Role of Glutamic Acid 216 in Cytochrome P450 2D6 Substrate Binding and Catalysis†

F. Peter Guengerich,*,‡ Imad H. Hanna,§ Martha V. Martin,‡ and Elizabeth M. J. Gillam||

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, Department of Drug Metabolism and Safety Assessment, Schering-Plough Research Institute, Kenilworth, New Jersey 07033-1300, and Physiology and Pharmacology, School of Biomedical Sciences, The University of Queensland, St. Lucia, Brisbane, Queensland, Australia 4072

Received October 30, 2002; Revised Manuscript Received December 5, 2002

ABSTRACT: Human cytochrome P450 (P450) 2D6 is an important enzyme involved in the metabolism of drugs, many of which are amines or contain other basic nitrogen atoms. Asp301 has generally been considered to be involved in electrostatic docking with the basic substrates, on the basis of previous modeling studies and site-directed mutagenesis. Substitution of Glu216 with a residue other than Asp strongly attenuated the binding of quinidine, bufuralol, and several other P450 2D6 ligands. Catalytic activity with the substrates bufuralol and 4-methoxyphenethylamine was strongly inhibited by neutral or basic mutations at Glu216 (>95%), to the same extent as the substitution of Asn at Asp301. Unlike the Asp301 mutants, the Gln216 mutant (E216Q) retained 40% enzyme efficiency with the substrate spirosulfonamide, devoid of basic nitrogen, suggesting that the substitutions at Glu216 affect binding of amine substrates more than other catalytic steps. Attempts to induce catalytic specificity toward new substrates by substitutions at Asp301 and Glu216 were unsuccessful. Collectively, the results provide evidence for electrostatic interaction of amine substrates with Glu216, and we propose that both of these acidic residues plus at least another residue(s) is (are) involved in binding the repertoire of P450 2D6 ligands.

P450[1] enzymes are monooxygenases that have a variety of functions. In microorganisms, they can enable growth on particular substrates (*2*) or produce useful products of secondary metabolism, e.g., antibiotics (*3*). Some of the mammalian P450s have critical functions, e.g., in sterol metabolism (*4*), while others appear to be present mainly for the metabolism of a wide variety of xenobiotic chemicals, i.e., drugs, pesticides, alkaloids, terpenes, and other substances not naturally found in the body (*5*). One of the human P450 enzymes in this latter group, P450 2D6, is involved in the oxidative metabolism of ∼30% of the drugs used today (*6*, *7*). Because of the polymorphism involving lack of P450 2D6 function (*8*, *9*), the development of new drugs for which P450 2D6 plays a major role in metabolism is often avoided (*6*, *7*).

Many of the substrates (*6*, *7*) and inhibitors (*10*) are amines or contain other basic nitrogen atoms. With our early realization that the alleged substrate phenacetin (*11*) was a specific substrate for P450 1A2 and not P450 2D6 (*12*), we presented a P450 2D6 pharmacophore model in which substrates were bound via hydrophobic interactions plus an electrostatic interaction between the basic nitrogen and a putative anionic charge located 5−7 Å away from the site of carbon oxidation (*13*). Subsequently, this pharmacophore model was further developed for inhibitors by our group (*14*) and for substrates by others (*15*−*30*). Several potential acidic amino acid residues had been considered for liganding in the modeling work. Ellis et al. (*31*) changed Asp301 using site-directed mutagenesis in a yeast expression system and interpreted the attenuated catalytic activity (90% decrease) in the context of a role of Asp301 in binding the substrates debrisoquine and metoprolol (and the inhibitor quinidine). Patterns of heme modification were used to argue that the Asp301 substitution caused minimal perturbation of the protein structure (*32*). Subsequently, most P450 2D6 homology models have been based upon the electrostatic interaction of a basic nitrogen in the substrate with Asp301.[2]

† This research was supported in part by U. S. Public Health Service Grants R01 CA90426 and P30 ES00267.

* To whom correspondence should be addressed: Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, 638 Robinson Research Bldg. (Medical Research Bldg. I), 23rd and Pierce Aves., Nashville, TN 37232-0146. Tel.: 615-322-2261; Fax: 615-322-3141; E-mail: guengerich@toxicology.mc.vanderbilt.edu.

‡ Vanderbilt University School of Medicine.

§ Schering-Plough Research Institute.

|| University of Queensland.

[1] Abbreviations: P450, cytochrome P450 (also termed heme-thiolate protein P450 by the Enzyme Commission, EC 1.14.14.1 (*1*)); HPLC, high performance liquid chromatography; di-12:0 GPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; K_d, dissociation constant; and K_s, dissociation constant estimated by spectral interaction.

[2] Statements such as the following are found in the literature: "All of our data [on propanolol derivatives] are consistent with the generally accepted model for binding of CYP2D6 [P450 2D6] substrates via formation of an ion pair of the protonated amine with the carboxylate anion of Asp301 in the enzyme active site and subsequent oxidation at a distant site in the molecule" and "The presence of basic nitrogens is a common requirement of high-affinity substrates [for P450 2D6]..." (*33*).

10.1021/bi027085w CCC: $25.00
Published on Web 01/17/2003

Table 1: Kinetic Parameters for Bufuralol Hydroxylation by P450 2D6 and Site-Directed Mutants

P450 2D6	product								
	1′-OH			6-OH			4-OH		
	k_{cat} min^{-1}	K_m μM	k_{cat}/K_m min^{-1} mM^{-1}	k_{cat} min^{-1}	K_m μM	k_{cat}/K_m min^{-1} mM^{-1}	k_{cat} min^{-1}	K_m μM	k_{cat}/K_m min^{-1} mM^{-1}
wild type	8.7 ± 0.2	15 ± 1	580	1.7 ± 0.1	16 ± 2	110	3.1 ± 0.2	21 ± 3	150
E216D	11 ± 0.5	46 ± 7	230	6.7 ± 0.4	66 ± 10	100	3.7 ± 0.3	160 ± 20	24
E216Q	1.0 ± 0.1	70 ± 5	15	0.38 ± 0.14	110 ± 64	3.6	0.44 ± 0.05	180 ± 66	2.4
E216A	1.2± 0.1	89 ± 8	14	0.59 ± 0.1	200 ± 26	2.9	0.68 ± 0.14	410 ± 190	1.7
E216H	1.4 ± 0.05	120 ± 10	12	0.25 ± 0.03	69 ± 20	3.6	0.72 ± 0.07	210 ± 55	3.5
D301N	0.68 ± 0.07	140 ± 35	4.9	0.54 ± 0.08	260 ± 93	2.1	0.20 ± 0.01	150 ± 17	1.4

However, the dogma about the role of Asp301 in electrostatic interaction with substrates has several caveats. In general, the pharmacophore and experimental studies have contained some bias in that amines may have been overrepresented in these studies. Many P450 2D6 substrates are oxidized by *N*-dealkylation, e.g., deprenyl (*34*, *35*), and regardless of the chemical mechanism of *N*-dealkylation used (*36*) the distance between the nitrogen atom and site of oxidation must be <5 Å. A possible deprotonation/migration gating mechanism has been proposed to explain this phenomenon (*34*). Others have postulated that Phe481 is involved in the docking of amine substrates that are *N*-dealkylated (*28*), but site-directed mutagenesis experiments have not supported a role for this residue (*37*). Another issue with the site-directed mutagenesis work on Asp301 is that removal of the negative charge at this site has the effect of reducing the incorporation of heme into the protein and the level of holoprotein, as observed in both yeast (*31*) and bacterial (*38*) expression systems. Further, Asp301 might also play a role in acting with a (conserved) I-helix Thr in proton delivery, as in the well-studied cases of rat P450 1A2 Asp318 (*39*) and bacterial P450 101 Asp251 (*40*, *41*). Finally, we (*42*) and others (*43*−*45*) have recently reported P450 2D6 substrates devoid of basic nitrogen, including spirosulfonamide (K_s 1.6 μM, k_{cat} 6.5 min^{-1} (*42*)).

One of the other acidic residues of P450 2D6 that has been mentioned in homology modeling studies is Glu216, which can be modeled as being in substrate recognition sequence 2 and the F-helix (*21*, *22*, *27*, *28*, *30*). Although this residue has been alluded to, no reports have appeared, to our knowledge, regarding experimental examination of its role, and, in this work, we mutated Glu216 to several other residues and examined the effects.[3]

EXPERIMENTAL PROCEDURES

Chemicals. Unlabeled 4-methoxyphenethylamine·HCl was recrystallized twice from C_2H_5OH (*46*). 4-[*Methyl*-d_3]-methoxyphenethylamine·HCl was prepared (and recrystallized) as described previously (*46*). Quinidine, (−)-sparteine, 1-naphthylacetic acid, 2-(1-naphthoxy)acetic acid, and the (4-methoxyphenyl)- and (4-hydroxyphenyl)-substituted carboxylic acids were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. (*S*)-Propranolol was from Fluka (Buchs, Switzerland). Codeine and amitriptyline were purchased from Sigma Chemical Co. (St. Louis, MO). The following chemicals were gifts from the indicated sources: (±)-bufuralol and 1′-hydroxybufuralol (Roche, Nutley, NJ); MPTP (N. Castagnoli, Jr., Virginia Polytechnic and State University, Blacksburg, VA); spirosulfonamide (D. A. Nicoll-Griffith, Merck-Frosst, Kirkland, Quebec, Canada); debrisoquine, encainide, and 2-methoxyphenamine (G. R. Wilkinson, Vanderbilt University, Nashville, TN); 3-(1-naphthoxy)lactic acid (P. E. B. Reilly, University Queensland, Brisbane, Australia); and metoprolol (C. Bäärnhielm, Astra-Zeneca, Mohndal, Sweden).

P450 2D6 Constructs and Vectors. The original P450 2D6 construct DB6 (*47*) had previously been modified to change the original Met374 to Val (*9*, *48*, *49*) and to introduce a C-terminal $(His)_5$ sequence (*38*). The construction of the D301N mutant had been reported previously (*38*).

Site-directed mutagenesis was performed following a two-step overlap extension PCR mutagenesis procedure (*50*). The sequences of mutagenic primers (purchased from Sigma Genosys, The Woodlands, TX) spanning the Glu216 codon are listed in Supporting Information. In addition to alteration of the codon for Glu216 (underlined), all the oligos contained a silent mutation (G → T; indicated in bold) to create an *Afl*II restriction enzyme site to facilitate subsequent rapid identification of mutants.

Briefly, individual sense oligos (Supporting Information) were used in conjunction with a complementary oligo containing an *Xba*I site (TCTAGATTAATGGTGATGGT-GATGGCGGGG) centered on the 3′-end of the P450 2D6 cDNA. Likewise, the complementary oligos (Table 1) were used in conjunction with a 5′-primer containing an *Nde*I site (AAAACATATGGCTCTTGAAGCACTTGTACC) centered on the P450 2D6 start codon. Individual PCR reactions were conducted using *Pfu* polymerase in an Applied Biosystems model 9600 thermocycler (Applied Biosystems, Foster City, CA). The resulting ~850 and ~700 bp products were resolved on 1.0% (w/v) agarose gels and purified using a gel extraction kit (Qiagen, Valencia, CA). The final DNA concentration of the purified fragments was estimated following visualization of ethidium bromide-stained bands. Approximately equimolar amounts (10 pmol) of the paired, overlapping products were combined with the external primers to amplify the full-length products again using *Pfu* polymerase. The resulting full-length fragments (~1500 bp) were purified by gel extraction, digested with *Nde*I and *Xba*I, and ligated into the pCW plasmid that was previously digested with the same endonucleases. Initial verification of the incorporation of the desired mutations was accomplished following *Afl*II digests. Subsequent verification of all indi-

[3] Portions of this work were reported at the joint meeting (Gemeinsame Herbsttagung) of the Gesellschaft für Biochemie und Molekularbiologie e. V. and the Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie e. V. (Halle, Germany, 7−10 September 2002).

vidual mutants and the integrity of the nucleotide sequences were done using an Applied Biosystems Prism version 3.0 sequencing kit.

Expression and Purification of Enzymes. Expression of wild-type P450 2D6 and the site-directed mutants was done in *Escherichia coli* MV1304 cells, using 500 mL cultures in 2.8 L Fernbach flasks, with shaking for 48 h at 28 °C (150 rpm in a New Brunswick Innova shaker, New Brunswick, NJ) (*38*). Chloramphenicol (1.0 mg mL^{-1}), 5-aminolevulinic acid (0.5 mM), and isopropyl β-D-thiogalactoside (1.0 mM) were present during the enzyme induction mode. Membranes were prepared from the cells and solubilized using a general procedure, with 0.5% sodium cholate and 0.2% Emulgen 913 (w/v) for solubilization. Purification was done using immobilized Ni^{2+} columns as described elsewhere (*38*, *51*).

Yields of the P450 2D6 E216 mutants were (per 500 mL culture): E216A, 140 nmol; E216D, 130 nmol; E216H, 190 nmol; E216K, 140 nmol; E216L, 210 nmol; E216N, 190 nmol; and E216Q, 140 nmol. Wild-type P450 2D6 and the E216A, E216D, E216H, E216K, and E216Q mutants were selected for purification. All purified P450s were >95% homogeneous as judged by SDS−polyacrylamide gel electrophoresis (*52*); when loaded on the basis of P450 content, the samples yielded similar staining intensities for the protein bands (*53*). P450 concentrations were estimated from Fe^{2+}·CO vs Fe^{2+} difference spectra (*54*).

Recombinant rat NADPH−P450 reductase was produced in *E. coli* cells and purified as described elsewhere (*55*).

Ligand Binding Spectra. Spectra were recorded using the approaches described in detail elsewhere (*56*), using a Cary 14/OLIS instrument (On-Line Instrument Systems, Bogart, GA). All stock ligands were prepared as aqueous solutions, except for spirosulfonamide (CH_3CN). Type I binding data ($\Delta A_{390-420}$ vs ligand concentration) were fit to hyperbolic plots using the program GraphPad Prism (GraphPad, San Diego, CA) or, when ligand concentrations were close to the P450 concentration, to a quadratic expression to correct for bound ligand (*46*, *57*).

Assays of Catalytic Activity. Bufuralol oxidation was analyzed as described elsewhere (*58*) using 0.4 μM P450 (and 0.8 μM NADPH−P450 reductase, plus 45 μM di-12:0 GPC), a reaction time of 5 min (37 °C), and reversed phase HPLC (*58*, *59*) with both fluorescence ($F_{252/300}$) and UV (A_{254}) detection. The fluorescence data were used for calculations of production of 1′- and 6-hydroxybufuralol; the UV data were used to estimate 4-hydroxybufuralol (*60*). The level of $\Delta^{1',2'}$-dehydrobufuralol formed (*58*, *61*) was too low to quantify accurately.

4-Methoxyphenethylamine *O*-demethylation was assayed using HPLC as described in detail elsewhere (*46*, *56*, *62*). Substrate concentrations of ≤300 μM were used because of the inhibition observed at higher concentrations (*46*) and the potential for further hydroxylation to dopamine (*56*).

The *O*-demethylation of 4-methoxybenzylamine was analyzed in the same manner as for the 4-methoxyphenethylamine reaction, with HPLC/fluorescence ($F_{277/300}$). The fraction of CH_3CN in the HPLC solvent mixture was decreased from 2 to 0% (v/v) during the first 3 min of the program (and then increased to 20%, v/v, over the time period of 3 to 10 min).

Spirosulfonamide methylene (syn-) hydroxylation was analyzed as described previously (*42*).

The assays of *O*-demethylation of 4-anisic acid (4-methoxybenzoic acid) and 4-methoxyphenyl acetic and propionic acids were patterned on the assay of 4-methoxyphenethylamine *O*-demethylation, using a 6.2 × 80 mm Zorbax octadecylsilane column (3 μm, MacMod, Chadds Ford, PA) and a linear gradient of 2 to 45% CH_3CN (v/v) in 20 mM aqueous $KClO_4$ (pH 2.5) over 8 min, with a flow rate of 3.0 mL min^{-1} and detection of the UV signal at 254 nm. The potential products 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, and 4-hydroxyphenylpropionic acid eluted with t_R 4−5 min under these conditions, with the substrates following later (6−8 min).

Results obtained in steady-state kinetic assays were fit to hyperbolic plots using the program GraphPad Prism.

Homology Modeling. Molecular modeling was performed using the Insight II suite of programs (v. 2000.1, Homology, Biopolymer, and Discover 3 modules) from Accelrys Inc. (San Diego, CA) on a Silicon Graphics O2 workstation. Models were constructed based on the published crystal structure of P450 2C5 (*63*). The amino acid sequence of P450 2D6 (specifying Val at position 374) was aligned with the P450 2C5 sequence from residue 34 onward, because the N-terminal "anchor" peptide is missing from the P450 2C5 crystal structure. The alignment (Supporting Information) was based on a multiple sequence alignment of both P450 2D6 and P450 2C5 with a number of other mammalian P450s as well as bacterial forms for which crystal structures are available. Where gaps and deletions were introduced between P450 2D6 and P450 2C5, an attempt was made to conserve possible secondary structural elements found across multiple P450 structures; thus, gaps and deletions were concentrated in turns and loops between conserved elements. Coordinates were assigned for designated structurally conserved regions; then loops (including the F−G loop which is not specified in the P450 2C5 structure) were built using the loop generator function in Homology. The coordinates for the heme group were taken directly from the P450 2C5 structure. After all loops and gaps were spliced, the resulting structure was subjected to energy minimization using Discover_3 and the consistent valence force field. Hydrogen atoms were included in the model and a pH of 7.4 was specified. Force field parameters for the heme were specified separately, and the heme and the Cys443 residue were kept fixed throughout all the minimizations. Minimization was initially performed using the steepest descent method (1000 steps) followed by a series of minimization stages (1000 steps each), using the conjugate gradient method until a local energy minimum was obtained.

RESULTS

Expression of P450 Glu216 Mutants. An issue in previous work with Asp301 mutants of P450 2D6 was the attenuated heme incorporation in neutral mutants and the total lack of heme in the mutants in which the residue had been changed to a basic residue (*38*). All of the Glu216 mutants could be expressed with levels of holoprotein (i.e., containing heme) similar to the wild-type enzyme (see Experimental Procedures). The purified proteins used in this work all contained ≤10% cytochrome P420 relative to P450 (with the exception of E216K, ∼30%), further indicating a generally preserved structure. Further, no noticeable differences were observed

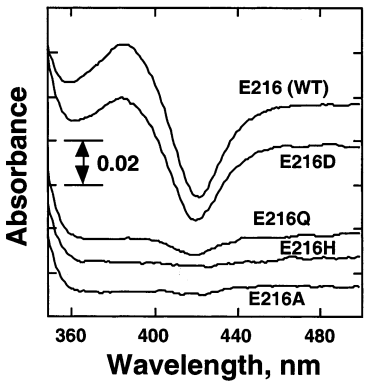


FIGURE 1: Binding of quinidine to P450 2D6 and Glu216 mutants. A spectrum of each P450 2D6 (either Glu (wild type), Asp, Gln, His, or Ala) was recorded with 2 μM P450 in 0.10 M potassium phosphate buffer (pH 7.0) containing 45 μM di-12:0 GPC. Quinidine was added to 20 μM and another spectrum was recorded. The first spectra were subtracted from the second using the OLIS software to yield the difference spectra shown here.

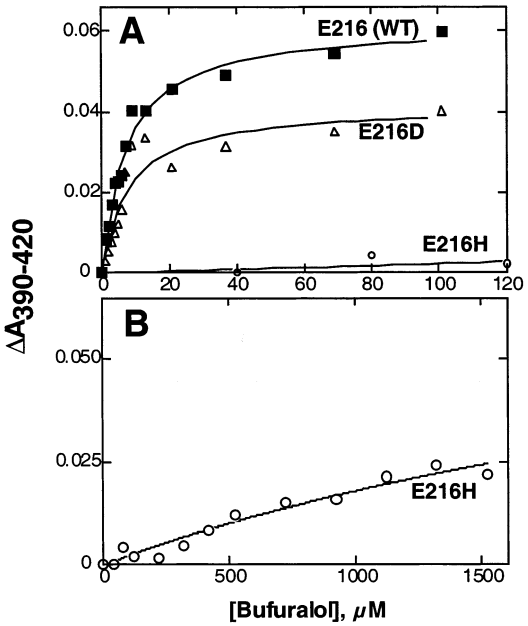


FIGURE 2: Concentration dependence of spectral changes observed in titration of P450 2D6 and mutants with bufuralol. See Supporting Information for some spectral traces. (A) Results are shown for wild-type P450 2D6 (Glu216, ■), P450 E216D (△), and P450 2D6 E216H (○). (B) Expanded graph of P450 2D6 E216H binding, from panel A. Data points were fit to a quadratic expression (for wild type and E216D) (*46*, *57*) and to a hyperbolic equation for E216H and yielded K_s = 7.3, 7.8, and 3420 μM for wild-type P450 2D6 and the E216D and E216H mutants, respectively.

in the ratio of P450 heme to apoprotein (detected in electrophoresis).

A preparation of wild-type P450 2D6 was made at the same time as the mutants and used directly in comparisons, to preclude any differences not due to the mutations.

Binding of Quinidine and Bufuralol. Wild-type P450 2D6 and all of the Glu216 mutants were found to be in the low-spin (ferric) iron form, as isolated. Addition of ligands often leads to displacement of the distal H_2O ligand, causing a partial shift to high-spin iron (Type I shift) (*64*). Preliminary spectral binding interactions were examined with quinidine, a competitive inhibitor (K_i < 0.1 μM with wild-type P450 2D6) (*10*) that is not oxidized by P450 2D6 (*46*, *65*). Quinidine produced the expected Type I difference spectra with wild-type P450 2D6 and the E216D mutant (Figure 1), but a change to a neutral or basic residue abolished the response.

The Type I binding spectra seen with bufuralol (*42*) was also attenuated in the neutral mutants. At higher concentrations of bufuralol, weak Type I difference spectra could be observed (see Supporting Information). Analysis of the data yielded a K_s of ~3.4 mM for the E216H mutant, compared to the values of 7.3 and 7.8 μM calculated for wild type P450 2D6 and the E216D mutant, respectively (Figure 2).

Bufuralol Oxidation Activity. Preliminary HPLC analysis indicated that production of all four oxidation products of bufuralol was compromised in the E216Q mutant (Supporting Information).[4] More extensive analysis indicated the attenuation of the three major oxidation activities of wild-type P450 2D6 by mutation of Glu216 (Table 1).[5] The activity of the E216D mutant was only compromised by ~1/2. All of the changes of Glu216 to nonacidic residues lowered most of the activities to ~3%, and the mutation D301N had a similar effect. These changes appeared to affect both k_{cat} and K_m.

4-Methoxyphenethylamine O-*Demethylation Activity*. This catalytic activity has one of the highest rates observed with P450 2D6 and has a number of advantages as an assay (*46*, *56*). As with bufuralol, this substrate contains a positively charged amine.

The substitution E216Q reduced the enzyme efficiency (k_{cat}/K_m) from 280 to 0.44 min^{-1} mM^{-1} (Figure 3), similar to the effect of the D301N mutation (0.27 min^{-1} mM^{-1}). (The high K_m values of the mutants make discernment of the k_{cat} and K_m parameters difficult.)

Wild-type P450 2D6 had been demonstrated to show a noncompetitive intermolecular deuterium isotope effect of 3.1−3.8 on both k_{cat} and k_{cat}/K_m (*46*). Even with the very low catalytic activity of the E216Q mutant, a similar kinetic deuterium isotope effect of 3.2 (on k_{cat}/K_m) could be demonstrated for this reaction (Figure 3) (the reaction of wild-type P450 2D6 had been determined to show an *intra*molecular isotope effect of 9.6, approximating the intrinsic isotope effect (*46*)).

Spirosulfonamide Hydroxylation Activity. Spirosulfonamide, a candidate from a cyclooxygenase-2 inhibitor pharmaceutical program, is of significance in that this P450 2D6 substrate (K_s 1.6 μM, K_m 6 μM) (*42*) is devoid of basic nitrogen. Other analogues devoid of basic nitrogen are also substrates for P450 2D6 (*42*). The mutation D301N did not alter the K_s or K_m of P450 2D6 but did reduce the k_{cat} by ~10-fold (*42*).

[4] Previously, a higher bufuralol 1′-hydroxylation rate (21 min^{-1}) had been reported (*38*, *42*). We found that a sample of the product standard used in that work (Research Biochemicals Int., Natick, MA) contained an incorrect stated concentration, as judged by UV analysis. The rate reported here is considered more accurate.

[5] The contributions of the individual enantiomers of (±)-bufuralol to the individual products have not been ascertained. P450 2D6 is known to convert both enantiomers to 1′-hydroxybufuralol (*12*). The (*S*)(−) enantiomer has been suggested to be the source of 4- and 6-hydroxybufuralol on the basis of studies with liver microsomes (*66*), although the contribution of human P450 1A2 to these bufuralol hydroxylations (*60*) makes the case more complex.

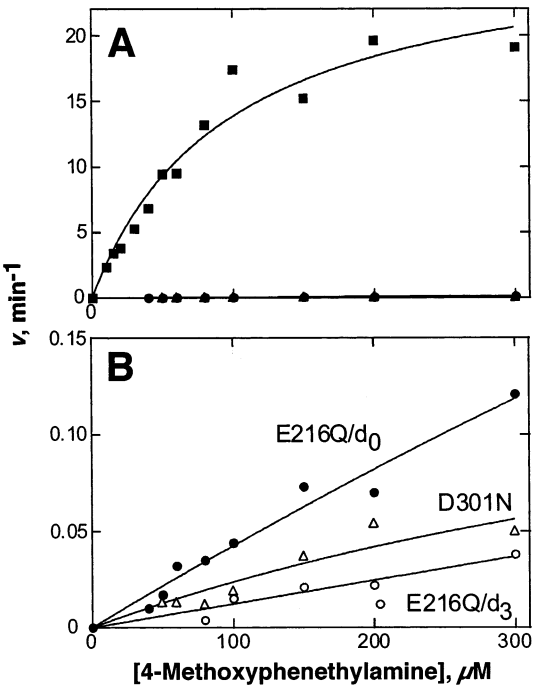
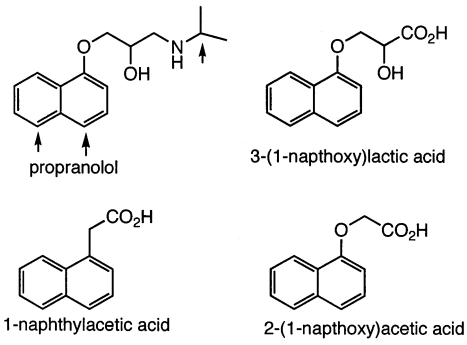


FIGURE 3: 4-Methoxyphenethylamine *O*-demethylation activity of P450 2D6 and mutants as a function of substrate concentration. Assays were done with 0.4 μM P450 for 10 min and 50−200 μL aliquots were analyzed by HPLC (fluorescence detection) after deproteinization. Results are shown (expanded scale in panel B) for wild-type P450 2D6 (■), P450 D301N (△), and P450 2D6 E216Q (●) using unlabeled 4-methoxyphenethylamine as substrate and also for P450 2D6 E216Q using 4-[*methyl*-d_3]methoxyphenethylamine as substrate (○). All fits were made to hyperbolic plots, with k_{cat} = 27 (± 3) min^{-1} and K_m = 97 (± 20) μM for wild-type P450 2D6 (k_{cat}/K_m = 280 min^{-1} mM^{-1}). The data points shown in panel B did not produce reliable fits with hyperbolic plotting; the respective k_{cat}/K_m estimates for D301N and E216Q (unlabeled substrate) and E216Q (trideuterated substrate) were 0.44, 0.27, and 0.14 min^{-1} mM^{-1}.

The substitution E216Q lowered the k_{cat} for spirosulfonamide methylene *syn*-hydroxylation from 4.1 (± 0.3) min^{-1} to 1.2 (± 0.1) min^{-1}; the respective K_m values were 16 (± 3) and 13 (± 3) μM for the wild-type enzyme and the E216Q mutant, respectively, in a direct comparison. Thus, the enzyme efficiency was reduced from 250 to 90 min^{-1} mM^{-1} by the neutral substitution. This decrease (60%) is significant but considerably less than the 10-fold change observed with the D301N substitution (*42*).

Effects of Mutation at Glu216 on Binding of Other Basic Amine Substrates. The results suggested that mutations at Glu216 (other than to Asp) compromised the ability of P450 2D6 to bind quinidine, bufuralol, and 4-methoxyphenethylamine but not necessarily spirosulfonamide. To more fully survey the role of Glu216 in binding ligands, we examined the effect of one of the substitutions of Glu216 on the binding of an additional nine P450 2D6 amine substrates, using (single) concentrations reported to yield optimal catalytic activity in the literature and comparing wild-type P450 2D6 as a control (Figure 4). The substrate spirosulfonamide (*42*), devoid of basic nitrogen, yielded a relatively strong (Type I) interaction relative to the amines and this binding was not decreased by the substitution at Glu216 (Figure 4A). The binding of most of these amines was markedly attenuated by the substitution E216H (Figure 4B−H, i.e., metoprolol, propranolol, debrisoquine, MPTP, encainide, sparteine, and amitriptyline). Less effect was seen on the binding of codeine and 2-methoxyphenamine (Figure 4I and J) under these conditions (with codeine and 2-methoxyphenamine the spectra were much weaker than most of the other ligands).

Scheme 1: Propranolol (with Sites of Oxidation by P450 2D6 Labeled) and Three Naphthyl Analogues Examined as Putative Ligands for P450 2D6 E216 K



Attempts to Alter Substrate Preferences of P450 2D6 by Mutation. Thus far in this report we have examined the effects of substitutions of P450 2D6 amino acids on catalytic activities toward known substrates. If electrostatic interaction of a ligand with an amino acid is the dominant factor in binding, then we might expect to be able to modify this or reverse it in a predictable way. Two attempts were made, one with Asp301 and one with Glu216.

One might expect the same enzyme complementarity between P450 2D6 D301E and 4-methoxybenzylamine as between P450 2D6 and 4-methoxyphenethylamine. Neither enzyme (wild type nor D301E) had particularly good catalytic activity toward 4-methoxybenzylamine (see Supporting Information). Wild-type P450 2D6 showed Type I difference spectra (Figure 1) in binding 4-methoxyphenethylamine (*56*) but did not show such spectral changes with 4- (or 3-) methoxybenzylamine.

The second case was an attempt at charge reversal. The E216K protein showed little binding of quinidine (added at 20 μM), similar to the E216Q, E216A, and E216H mutants (Figure 1) (data not presented). Six potential carboxylic acid ligands were added to wild-type P450 2D6 and to the E216K mutant: 4-methoxybenzoic acid (anisic acid), 4-methoxyphenylacetic acid, and 4-methoxyphenylpropionic acid (analogues of 4-methoxyphenethylamine (*46*, *56*)) and 1-naphthylacetic acid, 2-(1-naphthoxy)acetic acid, and 3-(1-naphthoxy)lactic acid, chosen for their potential similarity to the P450 2D6 substrate propranolol (*12*, *33*) (Scheme 1). None of these carboxylic acids produced a detectable Type I binding spectrum when added at concentrations of 10, 40, or 70 μM (data not shown). P450 2D6 E216K was incubated with NADPH−P450 reductase, di-12:0 GPC, and 4-methoxybenzoic acid, 4-methoxyphenylacetic acid, or 4-methoxyphenylpropionic acid, plus an NADPH-generating system. No products were detected, and the limit of detection of the *O*-demethylated products with 50−500 μM substrate concentrations corresponds to a k_{cat}/K_m value of <0.05 min^{-1} mM^{-1} (cf. 280 min^{-1} mM^{-1} for the *O*-demethylation of 4-methoxyphenethylamine by wild-type P450 2D6, Table 1).

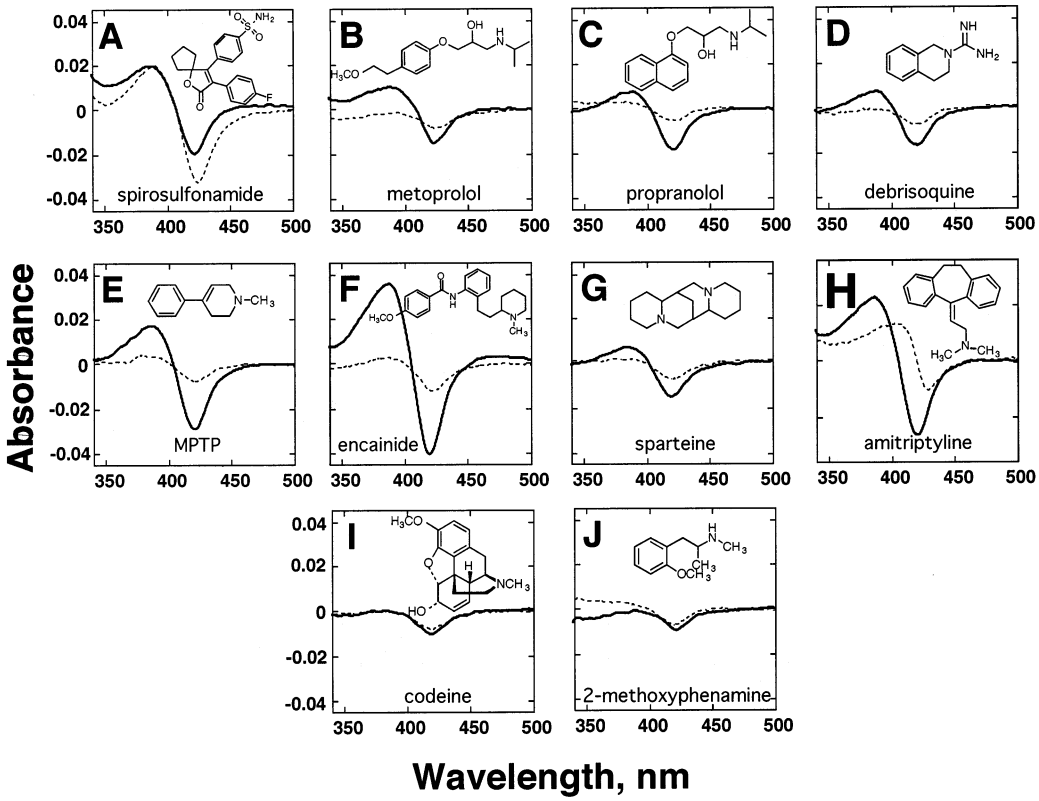


FIGURE 4: Survey of effect of substitution of Glu216 on binding of spirosulfonamide and known ligands with basic nitrogen to P450 2D6. Spectra were recorded as in Figure 1 with 1.5 μM P450 (P450 2D6 wild type = WT (—) or E216H (- - -)) in 0.10 mM potassium phosphate buffer (pH 7.4) containing the indicated ligands at the following concentrations (with references cited to previous literature). In each case, the initial spectrum was subtracted electronically from the spectrum recorded in the presence of ligand. Structures are shown for the ligands. (A) Spirosulfonamide, 20 μM (*42*); (B) metoprolol, 50 μM (*58*); (C) (*S*)-propranolol, 50 μM (*33*); (D) debrisoquine, 100 μM (*12*); (E) MPTP, 1.0 mM (*58*); (F) encainide, 200 μM (*12*); (G) (−)-sparteine, 1.0 mM (*67*); (H) amitriptyline, 500 μM (*68*); (I) codeine, 175 μM (*24*); and (J) 2-methoxyphenamine, 200 μM (*69*, *70*).

DISCUSSION

P450 2D6 has attracted considerable interest because of its role in the oxidative metabolism of a large fraction of the drugs used today and its extensive genetic polymorphism (*6*, *7*, *9*). Because of the potential for undesirable drug effects (*8*), efforts have been made to predict both inhibitors (*14*) and substrates (*13*, *15*−*29*). The docking of ligands to P450 2D6 has also been a matter of basic interest in the P450 field because this enzyme has often been considered to be one of the best understood of the microsomal P450s in terms of its interactions with small molecules. Almost all recent descriptions of P450 2D6−ligand interactions are based upon the role of electrostatic interactions between Asp301 and basic nitrogen atoms.[2] The results presented in this paper argue that Glu216 has at least as great a role in the binding of basic substrates.

The literature contains far more reports on P450 2D6 modeling than of actual site-directed mutagenesis. A list of P450 2D6 residues postulated to form the active site includes at least Asp100, Trp316, Pro371 (*18*), Pro103, Ile106, Thr107, Leu110, Pro114, Ser116, Ala122, Asp301, Ser304, Ala305, Thr309, Val370, Gly373, Val374, and Phe483 (*24*, *71*), Glu216 (*21*, *22*, *27*, *28*, *30*), and Gln117, Leu121, Leu213, Phe219, and Phe481 (*27*). Only six residues have been examined by site-directed mutagenesis to date. Changing Asp301 to any residue except Glu lowers catalytic activity >10-fold (*31*, *38*). Mutations changing Asp100 and Ser304 have been reported to have little effect, if any (*31*, *72*). Mutation of Phe483 to Ile produced some alteration of the pattern of testosterone metabolism by P450 2D6 (*71*). Changing Phe481 has been reported to lower k_{cat}/K_m 10-fold for some substrates but to produce no effect with others (*37*). Finally, P450 2D6 with Met at position 374 rather than Val (a Met 374-containing cDNA being the first P450 2D6 sequence characterized (*9*, *48*, *49*, *73*)) showed altered regioselectivity toward metoprolol.

The interpretation of the effects of the substitution at Asp301 requires some caveats, which have already been mentioned (*38*). Asp301 is eight residues away from the nearest Thr (>2 helix turns), and therefore its carboxylic acid may not be participating in O−O bond scission (*40*, *41*). However, Asp301 could be involved in a hydrogen bonding network with water molecules, such as that reported for substrate-bound P450 107A1 (*74*). Modi et al. (*26*, *71*) reported that binding of MPTP did not require Asp301 and invoked alternate residues. Residues other than Asp301 (e.g., Phe481) had also been proposed for binding of amine substrates that are *N*-dealkylated (*28*), although little evidence implicating any has been obtained. Recently, our group clearly showed that Asp301 was not needed to bind the spirosulfonamide, a substrate devoid of basic nitrogen, but that Asp301 was somehow needed otherwise for catalysis (*42*) and effective expression of holoenzyme (*38*).[6]

Our present results clearly show a role of an acidic residue at position 216 in binding of most basic substrates, as indicated by the spectral data (Figures 1, 2, and 4). Although

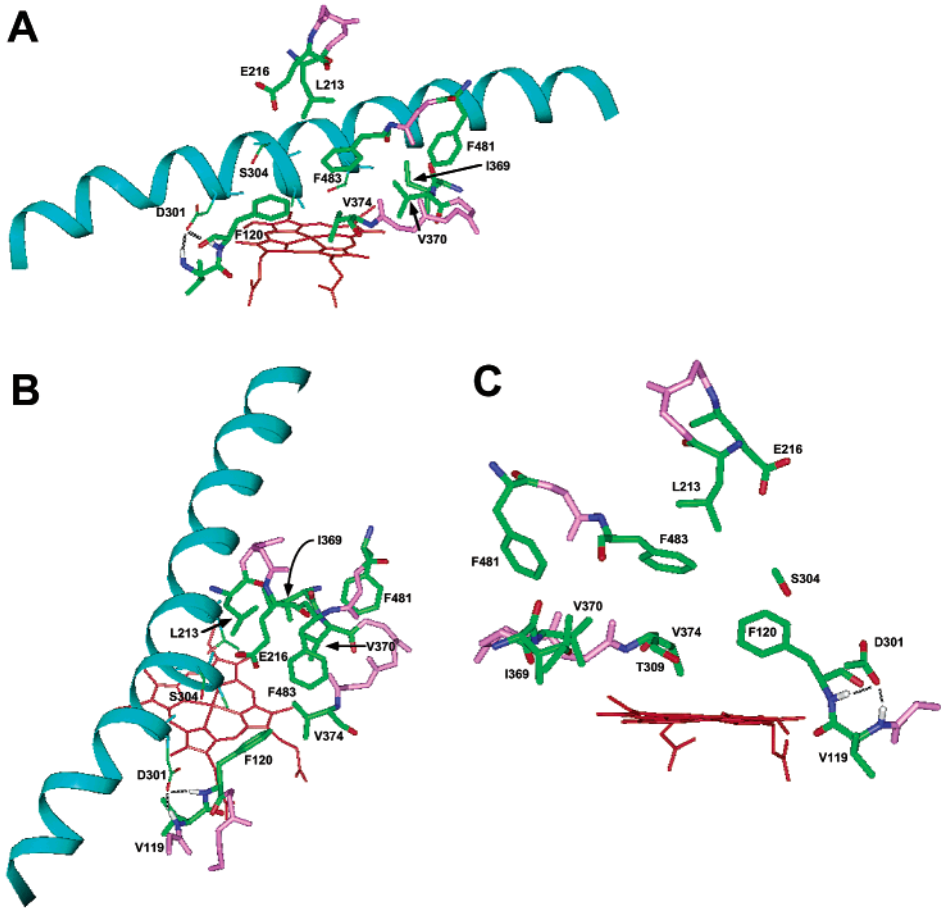


FIGURE 5: Model of P450 2D6 active site. Views of the substrate binding cavity in the P450 2D6 homology model are shown with only the side chains of the relevant residues, plus the heme (red), I-helix backbone (blue-green ribbon) and other areas of peptide backbone (purple) linking residues proposed to line the substrate binding site shown). For clarity, residues are labeled using the single letter code, and the heme is represented as thin sticks, as are side chains of I-helix residues where the I helix is shown (views A and B). Hydrogens are not shown except for the amide hydrogens of residues 119 and 120 hypothesized to hydrogen bond to the carboxylate oxygen atoms of Asp301 (possible hydrogen bonds are indicated as gray lines). (A) View from the side opposite the I-helix: the I-helix runs along the back of the view and Val374 is at the front of the view. (B) View looking down into the substrate binding site from above residue Phe120. (C) Side view of the active site from the perspective of the I-helix: I-helix residues have been cut away except the side chains of residues 301, 304, 309, and 313 shown at the front of the view.

the precise meaning of the K_m parameter is unknown in P450 2D6 reactions (*46*), it should be noted that this parameter was also greatly increased by the substitution of Glu216 with neutral residues (Figure 3, Table 1) (k_{cat} was also decreased; see ref *46* for discussion of what steps may control k_{cat}). The Glu216 mutants appeared to contain the same complement of heme as wild-type P450 2D6 (and little cytochrome P420), in contrast to the Asp301 mutants; moreover, no effect on the K_m was seen for the substrate spirosulfonamide (*42*) and the k_{cat} for oxidation to the *syn*-methylene alcohol was 40% of that seen with wild-type P450 2D6.

Examination of the homology model developed for P450 2D6 in the present study (Figure 5) shows that Glu216 is positioned at the top of the proposed substrate binding cavity, with its side chain pointing down toward the heme, consistent with a role in substrate binding. By contrast, the side chain of Asp301 is directed away from the heme and oriented in such a way as to form possible hydrogen bonds to the amide nitrogens of residues 119 and 120. The side chain of Asp301 is directed away from the active site and toward the amide protons of residues 119 and 120, such that hydrogen bonds of 1.74 and 1.96 Å could be proposed between one of the carboxylate oxygens of Asp301 and these amide protons (Figure 5). This proposal has also been raised recently by Kirtonet al. (*30*), who speculate that Asp301 may serve to stabilize the B−C loop in P450 2D6 and possibly other P450s that contain an acidic residue at this position. This structural role is consistent with mutagenesis studies showing compromised holoprotein stability upon mutation of Asp301 (*38*). In this putative role, Asp301 may also influence the orientation of Phe120, the side chain of which appears to be oriented into the active site in such a way that it may contribute to substrate binding. Thus, an alternative mechanism by which mutation of Asp301 may influence substrate specificity can be postulated, namely, reorientation of Phe120.

We summarize our views on the residues involved in the binding of P450 2D6 substrates, based on experimental

[6] During the preparation of this manuscript, an electronic preprint of a report by Kirton et al. (*30*) appeared suggesting a role for Glu216 in P450 2D6-ligand binding on the basis of homology modeling and principal component analysis. Kirton et al. (*30*) also pointed out that many P450s have an acidic residue at the position corresponding to P450 2D6 301 in their sequence alignments and do not preferentially bind amines; few P450s have an acidic residue corresponding to P450 2D6 Glu216, but these P450s do use amine substrates.

evidence. We reiterate our previous view that Asp301 has at least part of its role in the folding and structure of P450 2D6. The negative charges at the acidic residues at *both* positions 301 and 216 may both contribute in binding amines. For instance, replacing either residue 301 (*31*, *42*) or 216 (Figure 1) nearly abolishes quinidine binding, and the rest of this paper emphasizes the contribution of Glu216. However, other residues must also be involved in the binding of substrates: (i) P450 2D6 (both wild type and Asp301 mutants) binds spirosulfonamide and other substrates that are devoid of basic nitrogen, and (ii) in the present study we were unable to make simple changes at positions 216 and 301 to achieve results that would be predicted if only simple electrostatic interactions were involved.[7]

Our homology model (Figure 5) shows Asp301 with its oxygen atoms 10.7 and 10.8 Å from the ferric iron atom. Glu216 has the closer of its two oxygens 14.3 Å from the iron, but side chain movement may bring the carboxylate oxygen atoms somewhat closer than the default minimized model geometry shown in Figure 5. These distances might seem to argue against a role of Glu216 and in favor of one for Asp301, but the point should be made that the D301E (*31*, *38*) and E216D mutants (Figure 1, Table 1) retain much of their binding capability and catalytic activity. Further, models must all be considered imperfect. Even reengineering bacterial P450 101 ($P450_{cam}$) to utilize molecules other than camphor is not trivial (*75*). Finally, a major limitation of all models is the limited information about movement of key atoms in the course of the catalytic cycle (*76*), and the rate of catalysis is probably most dependent upon the tightness of substrate binding in the transition state (*77*). With these caveats presented, our model (Figure 5) suggests that the residues Phe120, Leu213, Phe483, and Val374 are most likely to interact with ligands, a proposal that is consistent with preliminary modeling of various substrates into the substrate binding site. Of these, only Phe483 and Val 374 have been examined and shown to have some effect on regioselectivity of testosterone and metoprolol metabolism, respectively (*49*, *71*). In contrast, Phe481 is more remote, consistent with site-directed mutagenesis experiments that have not supported a role for Phe481 in substrate binding (*37*).

We conclude that Glu216 has a substantial role in binding of basic substrates to P450 2D6. This interaction seems to be electrostatic, as judged by the effects of mutations to residues other than Asp (Figure 1, Table 1). The negative charge at Asp301 may also contribute to binding basic ligands, although the role of this residue is probably not only electrostatic (*38*, *42*) and may instead be indirect via an effect on Phe120. Other residues must also contribute to substrate binding, as shown by studies with uncharged substrates (*42*).

[7] In regard to this latter point about the lack of success in re-engineering P450 2D6 binding specificity, we did not attempt to mutate *both* Asp301 and Glu216 to basic residues, which might in principle be required to achieve binding of carboxylic acid substrates. Our experience with replacement of Asp301 with neutral and basic residues suggests that such mutants would probably not incorporate heme (*38*). Another point to make is that our attempts to find anionic ligands for P450 2D6 E216K (with Asp301 intact) are by no means exhaustive, and acidic ligands other than those considered in Scheme 1 might exist.

ACKNOWLEDGMENT

We thank G. P. Miller for helpful discussions and comments on the manuscript, W. A. McCormick for assistance in expression and purification of some of the P450 2D6 Glu216 mutants, and K. Trisler for assistance in preparation of the manuscript.

NOTE ADDED IN PROOF

During the review of this article, a separate paper appeared (*78*) in which the effect of mutating Glu216 was examined. The approaches and results differ in that P450 2D6 was not purified, ligand binding was not examined directly, the k_{cat} for bufuralol 1′-hydroxylation was an order of magnitude lower for wild-type P450 2D6 than reported here, and the k_{cat} was not reduced further by substitution of Glu216. However, the findings qualitatively support our own about the involvement of Glu216 in catalysis by P450 2D6.

SUPPORTING INFORMATION AVAILABLE

Sequences of oligonucleotides used for mutagenesis, alignment of P450 2C5 and P450 2D6 sequences used to generate the homology model developed in this work, difference spectra of binding bufuralol to P450 2D6 E216H, comparison of HPLC of bufuralol products of wild-type P450 2D6 and P450 2D6 E216Q, plots of v versus S for bufuralol hydroxylation by wild-type P450 2D6 and P450 2D6 E216Q, and plots of v versus S for *O*-demethylation of 4-methoxyphenethylamine and 4-methoxybenzylamine by wild-type P450 2D6 and P450 2D6 E216Q. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Palmer, G., and Reedijk, J. (1992) *J. Biol. Chem. 267*, 665−677.
2. Katagiri, M., Ganguli, B. N., and Gunsalus, I. C. (1968) *J. Biol. Chem. 243*, 3543−3546.
3. Shafiee, A., and Hutchinson, C. R. (1988) *J. Bacteriol. 170*, 1548−1553.
4. Keeney, D. S., and Waterman, M. R. (1993) *Pharmacol. Ther. 58*, 301−317.
5. Ortiz de Montellano, P. R. (1995) *Cytochrome P450*: *Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) 2nd ed., Plenum Publishing Corp., New York.
6. Guengerich, F. P. (1995) in *Cytochrome P450*: *Structure, Mechanism, and Biochemistry*, (Ortiz de Montellano, P. R., Ed.) 2nd ed., pp 473−535, Plenum Publishing Corp., New York.
7. Evans, W. E., and Relling, M. V. (1999) *Science 286*, 487−491.
8. Mahgoub, A., Idle, J. R., Dring, L. G., Lancaster, R., and Smith, R. L. (1977) *Lancet ii*, 584−586.
9. Daly, A. K., Brockmöller, J., Broly, F., Eichelbaum, M., Evans, W. E., Gonzalez, F. J., Huang, J. D., Idle, J. R., Ingelman-Sundberg, M., Ishizaki, T., Jacqz-Algrain, E., Meyer, U. A., Steen, V. M., Wolf, C. R., and Zanger, U. M. (1996) *Pharmacogenetics 6*, 193−201.
10. Otton, S. V., Inaba, T., and Kalow, W. (1984) *Life Sci. 34*, 73−80.
11. Al-Dabbagh, S. G., Idle, J. R., and Smith, R. L. (1981) *J. Pharm. Pharmacol. 33*, 161−164.
12. Distlerath, L. M., Reilly, P. E. B., Martin, M. V., Davis, G. G., Wilkinson, G. R., and Guengerich, F. P. (1985) *J. Biol. Chem. 260*, 9057−9067.
13. Wolff, T., Distlerath, L. M., Worthington, M. T., Groopman, J. D., Hammons, G. J., Kadlubar, F. F., Prough, R. A., Martin, M. V., and Guengerich, F. P. (1985) *Cancer Res. 45*, 2116−2122.
14. Strobl, G. R., von Kruedener, S., Stöckigt, J., Guengerich, F. P., and Wolff, T. (1993) *J. Med. Chem. 36*, 1136−1145.
15. Meyer, U. A., Gut, J., Kronbach, T., Skoda, C., Meier, U. T., and Catin, T. (1986) *Xenobiotica 16*, 449−464.
16. Islam, S. A., Wolf, C. R., Lennard, M. S., and Sternberg, M. J. E. (1991) *Carcinogenesis 12*, 2211−2219.

17. Koymans, L., Vermeulen, N. P. E., van Acker, S. A. B. E., te Koppele, J. M., Heykants, J. J. P., Lavrijsen, K., Meuldermans, W., and Donné-Op den Kelder, G. M. (1992) *Chem. Res. Toxicol. 5*, 211−219.
18. Koymans, L., Vermeulen, N. P. E., Baarslag, A., and Donne-Op den Kelder, G. (1993) *J. Comput.-Aided Mol. Des. 7*, 281−289.
19. de Groot, M. J., Bijloo, G. J., Hansen, K. T., and Vermeulen, N. P. E. (1995) *Drug Metab. Dispos. 23*, 667−669.
20. de Groot, M. J., Vermeulen, N. P. E., Kramer, J. D., van Acker, F. A. A., and Donné-Op den Kelder, G. M. (1996) *Chem. Res. Toxicol. 9*, 1079−1091.
21. Wiseman, H., and Lewis, D. F. (1996) *Carcinogenesis 17*, 1357−1360.
22. Lewis, D. F. V., Eddershaw, P. J., Goldfarb, P. S., and Tarbit, M. H. (1997) *Xenobiotica 27*, 319−340.
23. de Groot, M. J., Bijloo, G. J., Martens, B. J., van Acker, F. A. A., and Vermeulen, N. P. E. (1997) *Chem. Res. Toxicol. 10*, 41−48.
24. Modi, S., Paine, M. J., Sutcliffe, M. J., Lian, L. Y., Primrose, W. U., Wolf, C. R., and Roberts, G. C. K. (1996) *Biochemistry 35*, 4540−4550.
25. Ekins, S., de Groot, M. J., and Jones, J. P. (2001) *Drug Metab. Dispos. 29*, 936−944.
26. Modi, S., Gilham, D. E., Sutcliffe, M. J., Lian, L.-Y., Primrose, W. U., Wolf, C. R., and Roberts, G. C. K. (1997) *Biochemistry 36*, 4461−4470.
27. de Groot, M. J., Ackland, M. J., Horne, V. A., Alex, A. A., and Jones, B. C. (1999) *J. Med. Chem. 42*, 4062−4070.
28. de Groot, M. J., Ackland, M. J., Horne, V. A., Alex, A. A., and Jones, B. C. (1999) *J. Med. Chem. 42*, 1515−1524.
29. Lightfoot, T., Ellis, S. W., Mahling, J., Ackland, M. J., Blaney, F. E., Bijloo, G. J., de Groot, M. J., Vermeulen, N. P. E., Blackburn, G. M., Lennard, M. S., and Tucker, G. T. (2000) *Xenobiotica 30*, 219−233.
30. Kirton, S. B., Kemp, C. A., Tomkinson, N. P., St.-Gallay, S., and Sutcliffe, M. J. (2002) *Proteins 49*, 216−231.
31. Ellis, S. W., Hayhurst, G. P., Smith, G., Lightfoot, T., Wong, M. M. S., Simula, A. P., Ackland, M. J., Sternberg, M. J. E., Lennard, M. S., Tucker, G. T., and Wolf, C. R. (1995) *J. Biol. Chem. 270*, 29055−29058.
32. Mackman, R., Tschirret-Guth, R. A., Smith, G., Hayhurst, G. P., Ellis, S. W., Lennard, M. S., Tucker, G. T., Wolf, C. R., and Ortiz de Montellano, P. R. (1996) *Arch. Biochem. Biophys. 331*, 134−140.
33. Upthagrove, A. L., and Nelson, W. L. (2001) *Drug Metab. Dispos. 29*, 1377−1388.
34. Grace, J. M., Kinter, M. T., and Macdonald, T. L. (1994) *Chem. Res. Toxicol. 7*, 286−290.
35. Coutts, R. T., Su, P., and Baker, G. B. (1994) *J. Pharmacol. Toxicol. Methods 31*, 177−186.
36. Guengerich, F. P., Yun, C.-H., and Macdonald, T. L. (1996) *J. Biol. Chem. 271*, 27321−27329.
37. Hayhurst, G. P., Harlow, J., Chowdry, J., Gross, E., Hilton, E., Lennard, M. S., Tucker, G. T., and Ellis, S. W. (2001) *Biochem. J. 355*, 373−379.
38. Hanna, I. H., Kim, M.-S., and Guengerich, F. P. (2001) *Arch. Biochem. Biophys. 393*, 255−261.
39. Ishigooka, M., Shimizu, T., Hiroya, K., and Hatano, M. (1992) *Biochemistry 31*, 1528−1531.
40. Gerber, N. C., and Sligar, S. G. (1992) *J. Am. Chem. Soc. 114*, 8742−8743.
41. Gerber, N. C., and Sligar, S. G. (1994) *J. Biol. Chem. 269*, 4260−4266.
42. Guengerich, F. P., Miller, G. P., Hanna, I. H., Martin, M. V., Léger, S., Black, C., Chauret, N., Silva, J. M., Trimble, L., Jergey, J. A., and Nicoll-Griffith, D. A. (2002) *Biochemistry 41*, 11025−11034.
43. Niwa, T., Sato, R., Yabusaki, Y., Ishibashi, F., and Katagiri, M. (1999) *Xenobiotica 29*, 187−193.
44. Hiroi, T., Kishimoto, W., Yoshimura, K., Chow, T., Imaoka, S., and Funae, Y. (2000) *Front. Sci. Ser. 29*, 417−418.
45. Hiroi, T., Kishimoto, W., Chow, T., Imaoka, S., Igarashi, T., and Funae, Y. (2001) *Endocrinology 142*, 3901−3908.
46. Guengerich, F. P., Miller, G. P., Hanna, I. H., Sato, H., and Martin, M. V. (2002) *J. Biol. Chem. 277*, 33711−33719.
47. Gillam, E. M. J., Guo, Z., Martin, M. V., Jenkins, C. M., and Guengerich, F. P. (1995) *Arch. Biochem. Biophys. 319*, 540−550.
48. Gonzalez, F. J., Skoda, R. C., Kimura, S., Umeno, M., Zanger, U. M., Nebert, D. W., Gelboin, H. V., Hardwick, J. P., and Meyer, U. A. (1988) *Nature 331*, 442−446.
49. Ellis, S. W., Rowland, K., Ackland, M. J., Rekka, E., Simula, A. P., Lennard, M. S., Wolf, C. R., and Tucker, G. T. (1996) *Biochem. J. 316*, 647−654.
50. Higuchi, R. (1990) in *PCR Protocols*: *A Guide to Methods and Applications* (Innis, M. A., Gelfland, D. H., Sninsky, J. J., and White, T. J., Eds.), pp 177−183, Academic Press, San Diego.
51. Hanna, I. H., Reed, J. R., Guengerich, F. P., and Hollenberg, P. F. (2000) *Arch. Biochem. Biophys. 376*, 206−216.
52. Laemmli, U. K. (1970) *Nature 227*, 680−685.
53. Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) *Anal. Biochem. 118*, 197−203.
54. Omura, T., and Sato, R. (1964) *J. Biol. Chem. 239*, 2370−2378.
55. Hanna, I. H., Teiber, J. F., Kokones, K. L., and Hollenberg, P. F. (1998) *Arch. Biochem. Biophys. 350*, 324−332.
56. Miller, G. P., Hanna, I. H., Nishimura, Y., and Guengerich, F. P. (2001) *Biochemistry 40*, 14215−14223.
57. Lowe, L. G., and Guengerich, F. P. (1996) *Biochemistry 35*, 9840−9849.
58. Hanna, I. H., Krauser, J. A., Cai, H., Kim, M.-S., and Guengerich, F. P. (2001) *J. Biol. Chem. 276*, 39553−39561.
59. Kronbach, T., Mathys, D., Gut, J., Catin, T., and Meyer, U. A. (1987) *Anal. Biochem. 162*, 24−32.
60. Yamazaki, H., Guo, Z., Persmark, M., Mimura, M., Gonzalez, F. J., Sugahara, C., Guengerich, F. P., and Shimada, T. (1994) *Mol. Pharmacol. 46*, 568−577.
61. Hiroi, T., Chow, T., Imaoka, S., and Funae, Y. (2002) *Drug Metab. Dispos. 30*, 970−976.
62. Hiroi, T., Imaoka, S., and Funae, Y. (1998) *Biochem. Biophys. Res. Commun. 249*, 838−843.
63. Williams, P. A., Cosme, J., Sridhar, V., Johnson, E. F., and MeRee, D. E. (2000) *Mol. Cell 5*, 121−131.
64. Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967) *Mol. Pharmacol. 3*, 113−123.
65. Guengerich, F. P., Müller-Enoch, D., and Blair, I. A. (1986) *Mol. Pharmacol. 30*, 287−295.
66. Dayer, P., Leemann, T., Gut, J., Kronbach, T., Kupfer, A., Francis, R., and Meyer, U. A. (1986) *Biochem. Pharmacol. 34*, 399−400.
67. Otton, S. V., Inaba, T., Mahon, W. A., and Kalow, W. (1982) *Can. J. Physiol. Pharmacol. 60*, 102−105.
68. Coutts, R. T., Bach, M. V., and Baker, G. B. (1997) *Xenobiotica 27*, 33−47.
69. Coutts, R. T., Bolaji, O. O., Su, P., and Baker, G. B. (1994) *Drug Metab. Dispos. 22*, 756−760.
70. Geertsen, S., Foster, B. C., Wilson, D. L., Cyr, T. D., and Casley, W. (1995) *Xenobiotica 25*, 895−906.
71. Smith, G., Modi, S., Pillai, I., Lian, L.-Y., Sutcliffe, M. J., Pritchard, M. P., Friedberg, T., Roberts, G. C. K., and Wolf, C. R. (1998) *Biochem. J. 331*, 783−792.
72. Ellis, S. W., Hayhurst, G. P., Lightfoot, T., Smith, G., Harlow, J., Rowland-Yeo, K., Larsson, C., Mahling, J., Lim, C. K., Wolf, C. R., Blackburn, M. G., Lennard, M. S., and Tucker, G. T. (1999) *Biochem. J. 345*, 565−571.
73. Crespi, C. L., Steimel, D. T., Penman, B. W., Korzekwa, K. R., Fernandez-Salguero, P., Buters, J. T. M., Gelboin, H. V., Gonzalez, F. J., Idle, J. R., and Daly, A. K. (1995) *Pharmacogenetics 5*, 234−243.
74. Cupp-Vickery, J. R., Han, O., Hutchinson, C. R., and Poulos, T. L. (1996) *Nat. Struct. Biol. 3*, 632−637.
75. Chen, X., Christopher, A., Jones, J. P., Bell, S. G., Guo, Q., Xu, F., Rao, Z., and Wong, L. L. (2002) *J. Biol. Chem. 277*, 37519−37526.
76. Schlichting, I., Berendzen, J., Chu, K., Stock, A. M., Maves, S. A., Benson, D. E., Sweet, B. M., Ringe, D., Petsko, G. A., and Sligar, S. G. (2000) *Science 287*, 1615−1622.
77. Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.
78. Paine, M. J. I., McLaughlin, L. A., Flanagan, J. U., Kemp, C. A., Sutcliffe, M. J., Roberts, G. C. K., and Wolf, C. R. (2002) *J. Biol. Chem.*, electronic version (no. M209519200).

BI027085W