_{em}=302$ nm). Metabolites of bufuralol were identified by comparison with other studies [24].

MDMA metabolism: Reactions were carried out as described above with 0-200 µM MDMA (9 concentrations) and E. coli membranes (50 nM wild-type 2D6 or 25 nM 2D6[F120A]). Metabolites were separated using a C18 column (Phenomenex Inertsil ODS 150 x 4.6 mm) with a flow rate of 0.4 ml/minute. The mobile phase consisted of 23% acetonitril and 0.1% triethylamine, set to pH 3 with 70% HClO₄. Metabolites were detected by fluorescence ($\lambda_{ex}=280$ nm, $\lambda_{em}=320$ nm) and identified electrochemically (ECD, oxidation mode 0.8 V, for detection of 3,4-OH-MA), by LC-MS (3,4-OH-MA, MDA, N-OH-MDMA) and using synthesised reference compounds (MDA, N-OH-MDMA). Peak areas of all metabolites were quantified by Shimadzu Class VP 4.3 and analysed using GraphPad Prism 4.0.

**LC-MS**

To identify the metabolites of MDMA, dextromethorphan and dextrorphan, incubations were carried out for 10 min as described above with 100 µM MDMA, 100 µM dextrorphan or 25 µM dextromethorphan and E. coli membranes (50 nM wild-type 2D6 or 2D6[F120A]). Volumes of 100 µl supernatant were injected and separated using a phenyl column (Waters Novapak Phenyl 150 x 4.6 mm) with a flow rate of 0.4 ml/minute. The metabolites were eluted using a gradient starting with a 1% acetonitril eluens, supplemented with 10 mM ammonium acetate, increasing linearly to 95% acetonitril with 10 mM ammoniumacetate in 30 minutes and analysed by MS. APCI positive ionisation was used on a LCQ Deca mass spectrometer (Thermo Finnigan), vaporizer temperature 450 °C, N₂ as sheath (40 psi) and auxiliary gas (10 psi), needle voltage 6000 V, heated capillary 150 °C.

**Homology Modeling**

A protein homology model of P450 2D6 was constructed based on the crystal structures of dimethylsulphophenazole and diclofenac bound rabbit P450 2C5, PDB codes 1N6B and 1NR6 respectively [7, 25]. Homology modeling, model refinement and model validation was performed as described [9]. The final model of wild-type P450 2D6 was used as a template for modeling of the F120A mutant. The F120 residue was mutated to alanine using the homology module of InsightII (Biosym), after which an energy minimisation and a one picosecond position restrained molecular dynamics simulation was carried out.

**RESULTS**

**Expression of wild-type P450 2D6 and P450 2D6[F120A]**

P450 2D6[F120A] had consistently significantly lower expression levels compared to wild-type P450 2D6. An average culture contained about 70 nM of P450 for the mutant versus 200 nM for wild-type after 48 hours induction. In the difference spectrum taken from the culture
of the mutant enzyme a high absorbance was measured at 420 nm, showing the presence of large amounts of P420, the inactive form of P450, indicating that the mutation decreases the stability of the enzyme to some extent.

**Metabolism of model compounds**

O-demethylation of the P450 2D6 marker substrate MAMC (Figure 1) showed to be linear for over 40 minutes using *E. coli* membranes containing 40 nM of wild-type P450 2D6. Enzyme kinetics analysis revealed a $K_m$ of 49 µM and a $V_{max}$ of 3.7 min$^{-1}$ (Table 1). Using P450 2D6[F120A] under the same conditions did not lead to detectable HAMC formation. HPLC analysis of these incubations gave the same results and did not show any additional metabolites. Dextromethorphan was selectively O-demethylated to dextrorphan by wild-type P450 2D6 ($K_m$ 2 µM, $V_{max}$ 3.3 min$^{-1}$). P450 2D6[F120A] also formed dextrorphan, be it with a slightly lower $K_m$ and higher $V_{max}$ than the wild-type. The formation of dextrorphan showed to be linear for at least 20 minutes, indicating the mutant to be stable enough for studying the metabolism. Dextrorphan and other metabolites were identified by LC-MS. The N-demethylated metabolite of dextromethorphan, i.e. 3-methoxymorphinan (m/z 258, MS/MS: m/z 215), was detected for both the wild-type and the F120A mutant with a $t_r$ of 36 min. Because of overlap with the peak of dextromethorphan (m/z 272, MS/MS: m/z 215) at $t_r$ = 37 minutes, N-demethylation could

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**Figure 1:** Structures of the substrates (A: MAMC, B: MDMA, C: bufuralol, D: dextromethorphan) and ligands (E: quinidine (8R, 9S) and quinine (8S, 9R)) used, showing sites of oxidation by wild-type P450 2D6 (arrows) and chiral carbon atoms (*).
not be quantified. Interestingly, also a metabolite with m/z 244 (t_r = 31.1 minutes, MS/MS: m/z 147) was detected for the F120A mutant, apparently the double O- and N-demethylated metabolite of dextromethorphan, i.e. 3-hydroxymorphinan. The 3-hydroxymorphinan formation could also not be quantified under the conditions used because it is a secondary metabolite. When the F120A mutant was incubated with dextrophan, again 3-hydroxymorphinan was formed and two other metabolites with m/z 274 were detected (t_r = 275 minutes, MS/MS: m/z 199 and t_r = 303 minutes MS/MS: m/z 257 and 201), indicating hydroxylation of dextrophan. With the wild-type P450 2D6 no metabolites of dextrophan were detected.

Table I: Metabolism of model compounds by wild-type P450 2D6 and P450 2D6[F120A].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolites</th>
<th>K_m [µM]</th>
<th>V_max [min⁻¹]</th>
<th>K_m [µM]</th>
<th>V_max [min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMC</td>
<td>HAMC</td>
<td>49 ± 2</td>
<td>3.7 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dextromethorphan</td>
<td>dextrophan</td>
<td>2.0 ± 0.2</td>
<td>3.3 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3-OH-morphinan</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>dextrophan</td>
<td>3-OH-morphinan</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1'-OH</td>
<td>2.9 ± 0.4</td>
<td>54 ± 17</td>
<td>1.2 ± 0.2</td>
<td>51 ± 15</td>
</tr>
<tr>
<td></td>
<td>4-OH</td>
<td>3.2 ± 0.5</td>
<td>2.7 ± 0.8</td>
<td>1.5 ± 0.3</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>MDMA</td>
<td>3,4-OH-MA</td>
<td>1.9 ± 0.7</td>
<td>3.4 ± 1.0</td>
<td>55.4 ± 16</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>MDA</td>
<td>-</td>
<td>-</td>
<td>9.0 ± 2.5</td>
<td>6.0 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>N-OH-MDMA</td>
<td>-</td>
<td>-</td>
<td>11.0 ± 2.8</td>
<td>10.9 ± 3.8</td>
</tr>
</tbody>
</table>

All values are the means of at least three independent experiments ± S.D. K_m expressed in µM, V_max expressed in min⁻¹, not detectable, present but not quantifiable, V_max expressed in 1 x 10⁵ fluorescence units/min/nmol P450.

Upon incubation of bufuralol both with wild-type P450 2D6 and P450 2D6[F120A] four metabolites were detected; 6-OH bufuralol, 1'-OH bufuralol, 4-OH bufuralol and Δ² bufuralol, as identified before by others [24]. Under the conditions used only peak areas of the metabolites 1'-OH bufuralol and 4-OH bufuralol could be quantified. For both these metabolites the F120A mutant showed a two times lower K_m than wild-type P450 2D6 with a comparable turnover (Table 1).

Another known P450 2D6 substrate that was subjected to incubations with wild-type and the F120A mutant was MDMA [26]. In the case of wild-type P450 2D6 only demethylation of MDMA to the catechol 3,4-dihydroxymethylamphetamine (3,4-OH-MA) was observed with a K_m of 2 µM and a V_max of 3.4 x 10⁵ fluorescence units/min/mmol/P450. This metabolite was identified by ECD, LC-MS (measured as the quinone, t_r = 21.2 minutes, m/z 180) and it was described before by others [26]. The mutant enzyme also formed this catechol with a similar V_max but with a 30-fold higher K_m. In addition to demethylenation, MDMA was
also N-demethylated to MDA by the mutant enzyme, with a $K_m$ of 9 µM and a $V_{max}$ of $6 \times 10^5$ fluorescence units/min/nmol/P450, as identified by LC-MS ($t_r = 21.4$ minutes, $m/z = 180$) and co-elution with the synthetic reference (Figure 2). Furthermore, a third metabolite was formed by the F120A mutant with a $K_m$ of 11 µM, and a $V_{max}$ of $11 \times 10^5$ fluorescence units/min/nmol/P450. This metabolite was also identified by LC-MS. A $m/z$ of 210 was observed for this metabolite ($t_r = 21.9$ minutes), indicating hydroxylation of MDMA ($m/z = 194$). MS/MS data showed a mass over charge of 163, suggesting that hydroxylation took place on the nitrogen, as the $m/z$ of 163 is the propyl-methylenedioxyphenyl fragment, which is also a common fragment of both MDA and MDMA. In order to confirm the identity of this new metabolite of MDMA, N-OH-MDMA was synthesised and showed identical mass spectroscopic behaviour. Direct injection of this compound in the MS gave a $m/z$ 210, with as the major MS/MS fragment $m/z$ 163. In addition, the N-OH-MDMA also co-eluted with the new metabolite on both the C18 as the phenyl reversed phase columns, when spiked in an incubation sample, verifying it to have the same molecular structure.

**Figure 2:** Chromatograms of 100 µM MDMA incubations with membranes of E. coli cells expressing wild-type P450 2D6 (1) and P450 2D6[F120A] (2) in the presence (grey) and absence (black) of 20 µM quinidine (A). Metabolites are indicated by arrows and reference chromatograms of synthesised MDA (3) and synthesised N-OH-MDMA (4) are also shown. (B): Scheme of MDMA metabolism by wild-type P450 2D6 and P450 2D6[F120A] into 3,4-OH-MA (a), MDA (b), and N-OH-MDMA (c).
Inhibition of dextromethorphan metabolism

Inhibition of dextromethorphan O-demethylation by quinidine and quinine was determined for both wild-type P450 2D6 and P450 2D6[F120A] (Figure 3). In case of both the enzymes quinine inhibited dextrorphan formation with an IC_{50} of about 1 µM (Table 2), whereas quinidine was about six-fold less potent in inhibiting the F120A mutant (IC_{50} 240 nM) than the wild-type enzyme (IC_{50} 40 nM).

![Figure 3: Inhibition of dextromethorphan metabolism by quinine (A) and quinidine (B). Data are presented for the F120A mutant (triangles) and wild-type P450 2D6 (squares). Plotted values are the means of at least two independent experiments.](image)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>P450 2D6</th>
<th>P450 2D6[F120A]</th>
</tr>
</thead>
<tbody>
<tr>
<td>quinidine</td>
<td>0.04 ± 0.01</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>quinine</td>
<td>0.76 ± 0.17</td>
<td>1.3 ± 0.27</td>
</tr>
</tbody>
</table>

Table 2: IC_{50} values of the ligands quinine and quinidine for P450 2D6 and P450 2D6[F120A]. 8 µM dextromethorphan was used as substrate. All values are the means of at least two independent experiments.

Modeling

In a refined homology model of P450 2D6 used in this study, F120, E216 and F483 are positioned at approximately the same location as in the previously reported homology model [9]. D301 however, does not point into the active site, but is oriented such that it stabilises the B/C-
loop by hydrogen bonding to the backbone N-atoms of residues V199 and F120. D301 however may also play an electrostatic role in the binding of basic substrates by increasing the net negative charge within the active site [12]. Furthermore, the constructed homology model used in this study has a more 'closed' binding pocket as a result of the fact that the substrate-free P450 2C5 crystal structure template used for the old homology model is lacking the F/G-loop (unpublished data).

**Discussion**

Recently computer homology modeling studies suggested F120 to be a P450 2D6 active site residue involved in the binding of substrates via aromatic interactions [9, 11, 12]. The primary aim of this study was to evaluate the role of this residue in the P450 2D6 active site. The results presented in this study show that F120 is very relevant in P450 2D6 ligand binding, substrate selectivity and regiospecificity in catalysis.

The expression levels of the F120A mutant in *E. coli* were found to be lower than the wild-type enzyme, indicating that F120 may have a role in the stability of the enzyme. High levels of P420 were seen in the difference spectra, showing that the expression itself was not hampered, but that a large amount of enzyme formed was not functional as was reported before for several other P450 2D6 mutants [10, 27].

Four typical P450 2D6 substrates were selected to characterise the F120A mutant. Compared to wild-type P450 2D6 large substrate-dependent differences in metabolism were found after introducing a single mutation, not reported before for other P450 2D6 mutants. MAMC was not metabolised by the F120A mutant while bufuralol metabolism by the F120A mutant was similar to the metabolism by the wild-type enzyme. For both dextromethorphan and MDMA there were changes in regiospecificity after mutating F120 into an alanine. So despite the fact that all four substrates show structural similarity in having a basic N-atom at about 7 Å of the site of oxidation and in having an adjacent aromatic moiety [14], still the influence of the mutation differed between these substrates. The only time large substrate dependent differences were described for a P450 2D6 mutant was with a E216Q mutant, using the substrate spirosulfonamide [11] that lacks a basic N-atom and therefore does not fit into the classical pharmacophore model [14-16].

Neither HAMC nor other metabolites were detected after incubating MAMC with the F120A mutant so apparently F120 influences either the affinity, or the orientation of this substrate with respect to the heme. More experiments with other coumarin derivatives could give more information about the underlying mechanisms [28].

Bufuralol metabolism by P450 2D6 was studied previously using a series of D301 mutants [24] and also mutants of E216 to non-acidic residues were studied using this model substrate [10, 11]. Large effects were found on affinity and turnover of the mutants for this compound, showing that these acidic residues are more important for bufuralol metabolism than F120 as can be concluded from this study. The role of D301 was postulated also to be fixation of the B-C loop containing residue F120 [10-12] however the present results do not seem to support this role as the F120A mutation hardly shows an effect on bufuralol metabolism. Consequently,
the role of D301 does not seem to be merely the fixation of F120 into the active site [29], but creation of the net negative charge together with E216 may be of bigger importance. N-dealkylation of substrates is a possible route in P450 2D6 metabolism and F481 was predicted previously to be an important binding residue for these substrates [15]. However mutating the F481 residue into a glycine did not have a large influence on N-desisopropylation of metoprolol [17]. Both MDMA and dextrorphan were N-demethylated by the F120A mutant implicating a more promiscuous way of orientation of these two substrates in the active site of P450 2D6 and the involvement of the F120 residue in binding substrates undergoing N-dealkylation.

Besides N-dealkylation of MDMA, also N-hydroxylation occurred when MDMA was incubated with the F120A mutant. N-OH-MDMA has been described before as an MDMA metabolite in horse urine [30], however from this study it remained unclear what enzymes were playing a role in this reaction. Furthermore aliphatic N-hydroxylation has not been described before as a metabolic reaction catalysed by P450 2D6 wild-type or mutants for any substrate. The co-occurrence of both N-dealkylation and N-hydroxylation of a single substrate has been reported once before for N-methylbenzamidine by rabbit P450 2C3 [31]. Additional work should give more insights in the mechanism of these transformations by P450 2D6[F120A]. The role of F120 in the active site of P450 2D6 seems to be at least two-fold; firstly, the phenyl ring fills the active site cavity causing a reduction of its size and secondly, it may have a function as an aromatic interaction site. The metabolite 3-hydroxymorphinan is
detected exclusively in dextromethorphan incubations with the F120A mutant. In Figure 4 it is demonstrated how the mutation of the bulky phenylalanine into a smaller alanine residue allows the dextromethorphan N-demethylation binding mode to be accommodated more easily by creating more space in the P450 2D6 active site. As indicated earlier, F120 is considered important for interacting with quinidine via π-stacking [9]. The six-fold increase in IC50-value as determined for the F120A mutant validates the specific anchoring role of this residue. This role was confirmed by the inability of the F120A mutant to metabolise MAMC. The two roles, i.e. space filling and aromatic anchoring, however, only partly explain the substrate specific influence, as bufuralol, MDMA and MAMC are structurally similar substrates but showed different changes in metabolism when incubated with the F120A mutant. In the rat isoforms of the P450 2D family, no homologous aromatic residues are present in the B-C loop so F120 is a human P450 2D6 active site specific residue [9]. The data presented here introduce F120 as the fourth important residue in the binding of ligands to and catalysis by P450 2D6, next to E216, D301 and F483. The effects of removing the F120 phenyl-moiety are on the affinity, turnover and orientation of substrates, but the effects were quite different for structurally similar substrates. To rationalise the large differences found with the four chosen substrates advanced molecular modeling is needed. Combined with molecular dynamics it can give insights where space is a limiting factor or where specific interactions play a role in the P450 2D6 active site.
References


