Why Is Quinidine an Inhibitor of Cytochrome P450 2D6?

THE ROLE OF KEY ACTIVE-SITE RESIDUES IN QUINIDINE BINDING

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We have previously shown that Phe120, Glu216, and Asp301 in the active site of cytochrome P450 2D6 (CYP2D6) play a key role in substrate recognition by this important drug-metabolizing enzyme (Paine, M. J., McLaughlin, L. A., Flanagan, J. U., Kemp, C. A., Sutcliffe, M. J., Roberts, G. C., and Wolf, C. R. (2003) J. Biol. Chem. 278, 4021–4027 and Flanagan, J. U., Maréchal, J.-D., Ward, R., Kemp, C. A., McLaughlin, L. A., Sutcliffe, M. J., Roberts, G. C., Paine, M. J., and Wolf, C. R. (2004) Biochem. J. 380, 353–360). We have now examined the effect of mutations of these residues on interactions of the enzyme with the prototypical CYP2D6 inhibitor, quinidine. Abolition of the negative charge at either or both residues 216 and 301 decreased quinidine inhibition of bufuralol 1′-hydroxylation and dextrometorphan O-demethylation by at least 100-fold. The apparent dissociation constants (Kᵦ) for quinidine binding to the wild-type enzyme and the E216D and D301E mutants were 0.25–0.50 μM. The amide substitution of Glu216 or Asp301 resulted in 30–64-fold increases in the Kᵦ for quinidine. The double mutant E216Q/D301Q showed the largest decrease in quinidine affinity, with a Kᵦ of 65 μM. Alanine substitution of Phe120, Phe481, or Phe483 had only a minor effect on the inhibition of bufuralol 1′-hydroxylation and dextromethorphan O-demethylation and on binding. In contrast to the wild-type enzyme, a number of the mutants studied were found to be able to metabolize quinidine. E216F produced O-demethylated quinidine, and F120A and E216Q/D301Q produced both O-demethylated quinidine and 3-hydroxyquinidine metabolites. Homology modeling and molecular docking were used to predict the modes of quinidine binding to the wild-type and mutant enzymes; these were able to rationalize the experimental observations.

Human cytochrome P450 2D6 (CYP2D6) plays a central role in drug metabolism, metabolizing >30% of the most commonly prescribed drugs (1). The CYP2D6 gene is highly polymorphic, leading to wide interindividual and ethnic differences in CYP2D6-mediated drug metabolism (2–4). Cytochrome P450-Drug and drug-drug interactions involving CYP2D6 ligands are thus a prime consideration in the development of new drugs, emphasizing the importance of a detailed understanding of the factors that govern the substrate specificity of this enzyme.

Quinidine is not metabolized by CYP2D6 and has long been established as a potent competitive inhibitor of the enzyme (5–9). The fact that quinidine is an inhibitor rather than a substrate is intriguing because it produces a classical type I binding spectrum with CYP2D6 (10) that is usually associated with the binding of substrate molecules (11). In addition, quinidine possesses a number of features normally associated with CYP2D6 substrates, including a basic nitrogen atom, a flat hydrophobic region, and a negative molecular electrostatic potential (12). Studies of the relationship between structure and inhibitory activity for quinidine and its (less potent) stereoisomer quinine have been reported (13), but the protein-ligand interactions that are responsible for the fact that quinidine can bind tightly, but not in an orientation favorable for catalysis, have not hitherto been established.

Recent models of the active site of CYP2D6 (e.g. Ref. 14) suggest that two carboxylate groups (at Glu216 and Asp301) may play key roles in the recognition of substrates containing a basic nitrogen atom, and support for this has come from mutagenesis experiments (15–17). It has also been suggested that the aromatic residues Phe120, Phe481, and Phe483 may have roles in substrate binding through π-interactions with the planar hydrophobic regions common to many CYP2D6 substrates (10, 14, 18). Here, we describe studies of a series of mutants of these five residues aimed at investigating their role in quinidine binding and in determining whether quinidine is a substrate or an inhibitor of this important drug-metabolizing enzyme.

EXPERIMENTAL PROCEDURES

Materials—Terror broth, chloramphenicol, dithiothreitol, glucose 6-phosphate, NADP⁺, phenylmethylsulfonyl fluoride, sodium dithionite, cytochrome c, and quinidine were purchased from Sigma (Poole, UK). Ampicillin was obtained from Beecham Research (Welwyn Garden City, UK), isopropyl β-D-thiogalactopyranoside and 6-aminolevulinic acid from Melford Laboratories (Ipswich, UK), and glucose-6-phosphate dehydrogenase (type VII) from Roche Applied Science (Lewes, UK). HPLC-grade solvents were from Rathburn Scientific (Walkerburn, UK), and Agilent HPLC columns were from Agilent Technologies (Crawford Scientific (Lanarkshire, Scotland, UK). DNA-modifying enzymes were obtained from Invitrogen (Paisley, UK) and Promega Corp. (Southampton, UK). Bufuralol, 1′-hydroxybufuralol, and (3S,3′)-3-hydroxyquinidine were purchased from Ultrafine Chemicals (Manchester, UK). Quinidine N-oxide was a kind gift from Merck Sharp and Dohme (Harlow, UK). All other chemicals were from BDH (Poole, UK). Library efficient competent Escherichia coli strain JM109 was purchased from Promega Corp.

—Michael J. Sutcliffe, Ph.D.*
Role of Glu216/Asp301/Phe120 in 2D6-Quinidine Interactions

Mutagenesis and Expression in E. coli—The Glu216 and Asp301 mutants of CYP2D6 used in this study were constructed and expressed in E. coli along with human cytochrome P450 reductase as described previously (17). To obtain the remaining mutants, site-directed mutagenesis was performed following the single-stranded DNA template method (20) using pBS81 as a template and the dNTP ung E. coli strain CJ236 along with an appropriate mutagenic oligonucleotide: F120A, 3′-ata gcc gcg cag agc cac ccc ttg gga-5′; F481A, 3′-c-cag gag acc acc ccc tgg gga-5′; and F483A, 3′-c-cag gag acc gcc gag acc atg tgg-5′. Note that the oligonucleotide sequences are reverse-complemented. Once the presence of the desired mutation was confirmed by automated DNA sequencing, the mutants were coexpressed with human cytochrome P450 reductase as described above. Varying quantities of cytochrome P420, reflecting different degrees of stability in the presence of dithionite, were observed for the different mutants. E216Q, D301E, D301Q, F481A, and F483A had P450:420 peak area ratios of ~9:1; E216N, E216Q/D301Q, and F120A had ratios of ~1:1; and E216F, E216A, and D301N had ratios of ~1:5.

Quinidine Inhibition of Bufuralol 1′-Hydroxylation and Dextromethorphan O-Demethylation—Incubations were carried out in triplicate at 37°C with shaking in 300 μl of 50 mM potassium phosphate (pH 7.4) containing E. coli membranes equivalent to 10 pmol of CYP2D6 (wild-type or mutant), quinidine (0, 1, 10, or 100 μM), an NADPH-generating system (comprising 5 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 1 mM NADP+), and bufuralol or dextromethorphan at concentrations equivalent to the Km of each sample. The specific substrate concentrations used were as follows: CYP2D6, 1.1 μM bufuralol and 2.6 μM dextromethorphan; E216Q, 188 μM bufuralol and 51 μM dextromethorphan; E216D, 6 μM bufuralol and 13 μM dextromethorphan; E216F, 117 μM bufuralol and 30 μM dextromethorphan; E216A, 162 μM bufuralol and 63 μM dextromethorphan; E216K, 187 μM bufuralol and 312 μM dextromethorphan; D301E, 2 μM bufuralol and 11 μM dextromethorphan; D301Q, 142 μM bufuralol and 200 μM dextromethorphan; D301N, 160 μM bufuralol and 3598 μM dextromethorphan; E216Q/D301Q, 522 μM bufuralol and 438 μM dextromethorphan; F120A, 2.7 μM bufuralol and 1 μM dextromethorphan; F481A, 10 μM bufuralol and 11 μM dextromethorphan; F483A, 7.1 μM bufuralol and 9.5 μM dextromethorphan. After a 3-min preincubation at 37°C, reactions were initiated by the addition of the NADPH-generating system and were allowed to proceed for 6 min before being stopped by the addition of 15 μl of 60% perchloric acid. 100-μl aliquots of the stopped incubation mixture were used for HPLC, separating the authentic metabolite standards, with fluorescence detection at λex = 252 nm and λem = 302 nm.

Identification of the Novel Quinidine Metabolite—Further analysis of the novel quinidine metabolite was undertaken by HPLC with mass spectrometric detection. 25 μl of the stopped incubation mixture was separated on a Luna C18 column (3 μm, 150 × 2 mm; Phenomenex, Cheshire, UK) with a linear gradient of 5 mM ammonium formate (pH 3.5) (solvent A) and acetonitrile (solvent B) delivered by a Waters 2795 separations module. The gradient ran from 5 to 30% solvent A over 20 min at a flow rate of 200 μl/min before returning to the starting conditions. The eluent was introduced into the source of a Quattro micro mass spectrometer (Micromass, Manchester, UK) and was ionized by electrospray ionization in the positive ion mode. The main parameters were as follows: capillary voltage, 3.3 kV; cone voltage, 30 V; source and desolvation temperatures, 100 and 300°C, respectively; and cone and desolvation nitrogen gas flows, 90 and 300 liters/h, respectively. In collision-induced dissociation experiments, argon was used as the collision gas with a collision energy of 30 eV. Data were acquired and analyzed by MassLynx software.

Quinidine Binding—Quinidine binding was measured by optical difference spectroscopy of E. coli membranes containing CYP2D6 and NADPH-cytochrome P450 reductase (EC 1.6.2.4) using a Cary 4000 UV-visible spectrophotometer. E. coli membranes containing wild-type or mutant CYP2D6 enzymes were diluted in 100 mM potassium phosphate buffer (pH 7.4) to a final concentration of 0.5 μM cytochrome P450 and split into two matched black-walled quartz cuvettes. After running a base line, 1-μl aliquots of quinidine dissolved in deionized water were added to the sample cuvette, and equal volumes of water were added to the reference cuvette. The samples were left for 2 min between additions to equilibrate, and the difference spectrum was then run between 360 and 460 nm. The final volume of additions was kept to <2.5% of the total volume. Changes in absorbance as a function of quinidine concentration, at wavelengths selected on the basis of the spectral characteristics of the individual sample, were used to calculate binding constants using nonlinear regression analysis (Prism). Spectral determinations were performed at least twice for each mutant and found to be reproducible with respect to the spectral profile and the position of λmax and λmin.

Modeling and Molecular Docking—Homology modeling and molecular docking were used to predict the modes of quinidine binding to wild-type and mutant enzymes. The homology model of CYP2D6 was produced as described previously (14). In brief, the model was produced using the comparative modeling program Modeler (22) with five structural templates: cytochromes P450cam (23), P450terp (24), P450eryF (25), P450BM3 (26), and P4502C5 (27). Model structures for mutants E216F, E216Q/D301Q, and F120A were generated by replacing residues within SBYBL (28) and optimizing the positions of the new side chains with the rest of the protein held fixed. (Minimization of atoms in a 10-Å sphere around the mutated residue produced no noticeable changes; these results were therefore discarded.)

Docking studies were performed using the program GOLD (version 2.2) (29) with the ChemScore fitness function (30, 31) to generate 50 possible binding orientations for quinidine into both the deoxygenated (i.e. type I-like) and oxygenated (i.e. compound I-like) forms of each of and 1.6 ml of 60% perchloric acid dissolved in 5 liters of distilled H2O; solvent B) were mixed at a constant ratio of 15% solvent A to 85% solvent B (v/v) for the first 5 min, and then a linear gradient was applied over 4 min, ending at 31% solvent A to 69% solvent B (v/v), which was maintained for an additional 7 min. The retention times of (3S)-3′-hydroxyquinidine and quinidine N-oxide were established using authentic metabolite standards, with fluorescence detection at λex = 252 nm and λem = 302 nm.
the wild-type and mutant CYP2D6 models. The orientations were ranked according to the value of the ChemScore fitness function. In addition, "tethered" dockings were performed by applying constraints to the distances between the O-methyl group and 3-carbon atom of quinidine and the heme iron and between the basic nitrogen atom of quinidine and the carboxyl group of Glu216 (see TABLE TWO for details of the tethered dockings used). The docked energy of a solution that positioned the tethered groups farther than 4.5 Å apart was penalized, the size of the penalty being determined using a harmonic force constant of 5.0 kJ mol⁻¹ Å⁻².

RESULTS

Inhibition of CYP2D6 Mutants by Quinidine—We investigated the effects of mutations of the active-site residues Phe₁₂₀, Glu²¹⁶, Asp³⁰¹, Phe⁴₈₁, and Phe⁴₈₃ on the inhibition of CYP2D6 activity by quinidine. The inhibition profile of a panel of 12 mutants was examined by measuring bufuralol 1'-hydroxylation and dextromethorphan O-demethylation in the presence of 1, 10, or 100 μM quinidine (Fig. 1). Many of these mutants affect substrate binding; so, to isolate the effects of the mutations on quinidine inhibition, the substrate concentrations used were chosen to be equal to the measured Kᵣ for each protein. A, bufuralol 1'-hydroxylase; B, dextromethorphan O-demethylase.

FIGURE 1. Inhibition of bufuralol 1'-hydroxylase and dextromethorphan O-demethylase activities by 1, 10, and 100 μM quinidine. Samples were run in triplicate under the conditions described under “Experimental Procedures” at bufuralol and dextromethorphan concentrations equivalent to the Kᵣ for each protein. A, bufuralol 1'-hydroxylase; B, dextromethorphan O-demethylase.

The effects of the mutations were generally similar for quinidine inhibition of both bufuralol 1'-hydroxylation and dextromethorphan O-demethylation activities, although for most of the mutants, quinidine was found to be a somewhat better inhibitor with respect to dextromethorphan compared with bufuralol. These observations suggest that the negative charges at Glu²¹⁶ and Asp³⁰¹, but not the aromatic rings of the three phenylalanine residues, are important for the binding of quinidine; this is broadly consistent with the effects of mutation of these residues on the Kᵣ values of substrates containing a basic nitrogen atom (10, 16–18, 21, 32).

Quinidine Binding to the Glu²¹⁶ and Asp³⁰¹ Mutants—The effects of the mutations on quinidine binding were determined directly by measuring optical difference spectra upon addition of quinidine to bacterial membranes expressing cytochrome P450. Wild-type CYP2D6 showed a type I binding spectrum upon quinidine addition, with λ_max and λ_min of ~420 and ~390 nm, respectively (Fig. 2A), characteristic of the change from a low to high spin state of the ferric iron that is usually associated with the binding of substrate molecules (11). None of the mutants showed evidence of a type II spectrum, characteristic of direct coordination to the heme iron. The majority showed type I difference spectra (Fig. 2A) or variations thereof (Fig. 2C), but three showed a different form of spectrum with an increase in absorbance at shorter wavelengths (Fig. 2B), suggesting either a change in the heme environment or light scattering from membrane or protein aggregation.

The Kᵣ values for quinidine binding, derived from the dependence of the amplitude of the difference spectrum on quinidine concentration, are shown in TABLE ONE. The alanine substitutions of Phe₁₂₀, Phe⁴₈₁, and Phe⁴₈₃ led to no more than a factor of 2 decrease in binding affinity. However, removal of the negative charge from either Glu²¹⁶ or Asp³⁰¹ produced clear increases in the Kᵣ. Wild-type CYP2D6 and the

5 The difference spectra reported here for quinidine binding to the E216Q and E216A mutants appear to be different from those reported by Guengerich et al. (16). In our work, the difference spectra were recorded using E. coli membranes expressing CYP2D6, whereas Guengerich et al. (16) used detergent-solubilized purified enzyme, and this may account for the difference.
two conservative mutants E216D and D301E had $K_d$ values in the 0.4–0.5 μM range, whereas the $K_d$ values for the non-conservative substitutions were at least 30-fold higher, in the 15–65 μM range. Consistent with the inhibitory effects on bufuralol and dextromethorphan metabolism (Fig. 1), the largest increase in the $K_{d(app)}$ was observed with the double mutant E216Q/D301Q. These results are consistent with similar decreases in affinity indicated by $K_m$ estimates for bufuralol 1'-hydroxylation (17).

**Quinidine Metabolism**—Both the inhibition assays and the direct measurements of binding showed that removing the negative charges from Glu216 and/or Asp301 produced a major quantitative effect on quinidine binding to CYP2D6. Because quinidine produces a type I binding difference spectrum, typical of CYP2D6 substrates, but is not metabolized by wild-type CYP2D6 (5, 7–9), we carried out experiments to examine the possibility that some of the mutants might be able to metabolize quinidine. Quinidine was incubated for 15 min with 50 nM wild-type or mutant CYP2D6, and the incubation mixture was analyzed by HPLC. As shown by the chromatograms in Fig. 3, quinidine was clearly metabolized by the three mutants E216F, E216Q/D301Q, and F120A. Two clear metabolite peaks with retention times of 8.2 min (M1) and 8.8 min (M2), respectively, were observed; both were produced by E216Q/D301Q and F120A, whereas E216F produced only the metabolite eluting at 8.8 min. The peak with a retention time of 8.2 min comigrated with a standard of (3S)-3-hydroxyquinidine, demonstrating that, in contrast to wild-type CYP2D6, the E216Q/D301Q and F120A mutants, alone among the mutants studied, were able to catalyze the 3-hydroxylation of quinidine. The second metabolite, M2 (Fig. 3), formed by E216F, E216Q/D301Q, and F120A, did not comigrate with either of the quinidine metabolite standards available to us ((3S)-3-hydroxyquinidine and quinidine N-oxide). Quinidine 3-hydroxylation turnover rates for E216Q/D301Q and F120A were estimated from these experiments as 0.14 and 0.07 min$^{-1}$, respectively, somewhat slower than the typical rates of 1–5 min$^{-1}$ obtained for the wild-type enzyme for standard substrates such as bufuralol and dextromethorphan (17). Rates for O-demethylquinidine (metabolite M2) could not be measured due to lack of a metabolite standard.

**TABLE ONE**

| CYP2D6   | $K_{d(app)}$ $\mu M$ | $\lambda_{max}$ nm | $\lambda_{min}$ nm | $\Delta \lambda_{max}$ nm |
|----------|----------------------|---------------------|---------------------|--------------------------|
| Wild-type | 0.47 ± 0.03          | 389                 | 419                 | 0.044 ± 0.0003           |
| E216Q    | 25.72 ± 2.3          | 403                 | 424                 | 0.008 ± 0.0003           |
| E216D    | 0.38 ± 0.07          | 390                 | 422                 | 0.011 ± 0.0003           |
| E216F    | 15.13 ± 0.9          | 405                 | 427                 | 0.040 ± 0.0001           |
| E216A    | 21.76 ± 1.2          | 403                 | 427                 | 0.051 ± 0.001            |
| E216K    | 32.31 ± 2.2          | 404                 | 426                 | 0.010 ± 0.0003           |
| D301E    | 0.54 ± 0.04          | 389                 | 424                 | 0.013 ± 0.0003           |
| D301Q    | 18.00 ± 2.0          | 404                 | 425                 | 0.015 ± 0.0007           |
| D301N    | 24.70 ± 1.2          | 404                 | 427                 | 0.055 ± 0.0002           |
| E216Q/D301Q | 64.94 ± 9.1     | 402                 | 428                 | 0.037 ± 0.003            |
| F120A    | 0.8 ± 0.1            | 390                 | 423                 | 0.037 ± 0.002            |
| F481A    | 0.4 ± 0.07           | 390                 | 424                 | 0.012 ± 0.0007           |
| F483A    | 0.7 ± 0.06           | 390                 | 424                 | 0.014 ± 0.0005           |

**FIGURE 2.** Representative optical difference spectra produced by quinidine binding to wild-type and mutant CYP2D6. A, type I binding spectra typical of the wild-type enzyme and mutants E216F, E216A, D301E, D301N, F120A, F481A, and F483A; B, spectra typical of E216Q, E216K, and E216Q/D301E; C, spectra typical of E216D and D301Q. Experiments were performed as described under “Experimental Procedures.” Abs, absorbance.
suggesting the occurrence of a demethylation reaction (Fig. 4). Metabolite M2 was not present in the control reaction (Fig. 4A, upper chromatogram). Collision-induced dissociation generated the spectrum shown in Fig. 4B. The daughter ion at \( m/z \ 174.8 \) was assigned to the O-demethylated fragment 4-(hydroxymethyl)quinolin-6-ol, allowing us to identify metabolite M2 as O-demethylquinidine.

The observation that quinidine was metabolized by three of the mutants studied here, but not by the wild-type enzyme, clearly implies that the mode of quinidine binding, and not just its affinity, is affected by these mutations. To help us understand the structural basis of this, we carried out computational docking of quinidine into structural models of the active site of the wild-type and mutant enzymes.

**Modeling of Quinidine Binding to CYP2D6**—To help rationalize the experimental data, computational docking studies were performed using our previously described model of wild-type CYP2D6 (14). 50 solutions for quinidine binding to oxygenated (compound I-like) and deoxygenated (type I-like) CYP2D6 were obtained, leading to solutions with good ChemScore values (\(-39.0 \text{ kJ mol}^{-1}\)) (TABLE TWO), in keeping with the experimentally observed affinity (\( K_d = 0.4 \mu M \)). In all solutions, the quinidine was positioned away from the heme; the orientation of quinidine within the active site in the best ranked solution is shown in Fig. 5A. The results from these docking studies on the wild-type enzyme are consistent with the experimental data insofar as they produced no solutions for quinidine binding close to the heme in a position appropriate for catalytic turnover.

The predicted binding mode of quinidine in wild-type CYP2D6 appears to be influenced by interactions between the aromatic rings of quinidine and Phe\(^{120}\) and Phe\(^{483}\) and by a hydrogen bond between the basic nitrogen atom, tethered dockings were performed in which the amino group of Glu\(^{216}\) was constrained to the orientation consistent with formation of 3-hydroxyquinidine (metabolite M1) and O-demethylquinidine (metabolite M2). The resulting solutions had significantly poorer ChemScore values (31.7 and 36.3 kJ mol\(^{-1}\)) compared with the unconstrained dockings (39.0 kJ mol\(^{-1}\)) (TABLE TWO). These results suggest that such a binding mode is energetically unfavorable and that, even if such a binding mode were adopted, it would be incompatible with metabolism. To enable comparison with metabolites produced by the mutants studied (see below), tethered dockings were also performed to produce orientations consistent with formation of 3-hydroxyquinidine (metabolite M1) and O-demethylquinidine (metabolite M2). The resulting solutions had significantly poorer ChemScore values (31.7 and 36.3 kJ mol\(^{-1}\)) compared with the unconstrained dockings (39.0 kJ mol\(^{-1}\)). These results suggest that quinidine does not adopt a binding mode compatible with metabolism, consistent with its role as a competitive inhibitor of CYP2D6. In these tethered dockings, unfavorable contacts occurred with Phe\(^{120}\) and Ala\(^{305}\) in the orientation consistent with formation of 3-hydroxyquinidine and with Phe\(^{483}\), Leu\(^{213}\), and Glu\(^{216}\) in the orientation consistent with formation of O-demethylquinidine. This suggests that these residues are likely important in preventing metabolism of quinidine in wild-type CYP2D6.

We also carried out docking studies with a model of the F120A mutant. The 50 solutions obtained could be divided into two clusters; the best ranked solutions from each cluster are shown in Fig. 5 (B and C). The solution with the best ChemScore value (37.1 kJ mol\(^{-1}\)) (TABLE TWO) was used as the control reaction in the absence of NADPH. The assignment of the fragment ion at \( m/z \ 175 \) following collision-induced dissociation is shown on the inset.

**Mass spectrometric identification of quinidine metabolite M2.** A, extracted ion chromatograms (\( m/z \ 311 \)) of the O-demethylquinidine metabolite (M2) produced by the double mutant E216Q/D301Q; B, tandem mass spectra and structure (inset) of the metabolite. The upper chromatogram in A is the control reaction in the absence of NADPH. The assignment of the fragment ion at \( m/z \ 175 \) following collision-induced dissociation is shown on the inset.

**FIGURE 4.** Mass spectrometric identification of quinidine metabolite M2. A, extracted ion chromatograms (\( m/z \ 311 \)) of the O-demethylquinidine metabolite (M2) produced by the double mutant E216Q/D301Q; B, tandem mass spectra and structure (inset) of the metabolite. The upper chromatogram in A is the control reaction in the absence of NADPH. The assignment of the fragment ion at \( m/z \ 175 \) following collision-induced dissociation is shown on the inset.
TWO) positioned the quinidine too far from the heme iron to be metabolized. However, some solutions in the same cluster were consistent with 3-hydroxylation. The best ChemScore value of any such solution was 33.0 kJ/mol, i.e., only 0.1 kJ/mol poorer than the best overall ChemScore value) is consistent with O-demethylation, with both the basic nitrogen atom and the hydroxyl group of quinidine interacting with a carboxyl oxygen of Glu216. Thus, the docking studies with the F120A mutant provided results consistent with the experimental

| Model and docking type | ChemScore value of best ranked docked quinidine solution | Constraint energy of tether | Fe–X distance | Corresponding metabolism |
|-------------------------|--------------------------------------------------------|-----------------------------|--------------|--------------------------|
| Wild-type CYP2D6        |                                                        |                             |              |                          |
| Oxygenated              | −39.0                                                  | None                        | 6.5          | None                     |
| Deoxygenated            | −39.0                                                  | None                        | 5.3          | None                     |
| 3-Carbon tether         | −36.3                                                  | 0.8                         | 4.8          | 3-Hydroxylation           |
| O-Methyl tether         | −31.7                                                  | 0.0                         | 4.3          | O-Demethylation           |
| NH2–Glu216 tether       | −30.0                                                  | 0.3                         | 7.8          | None                     |
| F120A                   | −37.1                                                   | None                        | 6.5          | None                     |
| Oxygenated              | −37.0                                                   | None                        | 4.6          | O-Demethylation           |
| E216F                   | −37.8                                                   | None                        | 4.3          | 1'-Hydroxylation          |
| Oxygenated              | −37.3                                                   | None                        | 4.8          | O-Demethylation           |
| 3-Carbon tether         | −33.0                                                   | 1.3                         | 5.0          | 3-Hydroxylation           |
| E216Q/D301Q             |                                                        |                             |              |                          |
| Oxygenated              | −34.6                                                   | None                        | 5.5          | None                     |
| O-Methyl tether         | −27.8                                                   | 0.1                         | 4.7          | O-Demethylation           |
| 3-Carbon tether         | −30.2                                                   | 1.1                         | 5.0          | 3-Hydroxylation           |

a Distance from the heme iron to the nearest non-hydrogen atom in quinidine.

b Based on an Fe–X distance of up to 5 Å.

c Docking into protein containing oxygenated (i.e. compound I-like) heme iron.

d Docking into protein containing deoxygenated (i.e. type I-like) heme iron.

e Best ranked quinidine in the cluster with the lowest ChemScore value.

f A solution in this cluster with a lower ChemScore value (−33.0 kJ/mol) is compatible with 3-hydroxylation.

g Best ranked quinidine in the cluster with the second lowest ChemScore value.

FIGURE 5. Predicted binding modes of quinidine in wild-type and mutant CYP2D6. A, the best ranked docking of quinidine in the wild-type CYP2D6 model is shown. B, the highest ranked docking into the F120A CYP2D6 model having an orientation appropriate for formation of 3-hydroxyquinidine (metabolite M1) from the cluster of solutions containing the best ranked docking. C, the best ranked docking into the F120A CYP2D6 model from the cluster of solutions having an orientation appropriate for formation of O-demethylquinidine (metabolite M2). D, the best ranked docking into the E216Q CYP2D6 model from the cluster of solutions having an orientation appropriate for formation of 3-hydroxyquinidine (metabolite M1). E, the best ranked docking into the E216Q CYP2D6 model from the cluster of solutions having an orientation appropriate for formation of O-demethylquinidine (metabolite M2). Predicted hydrogen bonds are denoted as dotted lines.
observation that this mutant was able to metabolize quinidine to its O-demethyl and 3-hydroxy derivatives.

In our model of the E216F mutant, the most energetically favorable position of this substituted side chain forms a face-on-face interaction with Phe₁²¹⁹ and an edge-on-face interaction with Phe₁⁴⁸. The 50 solutions obtained could be divided into two clusters. The solution with the best ChemScore value (37.8 kJ/mol⁻¹) (TABLE TWO) is consistent with 1'-hydroxylation, i.e. formation of neither metabolite M1 nor M2. The solution in the second cluster with the best ChemScore value (37.2 kJ/mol⁻¹, i.e. only 0.6 kJ/mol⁻¹ poorer than the best overall ChemScore value) is consistent with O-demethylation (Fig. 5D). This orientation suggests an interaction between the aromatic rings of quinidine and Phe₂¹⁶ and Phe₁²⁰ and a hydrogen bond between the basic nitrogen atom of quinidine and the side chain of Ser³⁰⁴. To investigate the possibility of forming 3-hydroxyquinidine, tethered dockings were performed. This distance constraint is difficult to satisfy, producing unfavorable contacts with Phe₁²⁰, Val³⁰⁸, and Phe₁⁴⁸. However, Asp³⁰¹ is thought to contribute to the stabilization of the B'-C loop through a hydrogen bond with the backbone of Val¹¹⁵ (14), and therefore, mutation of this residue to Gln could lead to a conformational change in the B'-C loop, thus impacting more globally on the nature of the active site.

DISCUSSION

Quinidine is a well established and potent competitive inhibitor of CYP2D6 (5, 7–9); indeed, inhibition by quinidine is often used as a diagnostic for involvement of CYP2D6 in drug metabolism. However, it has not been clear why quinidine is not a substrate because it has many of the features that are regarded as characteristic of CYP2D6 substrates, including a basic nitrogen atom and a flat hydrophobic region (12). Docking quinidine into our homology-based model of CYP2D6 (Fig. 5) led to a predicted mode of binding in which it fits into the active site, but is too far from the heme for catalytic turnover. Quinidine binding to CYP2D6 produces a classical type I optical difference spectrum (cf. Fig. 2), indicative of the change from a low to high spin state of the ferric iron that usually accompanies the binding of substrate molecules (11) and that is associated with the displacement of the water molecule bound to the iron in the “resting” enzyme, converting the iron from six- to five-coordinate. In the proposed mode of binding of quinidine, the inhibitor is too far from the heme iron to displace the bound water molecule directly. However, it is clear (for example, from NMR and crystallographic studies of CYP102A (33, 34)) that the binding of ligands relatively distant (~9 Å) from the heme iron of cytochromes P450 can lead to the displacement of the bound water molecule.

Previous modeling and mutagenesis work had suggested that two carboxylate groups (at Glu²¹⁶ and Asp³⁰¹) and three phenylalanine residues (Phe₁²⁰, Phe₁⁴⁸, and Phe₁⁴⁸) play important roles in determining the binding of substrates in the active site of CYP2D6 (e.g. Refs. 10 and 14–19). This work has shown that several of these residues also play significant roles in binding the inhibitor quinidine. In terms of the binding constants, the two carboxylate groups are clearly the most important; abolition of one or both of these charges increased the $K_d$ for quinidine by 30–100-fold. In the best scoring docked orientation of quinidine in the active site of the wild-type enzyme, the basic quinuclidine nitrogen was closer to Glu²¹⁶ (5.4 Å) than to Asp³⁰¹ (8.8 Å), although the effects of substituting either residue with the corresponding amide were very similar (TABLE ONE). The fact that the double mutant E216Q/D301Q showed significantly weaker binding than either single mutant supports the idea that the electrostatic field of both residues is significant for quinidine binding. A recent study of a number of analogs of quinidine demonstrated that alkylation of the quinuclidine nitrogen with groups as bulky as naphthyl had no effect on the measured $IC_{50}$, and it was concluded that the proposed charge-charge interaction with Asp³⁰¹ does not make a major contribution to binding (13). The ~30-fold increase in the $K_d$ for E216F, corresponding to a decrease in binding energy of ~9 kJ/mol⁻¹, is consistent with the loss of a hydrogen bond and/or a weak charge-charge interaction. This is consistent with our prediction that the hydroxyl group (rather than the basic nitrogen atom) of quinidine forms the dominant interaction with Glu²¹⁶. More generally, it must be recognized that, particularly for the cytochromes P450, interpretations of the effects of altering either the protein or the ligand are complicated by the possibility (indeed, the likelihood) that these changes will lead to an altered mode of binding.

For several of the mutants studied here, it is clear that there was indeed a change in the mode of binding of quinidine because it became a substrate rather than an inhibitor. The F120A mutant and the double mutant E216Q/D301Q each formed both 3-hydroxyquinidine and O-demethylation of quinidine in significant quantities. 3-Hydroxyquinidine is a major product of quinidine metabolism by CYP3A4 (7) and, indeed, this reaction has been suggested as a specific marker for CYP3A4 in human liver microsomes (35). We have shown previously that this double mutant of CYP2D6 is able to catalyze another characteristic CYP3A4 reaction, the $N$-oxidation of nifedipine (17), and concluded that Glu²¹⁶ and Asp³⁰¹ have central roles in defining the specificity of CYP2D6. We also noted the possibility that the effects of mutation of Asp³⁰¹ are indirect because, in our model (14), the side chain of this residue interacts with the backbone of the B'-C loop, thus helping to position this loop, including Phe₁²⁰, in the active site and, indeed, the F120A mutant metabolizes quinidine to the same two products as the E216Q/D301Q mutant. However, substitution of Asp³⁰¹ alone is not sufficient to enable CYP2D6 to metabolize quinidine, and Glu²¹⁶ clearly plays an important role in determining the mode of binding. This is emphasized by the fact that substitution of this residue with a bulky side chain in the E216F mutant confers on CYP2D6 the ability to catalyze the O-demethylation of quinidine and also, to some extent, the 6β-hydroxylation of testoster one, another characteristic CYP3A4 reaction (17).

To obtain a structural picture of the possible changes in the binding mode of quinidine in the mutants, we docked quinidine into models of the mutants, obtained by side chain substitution in our earlier model of the wild-type enzyme (14). The model of the F120A mutant gave results consistent with the experimental observations in that quinidine was predicted to bind closer to the heme than in the wild-type model, with the most favorable modes of binding predicted to be those corresponding to 3-hydroxylation and O-demethylation, the observed routes of metabolism (Fig. 5, B and C). Within the limitations of the model, this indicates a direct role of the Phe₁²⁰ side chain in determining the unpro-
Role of Glu^{216}/Asp^{301}/Phe^{120} in 2D6-Quinidine Interactions

ductive mode of binding of quinidine to CYP2D6. The model of the E216F mutant also gave results consistent with the experimental observations.

On the other hand, with the E216Q/D301Q mutant, simple docking calculations did not predict a productive mode of binding for quinidine. However, when appropriate distance constraints were introduced into the docking calculations, solutions were obtained that were consistent with the formation of the experimentally observed metabolites. The calculated ChemScore values of these solutions were somewhat poorer than those of the unconstrained solutions due largely to unfavorable interactions with the side chains of Phe^{120} and Phe^{483}. It is possible that, in these mutants, quinidine binds most of the time in a nonproductive mode, but that productive mode(s) of binding are accessible and lead to the observed turnover. However, the limitations of the models should be recognized. Phe^{120} and Phe^{483}, which appear to interact unfavorably with quinidine in the models of these two mutants, are both located in loops, in SRS1 and SRS6, respectively. Loop regions are often highly flexible and therefore difficult to represent with either a single model or a single crystal structure. The docking program we have used (GOLD Version 2.2) (29, 36) allows the ligand full translation and rotational freedom, but, like most available docking programs, it treats the protein as rigid. Thus, it may be that the flexibility in these loops is such that the two phenylalanine rings can move away from the bound quinidine, making the productive mode of binding the most favorable. Particularly in cytochromes P450, the problem of receptor flexibility is a significant challenge in predicting ligand binding.

The results of these mutagenesis experiments clearly show that Phe^{120}, Glu^{216}, and Asp^{301} are important in determining the mode of binding of quinidine to CYP2D6, particularly in determining whether it binds in a nonproductive mode, as in the wild-type enzyme, and is thus an inhibitor, or whether it binds productively. They also demonstrate that changes in affinity and binding mode do not necessarily go hand-in-hand: in the F120A mutant, a change in the mode of binding (indicated by the ability to metabolize quinidine) was not accompanied by any change in \( K_a \), whereas in the E216K mutant, a 70-fold increase in \( K_d \) was accompanied by a change in the mode of binding sufficient to allow quinidine to be metabolized. A particularly important role is indicated for Phe^{120} because substitution of this single residue allowed CYP2D6 to metabolize its “classical” inhibitor quinidine with, however, with a decrease in binding affinity, and the docking calculations strongly suggest that this residue has a direct effect in forcing quinidine to bind in an unproductive mode to CYP2D6.

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