Oxidation of Phenethylamine Derivatives by Cytochrome P450 2D6: The Issue of Substrate Protonation in Binding and Catalysis†

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ABSTRACT: Cytochrome P450 (P450) 2D6 oxidizes a wide variety of drugs typically at a distance of 5−7 Å from a basic nitrogen on the substrate. To investigate the determinants of P450 2D6 catalysis, we analyzed the binding and oxidation of phenethylamine substrates. P450 2D6 discriminated between the various phenethylamines, as evidenced by binding and steady-state results. Whereas the spectral binding affinity for 3-methoxyphenethylamine and 4-methoxyphenethylamine was similar, the affinity for 4-hydroxyphenethylamine was 12-fold weaker than for 3-hydroxyphenethylamine at pH 7.4. The binding of 3,4-dihydroxyphenethylamine was equally poor. These equilibrium dissociation constants were based on the observation of both type I and type II perturbation difference spectra; the former involves displacement of the proximal H_2O ligand, yielding an iron spin state change, and the latter requires nitrogen ligation to the heme iron. One explanation for the observed type II binding spectra is the presence of both protonated and unprotonated forms of these compounds. To address this possibility, the K_S values for 3-methoxyphenethylamine and 4-methoxyphenethylamine were determined as a function of pH. Two apparent pK_a values were determined, which corresponded to a P450 2D6 residue involved in binding and to a lowered pK_a of a substrate amine group upon binding P450 2D6. The apparent pK_a of the enzyme residue (6.6) is much higher than the expected pK_a of Asp301, which has been hypothesized to play a role in binding. Interestingly, the apparent pK_a for the methoxyphenethylamine derivatives decreased by as much as 2 pH units upon binding to P450 2D6. 3-Methoxyphenethylamine and 4-methoxyphenethylamine underwent sequential oxidations with *O*-demethylation and subsequent ring hydroxylation to form 3,4-dihydroxyphenethylamine (dopamine). At higher substrate concentrations, the second oxidation was inhibited. This result can be explained by the increasing concentration of the inhibitory unprotonated substrate. Nevertheless, the rates of methoxyphenethylamine oxidations are the highest reported for P450 2D6 substrates.

P450s[1] encompass a large family of heme−thiolate monooxygenase enzymes found throughout nature. These enzymes catalyze the oxidation of the majority of drugs, pollutants, pesticides, and carcinogens, as well as endogenous compounds such as steroids, alkaloids, and eicosanoids. One of these enzymes, P450 2D6, is a minor component of the hepatic P450 content and is even absent in 5−10% of the Caucasian population (*2*). Nevertheless, P450 2D6 is capable of metabolizing a wide variety of important drugs (*3*), including antipsychotics, antidepressants, and opioid prodrugs. Thus, an understanding of its activity is an important goal in drug metabolism research.

Despite structural variability, there are common features among P450 2D6 substrates. The preferred substrates possess typically a positive charge, usually a basic nitrogen atom, e.g., an amino or guanidinium group. With some exceptions, the major site of oxidation occurs at a distance of 5−7 Å from the positive charge (*4*−*7*). This prospect has led to the speculation that an amino acid residue is responsible for interacting with the positively charged amine group to coordinate the site oxidation. On the basis of active site modeling (*4*−*7*) and site-directed mutagenesis (*8*, *9*), Asp301 is believed to fulfill this role, although other residues may also be involved in binding (*7*, *10*, *11*). The variable binding modes, coupled with the catalytic potential of the activated iron−oxygen species, lead to a common characteristic of P450 2D6 reactions, namely, the possibility for multiple oxidations. How the introduction of multiple reactions affects the overall turnover of substrate is a complex, less understood facet of P450 catalysis.

β-Arylethylamines constitute an important class of P450 2D6 substrate. The metabolism of amphetamines involves

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[1] Abbreviations: P450, cytochrome P450 [also termed "heme−thiolate P450" by the Enzyme Commission (EC 1.14.14.1) (*1*)]; NPR, NADPH−P450 reductase; DLPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine; CHAPS, 3-[(3-choloamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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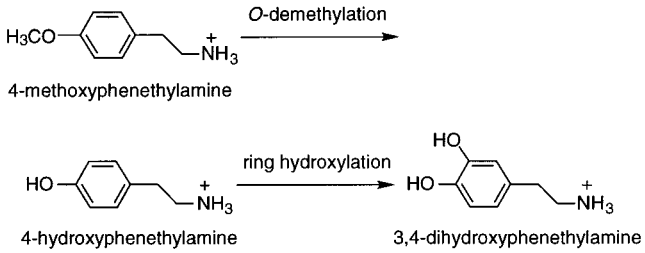
Scheme 1: Oxidation of 4-Methoxyphenethylamine by P450 2D6



N-dealkylation (*12*, *13*), ring hydroxylation (*14*, *15*), and *O*-demethylation (*12*, *14*). These types of reactions depend on the size of the *N*-alkyl group as well as the position of the hydroxyl or methoxy group. Oxidation of these compounds may be linked to their clearance, and several in vitro studies have suggested that P450 2D6 mediates the metabolism of amphetamines in man. Typical reactions include *O*-demethylation of methoxyamphetamines [which possess hallucinogenic properties (*16*, *17*)] and the *N*-dealkylation of some analogues of *N*-methylamphetamine (*18*). Hiroi and co-workers examined the oxidation of 4-hydroxyphenethylamine (tyramine) (Scheme 1) by microsomal P450 2D6 (*19*). Interestingly, this study indicated that both 3- and 4-hydroxyphenethylamines are readily oxidized to dopamine, a neurotransmitter and precursor of norepinephrine and epinephrine. The oxidation of 3-hydroxyphenethylamine to dopamine is in contrast to the observation for amphetamines, where ring hydroxylation did not occur at the 4 position.

To investigate the determinants of P450 2D6 catalysis, we utilized the model substrates 3-methoxyphenethylamine and 4-methoxyphenethylamine, which can undergo either ring hydroxylation or *O*-demethylation (Scheme 1). These compounds both possess a protonated amine 5−7 Å from the site of oxidation. Unlike the former study (*19*), we investigated P450 2D6 catalysis in the context of both binding and steady-state studies. Equilibrium binding constants were obtained by monitoring shifts in the Soret spectrum of P450 2D6 upon formation of the binary complex for all compounds of this study. Another important distinction of this study is the broadening of the scope of the catalyzed reaction to include both *O*-demethylation and ring hydroxylation reactions. The oxidation of 3-methoxyphenethylamine and 4-methoxyphenethylamine to the respective hydroxyphenethylamine derivatives and eventually 3,4-dihydroxyphenethylamine was assessed using HPLC techniques. In addition, the ring hydroxylation of the hydroxyphenethylamines to 3,4-dihydroxyphenethylamine was also monitored to complement the former studies. The simplicity of the substrates limits multiple oxidations to *O*-demethylation and ring hydroxylation, provides higher solubility, and offers a potentially rapid rate of turnover, an attractive quality for a marker substrate for P450 2D6 activity.

EXPERIMENTAL PROCEDURES

Materials. All reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO). 3-Methoxyphenethylamine, 4-methoxyphenethylamine, 4-hydroxyphenethylamine (tyramine), and 3,4-dihydroxyphenethylamine (dopamine) were purchased from Aldrich (Milwaukee, WI). 3-Hydroxyphenethylamine was prepared by the hydrolysis of 3-methoxyphenethylamine in concentrated HI (instead of HCl) under reflux for 2 h (*20*). All of the compounds were recrystallized from C_2H_5OH as HCl salts and used to prepare aqueous stock solutions (see Supporting Information for analyses).

Expression of P450 2D6 in Escherichia coli and Purification. The cDNA sequence of full-length P450 2D6 construct DB#6 was modified by PCR mutagenesis to insert a $(His)_5$ sequence at the C-terminus (*21*). In addition, a Met374Val substitution was introduced into the cDNA by site-directed mutagenesis to reflect the common (wild-type) P450 2D6 sequence. Expression of P450 2D6 in *E. coli* strain MV1304 in the presence of 1.0 mg of chloramphenicol L^{-1} was accomplished, and the protein was purified using a Ni^{2+}-nitrilotriacetate column essentially as described (*21*, *22*).

Binding Spectra. Binding spectra were recorded on an Aminco DW2a spectrophotometer equipped with an OLIS operating system (On-Line Instrument Systems, Bogart, GA) using the general procedure as described (*23*). P450 2D6 was suspended to a final concentration of 0.5−2.0 μM in reaction buffer (100 mM potassium phosphate, pH 7.4) and divided between two tandem cuvettes (sample and reference). These cuvettes were used to correct for both dilution of the enzyme and the absorbance of the titrant. Spectra were recorded between 360 and 500 nm after each addition. For type I binding spectra the λ_{max} was 390 nm and the λ_{min} was 420 nm. For type II binding spectra the λ_{max} was 435 nm and the λ_{min} was 416 nm. The resulting data were fit to the typical hyperbolic curve (eq 1) using the Graph Pad PRISM computer program, where B_{max} is the amplitude of the observed absorbance change and [L] is the concentration of ligand.

$$\text{signal} = \frac{B_{max}[L]}{[L] + K_D} \quad (1)$$

Catalytic Assays. Oxidation assays were conducted in 0.5 mL reaction volume at 37 °C for 10 min in 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (1.0 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase mL^{-1}). Reactions contained P450 2D6 (200 pmol), reconstituted with NADPH−P450 reductase (500 pmol) and 30 μg of DLPC for 10 min at room temperature. Mixtures were also supplemented with 1000 units of bovine liver catalase (dialyzed to remove thymol) and 20 μg of bovine erythrocyte superoxide dismutase in order to destroy partially reduced oxygen species (*24*). Reaction mixtures were quenched with 50 μL of 60% $HClO_4$ and incubated on ice for 10 min (to precipitate protein and salts), followed by centrifugation at 3000*g* for 10 min. Fifty microliter aliquots of the recovered supernatants were injected onto a YMC octadecylsilane C_{18} HPLC column (5 μm, 2.5 × 150 mm; YMC, Wilmington, NC), and eluted compounds were detected using fluorescence measurements (λ_{ex} 277 nm, λ_{em} 300 nm) (*19*). Chromatography was done at a flow rate of 1.0 mL min^{-1} utilizing 20 mM $NH_4CH_3CO_2$ (pH 4.5) buffer containing CH_3CN (5%−30%, v/v, depending on the products being analyzed). Quantitation of the metabolites formed was achieved by running standard curves generated using 3-hydroxyphenethylamine, 4-hydroxyphenethylamine, and 3,4-dihydroxyphenethyalmine standards. Where appropriate,

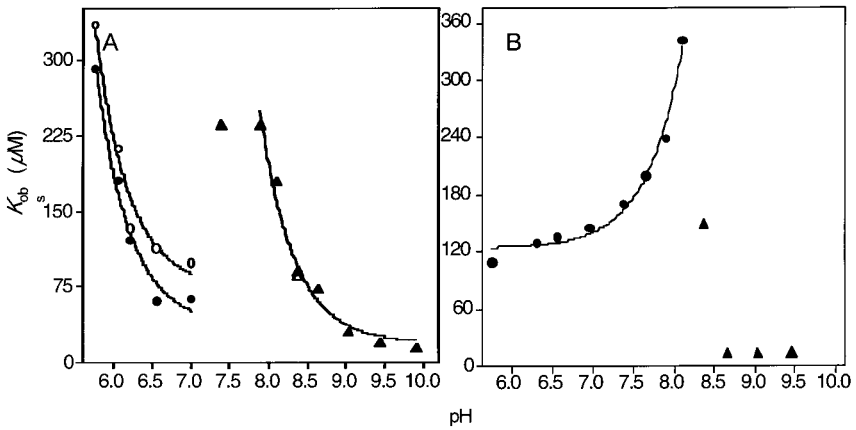


FIGURE 1: Effect of pH on K_{obs}. (A) Plot of observed dissociation constants for 3-methoxyphenethylamine as a function of pH. Data include values taken at constant buffer conditions, which yielded type I (●) and type II (▲) binding spectra, as well as values obtained at constant ionic strength (○), where KCl was added to maintain $\mu = 0.19$ (same as 100 mM potassium phosphate, pH 7.4). (B) Plot of observed dissociation constants for 4-methoxyphenethylamine as a function of pH. Data include values taken at constant buffer conditions, which yielded type I (●) and type II (▲) binding spectra. The binding data were determined in a system containing 2.0 μM purified recombinant P450 2D6 in 100 mM potassium phosphate, variable pH, with 50 μM DPLC at 25 °C.

the data were fit to the standard Michaelis−Menten equation using the Graph Pad PRISM computer program. Equation 2 is the standard Michaelis−Menten equation where the velocity of the reaction is a function of the rate-limiting step in turnover (k_{cat}), the enzyme concentration ([E]), substrate concentration ([S]), and the Michaelis constant (K_M).

$$v = \frac{k_{cat}[E][S]}{[S] + K_M} \quad (2)$$

RESULTS AND DISCUSSION

Equilibrium Binding of Ligands. An important component in understanding enzymatic catalysis is the nature of the enzyme−substrate complex. The first step in a catalytic cycle is the formation of the binary complex, as measured by equilibrium studies. A useful parameter for these studies relates to the spin state of the heme iron. In the high-spin state, heme iron is pentacoordinate, whereby the heme provides four nitrogen ligands to heme and a cysteinate from the enzyme forms the fifth coordinate. The characteristic absorbance of this complex changes upon the ligation of water to the heme iron with the concomitant shift of the iron to the low-spin state. The creation of a hexacoordinate system has a characteristic spectrum that differs from the high-spin state. The availability of P450 2D6 in the low-spin state for the heme iron provides a suitable parameter to monitor the formation of the binary complex. A binding event displaces the water molecule coordinated to the heme iron to produce a shift to the high-spin state (type I difference spectrum) (*23*). Alternatively, the binding event can involve the replacement of the heme-bound water molecule with a nitrogenous ligand, likely from the titrant itself. In this case the heme iron remains in the low-spin state; however, the change in ligation induces a red shift in the spectrum to produce a type II difference spectrum.

Titrations with phenethylamines yielded either type I or type II difference spectra (Figure 1). Interestingly, P450 2D6 discriminated between the compounds of this study on the basis of the position of the methoxy or hydroxy substituent (Table 1). Whereas the K_S values for 3-methoxyphenethylamine and 3-hydroxyphenethylamine are similar, the loss of the methyl group from 4-methoxyphenethylamine to form 4-hydroxyphenethylamine results in a 13-fold drop in affinity. Possibly the binding contacts from the methyl group are more critical in binding 4-methoxyphenethylamine than 3-methoxyphenethylamine. Given that the P450 2D6 binding site can accommodate molecules such as quinidine, bufuralol, and metropolol (*3*), which are larger than the methoxyphenethylamines, it seems unlikely that such a large effect on binding results from loss of hydrophobic contacts from a single methyl group. Alternatively, the interaction of 3-hydroxyphenethylamine with P450 2D6 might be inherently different than 4-hydroxyphenethylamine, as suggested by different binding spectra for these compounds. When hydroxyl groups are present at both the 3 and 4 positions, as in the case of 3,4-dihydroxyphenethyalmine, binding is similar to that of 4-hydroxyphenethylamine, with a K_S of 3600 μM resulting from a type II binding spectrum.

Table 1: Equilibrium Dissociation Constants of Phenethylamines

titrant	K_S (μM)	spectrum
3-methoxyphenethylamine	221 ± 16	type II
3-hydroxyphenethylamine	181 ± 15	type I
4-methoxyphenethylamine	170 ± 20	type I
4-hydroxyphenethylamine	2400 ± 270	type II
3,4-dihydroxyphenethylamine	3600 ± 260	type II

The observation of both type I and type II binding spectra for these compounds was surprising (Table 1). Since P450 2D6 exists mainly in the low-spin state, it was expected that the binding of these compounds would displace the water molecule bound to the heme iron to produce type I binding spectra. On the other hand, the production of type II binding spectra was unexpected. This type of binding spectrum requires the coordination of unbonded electrons from nitrogen to the heme iron. The basic nitrogen for these phenethylamine derivatives is presumably protonated under assay conditions. It is possible that a contaminant may influence the binding spectra, although the compounds of this study were recrystallized (HCl salts). To address this issue, the compounds of this study were subjected to further analyses (Supporting Information).

In the absence of a contaminant, the titrants that produced a type II spectral shift must exist, at least partly, in the

Table 2: Apparent pK_a Values of Phenethylamines Free in Solution

compound	phenol	amine
3-methoxyphenethylamine		9.89[a]
3-hydroxyphenethylamine	9.58[a]	10.50[a]
4-methoxyphenethylamine		9.79[b]
4-hydroxyphenethylamine	9.74[a]	10.52[a]
3,4-dihydroxyphenethylamine	8.87, 8.92[c]	10.63[c]

[a] From ref *25*. [b] From ref *26*. [c] From ref *27*.

unprotonated state. This observation raises the question of what the concentrations of the unprotonated phenethylamine derivatives are in solution. The pK_a values for these compounds free in solution have been determined spectrophotometrically and potentiometrically (Table 2) (*25*−*27*). On the basis of these values, the concentration of the unprotonated phenethylamine derivatives should be extremely low. For example, for 3-methoxyphenethylamine, the ratio of protonated to unprotonated forms is 309:1, or only 0.32% of the 3-methoxyphenethylamine is unprotonated at pH 7.4 in the absence of protein. However, the observed type II spectral shifts implicate a more complex scenario for interactions between P450 2D6 and the phenethylamine derivatives. The apparent pK_a for the amine groups must be shifted to lower pH to provide a sufficient concentration of titrant to induce the observed type II spectra. If lowering the apparent pK_a results in the presence of both forms of the phenethylamine derivatives at pH 7.4, then changes in the populations of these species would be readily observable as the pH is varied.

Effect of pH on the Binding of Phenethylamines. Of the compounds of this study, the methoxyphenethylamine derivatives are best suited to investigate the effects of pH on binding, because these compounds possess only one ionizable group. The potential to observe the shift of titrant between a protonated and an unprotonated state upon binding to P450 2D6 introduces an interesting scenario. At sufficiently low pH the methoxyphenethylamine derivative is protonated, resulting in a type I binding spectrum, because the positively charged amine group forms a putative ionic−ionic interaction with Asp301. This binding interaction effectively displaces the water molecule ligated to the heme iron. However, at higher pH the methoxyphenethylamine derivative becomes unprotonated to produce a free pair of electrons on the nitrogen atom. The ligation of the nitrogen to the heme iron produces a type II spectrum. Taken together, the shift from protonated to unprotonated methoxyphenethylamine as a function of pH produces a shift from type I to type II binding spectrum.

Whereas the protonation state of the methoxyphenethylamine determines the type of binding spectrum observed, the actual titrant concentration determines the observed K_S value (*28*). The true K_S value for protonated methoxyphenethylamine is obtained at sufficiently low pH (absence of the unprotonated form). As the pH increases, the actual concentration of protonated methoxyphenethylamine decreases, resulting in a rising observed K_S value, whose asymptote is determined by the pK_a of the basic amine. This relationship is described by eq 3, where rising pH results in

$$K_{S,obs} = K_{S,cor} + K_{S,cor}10^{(pH-pK_a)} \quad (3)$$

a loss in binding. This equation provides the true K_S or corrected K_S value ($K_{S,cor}$) and the apparent pK_a of the functional group responsible for the pH dependence in binding. For unprotonated methoxyphenethylamine an analogous trend exists except that as the pH decreases, the observed K_S value for unprotonated methoxyphenethylamine increases to an infinite value at the respective pK_a value of the basic nitrogen. The corrected K_S value can only be obtained at a high enough pH to exclude the presence of protonated methoxyphenethylamine. This reversal of the observed trend is described by eq 4.

$$K_{S,obs} = K_{S,cor} + K_{S,cor}10^{(pK_a-pH)} \quad (4)$$

The pH dependence of enzyme specificity has precedence in studies of monoamine oxidase, which selectively binds and deaminates unprotonated substrates (*28*, *29*). For monoamine oxidase, eq 4 has been used successfully to analyze issues in binding and catalysis. In contrast, both protonated and unprotonated methoxyphenethylamine bind to P450 2D6, and thus the measured pK_a could be determined from either the type I or type II binding spectra. In practice, however, the ability to determine $K_{S,obs}$ depends on the magnitude of the spectral amplitude. Because type I and type II binding spectra overlap, binding titrations near the pK_a contain sufficient concentrations of both protonated and unprotonated methoxyphenethylamine, resulting in interference between the corresponding type I and type II binding spectra. The higher $K_{S,obs}$ values could be defined by the binding spectra with the larger change in absorbance, and thus only one set of data could be fit to either eq 3 or eq 4.

Consistent with this model, two trends were evident as shown in Figure 1. First, lower pH favored type I binding spectra, whereas higher pH favored type II binding spectra. Second, the observed K_S values demonstrated a pH-dependent increase. At low pH the addition of methoxyphenethylamine resulted in the formation of a typical type I spectral shift, with a peak at 390 nm and a trough at 420 nm (Figure 2). The observed K_S values decreased until an intermediate pH, where both type I and type II binding spectra were observed (Figure 3). For example, with 3-methoxyphenethylamine (pH 7.0), the addition of low concentrations of titrant resulted in a typical type I binding spectrum; however, as the concentration of titrant increased, the maximal and minimal absorbance peaks shifted to typical type II binding spectrum. At higher pH values, only type II binding spectra were observed with a peak at 435 nm and a trough at 416 nm.

Surprisingly, 3-methoxyphenethylamine binding as a function of pH revealed two pK_a values when each data set was fit to eq 4 (Table 3). On the basis of the model, the transition from type I to type II spectra at pH ~7.5 marked the shift from protonated methoxyphenethylamine to unprotonated methoxyphenethylamine (pK_a = 8.9). The corrected K_S value (22 μM) indicates high affinity for unprotonated 3-methoxyphenethylamine. The lower pK_a (6.6) is derived solely from type I spectra; thus this pK_a reflects the titration of an enzyme group involved in the formation of the binary complex for protonated 3-methoxyphenethylamine. This apparent pK_a of an enzyme residue is much higher than the expected pK_a of Asp301 [3.09 for the free acid (*30*)] that has been hypothesized to play a role in binding. In the absence of any structural information, the identity of this enzyme residue is

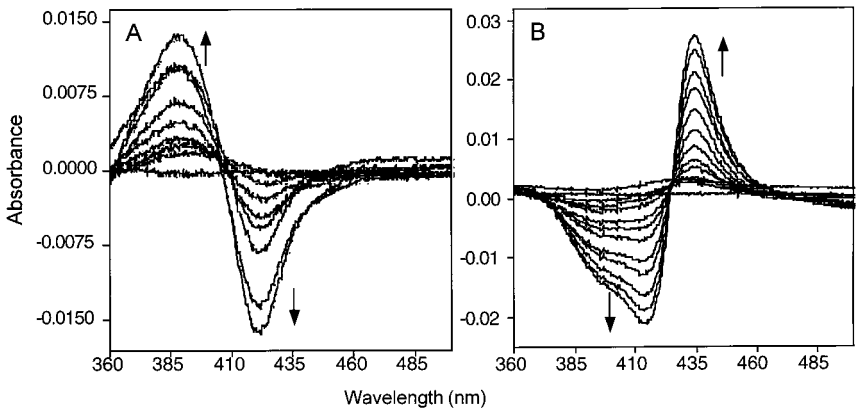


FIGURE 2: Binding spectra resulting from the spectral shift of heme absorbance upon formation of the binary complex. (A) Type I binding spectrum reflects titration of 4-methoxyphenethylamine with 0, 4, 12, 24, 44, 74, 114, 164, 224, 324, 474, 674, 874, and 1170 μM titrant. (B) Type II binding spectra reflects titration of 3-methoxyphenethylamine with 0, 4, 12, 24, 44, 74, 119, 164, 263, 364, 514, 714, and 1010 μM titrant. The spectra were measured in a system containing 2.0 μM purified recombinant P450 2D6 in 100 mM potassium phosphate, pH 7.4, containing 50 μM DPLC at 25 °C.

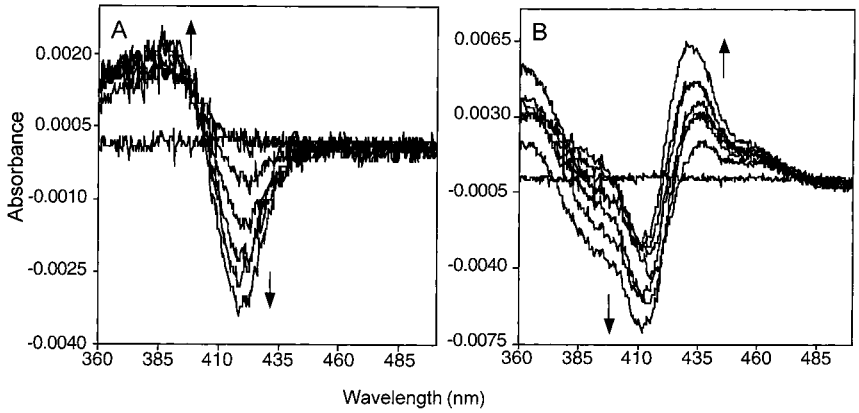


FIGURE 3: Spectral shift of heme absorbance upon 3-methoxyphenethylamine binding at pH 7.0. Initially, a type I binding spectra (A) was observed at low substrate concentrations (4, 10, 18, 28, 40, 60, 90, 130, and 190 μM), which shifted to a type II binding spectra (B) at higher titrant concentrations (290, 440, 590, 790, 990, 1190, and 1390 μM). The spectra were measured in a system containing 2.0 μM purified recombinant P450 2D6 in 100 mM potassium phosphate, pH 7.0, containing 50 μM DPLC at 25 °C.

Table 3: Binding Parameters for Methoxyphenethylamines As Determined (Equations 3 and 4)

titrant	form	$K_{S,cor}$	pK_a
3-methoxyphenethylamine	protonated	37 ± 1	6.6 ± 0.1
	($\mu = 0.19$)	(74 ± 5)	(6.3 ± 0.1)
	unprotonated	22 ± 1	8.9 ± 0.2
4-methoxyphenethylamine	protonated	125 ± 5	7.9 ± 0.1
	unprotonated	15 ± 1[a]	

[a] $K_{S,cor}$ reflects the average of three K_S values determined at pH $\gg$ pK_a (Figure 1).

unclear at this time. The loss in binding is not likely attributable to protein unfolding as evidenced by the absence of this effect during 4-methoxyphenethylamine binding (vide infra). The corrected K_S for protonated 3-methoxyphenethylamine is almost 2-fold the value determined for unprotonated 3-methoxyphenethylamine, 37 versus 22 μM, respectively. In other words, the nonsubstrate unprotonated 3-methoxyphenethylamine has a higher affinity for P450 2D6 than the protonated substrate.

As implicated in the model, 4-methoxyphenethylamine binding as a function of pH revealed a single pK_a relating to the transition from type I binding spectra to type II binding spectra (Figure 1). Unlike the binding of 3-methoxyphenethylamine, the spectral amplitudes for the type I and type II spectra for 4-methoxyphenethylamine were very different, 0.060 versus 0.002, respectively. The greater type I amplitude essentially masked the type II spectral amplitude. The $K_{S,cor}$ value for unprotonated 4-methoxyphenethylamine (type II binding spectra) is based on the average of three $K_{S,obs}$ values determined at pH $\gg$ pK_a, which differed by less than 5%. These data were fit to eq 3, and the results are summarized in Table 3. On the basis of the nature of the spectral shift during binding, this pK_a reflects the basic amine of 4-methoxyphenethylamine, which is lower than for 3-methoxyphenethylamine, 7.9 versus 8.9, respectively. The more pronounced effect for 4-methoxyphenethylamine reflects subtle differences between their respective enzyme−titrant interactions. This conclusion is further substantiated by the lack of a second titratable group at lower pH as observed during 3-methoxyphenethylamine binding. Furthermore, the difference in affinity of P450 2D6 for protonated and unprotonated 4-methoxyphenethylamine is more significant than that observed for 3-methoxyphenethylamine. Although the affinity for unprotonated 4-methoxyphenethylamine is relatively high ($K_{S,cor} = 15$ μM), the affinity for protonated 4-methoxyphenethylamine is 8-fold lower ($K_{S,cor} = 125$ μM). Similar to 3-methoxyphenethylamine, P450 2D6 displays a higher affinity for the unprotonated nonsubstrate over the protonated substrate.

Whereas the accepted paradigm involves substrates possessing a basic nitrogen, it is clear that P450 2D6 not only binds the unprotonated substrates but also facilitates the formation of the unprotonated species by decreasing the pK_a for the amine group. These values are significantly lower than those reported for these compounds in solution (Table 2). A mechanism for this effect relates to the formation of the enzyme−titrant complex, specifically the milieu of the P450 2D6 binding site. On the basis of models of P450 2D6 (*4*−*7*), the binding pocket involves a hydrophobic cavity interspersed with hydroxyl groups originating from serines and threonines. It is possible that these hydroxyl groups could act as general bases to remove the proton and shuttle it out of the active site, or even the ligation of the free amine with the heme may drive the equilibrium to the unprotonated species. If the shift in pK_a was related to the interaction with the heme, then the titration of heme alone with methoxyphenethylamines as a function of pH may reveal a shift in the observed pK_a for the methoxyphenethylamines of this study. However, a titration with unbound heme was inconclusive due to poor binding (K_S > 10 mM; data not shown). In addition, P450 2D6 could also perturb the pK_a for the hydroxyl groups of the hydroxyphenethylamines, although evidence for this prospect is not possible with these types of experiments. This effect has no precedent in P450 2D6 studies or P450 studies in general and thus requires careful consideration in studies concerning catalysis and inhibition.

Another consideration for these studies is the issue of ionic strength. Many P450 studies (and most if not all P450 2D6 studies) assay P450 activity in 100 mM potassium phosphate buffer at pH 7.4, which serves as a model for physiological conditions. Varying the pH alters the ionic strength of the medium if the concentration of the buffer is held constant. The ionic strength of a 100 mM potassium phosphate solution varies from 0.110 at pH 6.0 to 0.245 at pH 10.0. Binding interactions that involve charges and polar contacts, such as between the protonated amine of the phenylamines and an enzyme residue(s), would be more sensitive to changes in ionic strength. Since the unprotonated form of the phenethylamines coordinates through an uncharged amine, the affinity for P450 2D6 should not be significantly affected by changes in ionic strength.

The different binding modes for the two forms of the phenethylamines were confirmed through binding studies for 3-methoxyphenethylamine, where the ionic strength was increased upon addition of KCl (Figure 1). On the basis of the type I binding spectra, the observed K_S values were dependent on the ionic strength. At pH 6.06 the addition of 80 mM KCl increased $K_{S,obs}$ from 181 to 212 μM, where μ increased from 0.11 to 0.19. At 200 mM KCl (final μ = 0.31), $K_{S,obs}$ was 256 μM. Since the ionic strength varies from 0.105 to 0.127 as the pH increases from 5.76 to 6.56, $K_{S,obs}$ was determined at a constant ionic strength of 0.19 (the same value for reaction buffer, 100 mM potassium phosphate at pH 7.4). These data were then fit to eq 4, yielding $K_{S,cor}$ = 74 μM and pK_a = 6.3. On the basis of these values, the K_S for the protonated form of 3-methoxyphenethylamine is 80 μM, not 221 μM shown in Table 1, because this value was derived from a type II binding spectrum. As for 4-methoxyphenethylamine binding, the observed type I binding spectra at pH 7.4 provide the apparent binding constant for this substrate. As expected, monitoring the formation of type II binding spectra at pH 8.37 revealed no effect on $K_{S,obs}$ when the ionic strength was increased from 0.24 to 0.44 upon addition of 200 mM KCl.

The corrected K_S values introduce an important consideration for catalytic studies, typically performed in 100 mM potassium phosphate buffer at pH 7.4. Substrate oxidation by P450 2D6 apparently relies on the protonation of the amine to properly orientate the substrate; however, the unprotonated form of the substrate ligates the heme iron and thus acts as a competitive inhibitor. In the case of methoxyphenethylamine, the oxidation of the protonated substrate occurs in the presence of the unprotonated inhibitor whose concentration depends on pH. Under typical buffer conditions (100 mM potassium phosphate, pH 7.4), the amount of inhibitor present (i.e., unprotonated form) during 3-methoxyphenethylamine oxidation is 3% of the total concentration of 3-methoxyphenethylamine. More significantly, the lower pK_a for 4-methoxyphenethylamine indicates that 24% of the substrate is unprotonated under assay conditions.

This scheme introduces two important considerations for catalytic studies. First, without prior knowledge of the equilibrium state of the substrate, the determination of steady-state parameters yields only apparent values, because the presence of unprotonated inhibitor masks the correct steady-state parameters. Second, the observed steady-state parameter is sensitive to the ionic strength of the reaction medium. As indicated by the KCl studies, binding of the protonated substrate is more sensitive to the ionic strength than the unprotonated inhibitor. Thus, changes in ionic strength modulate the formation of enzyme−substrate and enzyme−inhibitor complexes.

A complement to this work would be the demonstration of a similar pK_a shift during catalytic turnover (*31*, *32*). Unfortunately, the complexity of the catalytic cycle makes the interpretation of such data very difficult. An analysis of steady-state turnover as a function of pH would reveal titratable groups involved in the oxidation of NADPH by NPR, in facilitating the interaction between NPR and P450, and in the oxidation of substrate by P450, which involves multiple proton transfer steps to form the activated perferryl species.

Steady-State Kinetics of Phenethylamine Oxidation by P450 2D6. Although P450 2D6 is capable of both *O*-demethylation and ring hydroxylation reactions, the oxidation of methoxyphenethylamines involves the sequential *O*-demethylation of these substrates to the corresponding hydroxyphenethylamines, followed by ring hydroxylation to 3,4-dihydroxyphenethylamine (Scheme 1, Figures 4 and 5, Table 4). The ring hydroxylation of methoxyphenethylamines did not occur at a rate sufficient for detection, which may reflect the role of steric factors or energetics. Despite similar parameters for the *O*-demethylation of the methoxyphenethylamines, the oxidation of the hydroxyphenethylamines demonstrated different steady-state kinetics.

Data from both the *O*-demethylation of 3-methoxyphenethylamine and the ring hydroxylation of the hydroxyphenethylamine product are displayed in Figures 4 and 5. Only the data for the *O*-demethylation of substrate could be fit to the Michaelis−Menten equation due to a sharp decrease in the observed rate of 3,4-dihydroxyphenethylamine production. Rates for 3-methoxyphenethylamine *O*-demethylation began to decrease at high substrate concentrations; thus only

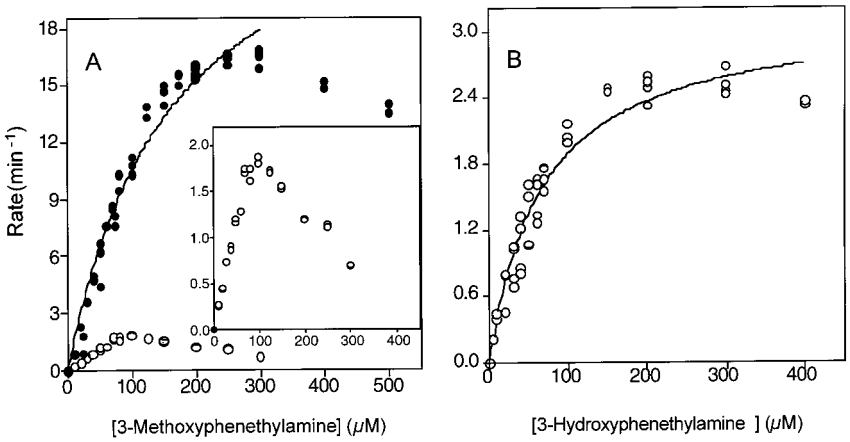


FIGURE 4: (A) Saturation curve of P450 2D6-mediated oxidation of 3-methoxyphenethylamine to 3-hydroxyphenethylamine (●) and 3,4-dihydroxyphenethylamine (○). The data were fit to the standard Michaelis−Menten equation (solid line). (B) Saturation curve of P450 2D6-mediated oxidation of 3-hydroxyphenethylamine following the formation of 3,4-dihydroxyphenethylamine (○). The data were fit to the standard Michaelis−Menten equation (solid line). The activity was measured in a system containing 0.4 μM purified recombinant P450 2D6 and 1.0 μM rat NPR in 100 mM potassium phosphate buffer, pH 7.4, with 50 μM DPLC at 37 °C.

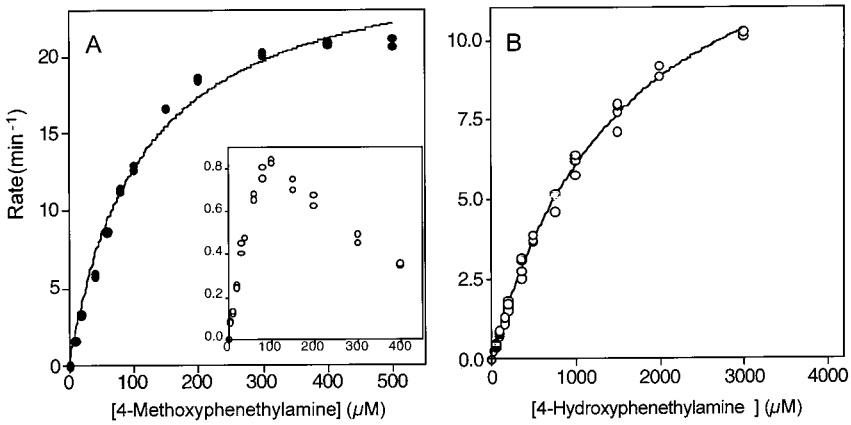


FIGURE 5: (A) Saturation curve of P450 2D6-mediated oxidation of 4-methoxyphenethylamine following the formation of 4-hydroxyphenethylamine (●) and 3,4-dihydroxyphenethylamine (○). The data were fit to the standard Michaelis−Menten equation (solid line). (B) Saturation curve of P450 2D6-mediated oxidation of 4-hydroxyphenethylamine following the formation of 3,4-dihydroxyphenethylamine (○). The data were fit to the standard Michaelis−Menten equation (solid line). The activity was measured in a system containing 0.4 μM purified recombinant P450 2D6 and 1.0 μM rat NPR in 100 mM potassium phosphate buffer, pH 7.4, with 50 μM DPLC at 37 °C.

Table 4: Michaelis−Menten Kinetic Parameters for the Oxidation of Phenethylamines

substrate	product	k_{cat} (min^{-1})	K_M (μM)
3-methoxyphenethylamine	3-hydroxyphenethylamine[a]	27 ± 1	160 ± 17
3-hydroxyphenethylamine	3,4-dihydroxyphenethylamine	3.2 ± 0.1	67 ± 7
4-methoxyphenethylamine	4-hydroxyphenethylamine	32 ± 1	111 ± 5
4-hydroxyphenethylamine	3,4-dihydroxyphenethylamine	16 ± 1	1630 ± 80

[a] Data used in the fit were taken at substrate concentrations <400 μM.

rates measured with <400 μM substrate were used in the fit. The k_{cat} values for these substrates were 32 min^{-1} and 27 min^{-1}, and the K_M values were 160 μM and 110 μM, respectively (Table 4). In contrast, the use of 3-hydroxyphenethylamine as the sole substrate did not produce the decrease in turnover rate as observed during 3-methoxyphenethylamine oxidation within a similar range of assayed concentrations. A higher maximal rate for 3,4-dihydroxyphenethylamine production was observed during 3-methoxyphenethylamine oxidation rather than 4-methoxyphenethylamine oxidation, even though the k_{cat} value for 4-hydroxyphenethylamine hydroxylation was 5-fold the respective value for 3-hydroxyphenethylamine.

An explanation for the catalytic inhibition is not forthcoming in the absence of further study. The confirmation of substrate purity rules out the possibility of a contaminant (Supporting Information). Most likely, the key lies in the microscopic rates that comprise the complex steady-state cycle for P450 2D6. Possible rate-limiting steps in P450 catalysis include substrate binding, reduction, oxygen binding to ferrous P450, addition of the second electron to the system, rearrangement to the final active oxygen species, C−H bond cleavage, product release, and any protein rearrangements (*33*, *34*). Little information is available about the rate-limiting step(s) in the P450 2D6 catalytic cycle. A more in-depth analysis of microscopic rates contributing to steady-state turnover is the focus of future studies in this laboratory.

The observation of 3,4-dihydroxyphenethylamine formation during oxidation of the methoxyphenethylamines is striking in light of the data for hydroxylation of the

hydroxyphenethylamines. For example, the rate of 3,4-dihydrophenethylamine formation at 100 μM 3-methoxyphenethylamine (about half the K_M for 3-methoxyphenethylamine) was ~1.8 min^{-1}. To obtain this rate based on steady-state studies of 3-hydroxyphenethylamine oxidation, the effective concentration of 3-hydroxyphenethylamine must be 48 μM. At its maximal rate P450 2D6 produces 11 μM 3,4-dihydroxyphenethylamine from 3-methoxyphenethylamine. The data for 3,4-dihydroxyphenethylamine formation during 4-methoxyphenethylamine demonstrate a similar trend. In effect, the hydroxyphenethylamines are oxidized more rapidly during the sequential oxidative mechanism than the independently determined oxidation of hydroxyphenethylamines. There is a distinct difference between the two mechanisms. The rate of 3,4-dihydroxyphenethylamine formation during oxidation of the methoxyphenethylamines does not include any steps prior to formation of the Michaelis complex. The observed rate includes the chemistry step and any steps occurring after chemistry to recycle the enzyme. In contrast, the rate of 3,4-dihydroxyphenethylamine formation from the hydroxyphenethylamines includes all steps of the catalytic cycle. Unfortunately, the inhibition of the reaction at higher substrate concentrations prevents an accurate determination of the rate difference, although it is clear that steps prior to chemistry contribute to the observed rate of substrate turnover.

Another interesting observation is the decrease in product formation rates at higher substrate concentrations, especially during 3-methoxyphenethylamine oxidation (Figures 4 and 5). Although a contaminant could explain the observed trend, these substrates had been carefully recrystallized, which would lessen the likelihood of this possibility. Alternatively, substrate may inhibit catalytic turnover by binding in an unproductive conformation. In light of the binding studies, we hypothesize that as the concentration of methoxyphenethylamines increases, the amount of unprotonated methoxyphenethylamine also increases such that it effectively outcompetes the poorer binding protonated for of the methoxyphenethylamine. As expected, this effect is more significant for 3-methoxyphenethylamine turnover than 4-methoxyphenethylamine turnover.

The oxidation of the hydroxyphenethylamines demonstrated a high selectivity with respect to the position of the hydroxyl group (Table 4). Plots of the rates of 3,4-dihydroxyphenethylamine formation as a function of 3-hydroxyphenethylamine and 4-hydroxyphenethylamine concentration are shown in Figures 4 and 5, respectively. The maximal turnover rate of 4-hydroxyphenethylamine was 5 times the rate measured for 3-hydroxyphenethylamine; however, the Michaelis constant (K_M) for 4-hydroxyphenethylamine was 24 times greater than the value for 3-hydroxyphenethylamine. In other words, 3-hydroxyphenethylamine was more efficiently oxidized than 4-hydroxyphenethylamine, as reflected by k_{cat}/K_M. Without further study it is not clear what may account for this observation, although the differences in formation of the Michaelis complex may relate to binding as observed for the methoxyphenethylamines. This trend was not observed in an earlier study of the oxidation of the hydroxyphenethylamines to 3,4-dihydroxyphenethylamine by a microsomal P450 2D6 prepared from yeast (*19*). The discrepancy may reflect the coexpression of P450 2D6 with NADPH−P450 reductase, where only the P450 content was quantitated. It is unclear what the ratio of P450 to reductase was in their studies. In contrast, we reconstituted individually purified P450 2D6 and rat reductase at a ratio of 1:2.5.

P450 2D6-Catalyzed N-Dealkylations. Although typical P450 2D6 oxidations occur 5−7 Å from the basic nitrogen of the substrate, there are exceptions in which P450 2D6 catalyzes *N*-dealkylations (*14*). For example, L-(−)-deprenyl undergoes both *N*-demethylation and *N*-propargylation by P450 2D6 (*35*). The authors hypothesized that the protonated form of the substrate binds P450 2D6 but is not in a productive conformation for oxidation to occur; however, the equilibrium of the two forms of the substrate produces the unprotonated species, which undergoes oxidation. This proposition was founded on the low basicity and low redox potential of tertiary amines. Our study indicates that the binding event itself is able to decrease the basicity of the substrate amine (in this case an unsubstituted amine), thereby enhancing the availability of the unprotonated form of the substrate. It is conceivable that this effect also occurs with the substituted amine groups of substrates, thereby facilitating *N*-dealkylation reactions. The variability on the magnitude of the pK_a shift as demonstrated by the methoxyphenethylamines indicates complex enzyme−substrate interactions, such that predictions for this effect would be difficult.

Concluding Remarks. The role of P450 2D6 in drug metabolism has made understanding P450 2D6 catalysis a focus of intense research. In this study we investigated two characteristics of P450 2D6 activity using methoxyphenethylamines as model substrates. First, the oxidation of these substrates was relatively rapid and efficient. The k_{cat} of 32 min^{-1} is higher than any other reported P450 2D6 reaction. The surprising finding of both protonated and unprotonated forms of potential substrates marks a significant consideration for P450 studies in general. In this study, the unprotonated form of the substrate would act as a competitive inhibitor during steady-state turnover. Second, P450 2D6 catalyzed both *O*-demethylation and ring hydroxylation reactions in a selective manner, where the former reaction was favored by the latter. These types of multiple oxidations are common for P450 2D6. The kinetic and binding properties investigated in this report are of interest in the characterization of other substrates oxidized by P450 2D6 and the oxidation of amines by P450s in general.

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SUPPORTING INFORMATION AVAILABLE

Further analysis of all compounds by melting point, HPLC, NMR, and mass spectrometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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