Oxidation of Methoxyphenethylamines by Cytochrome P450 2D6

ANALYSIS OF RATE-LIMITING STEPS*[5]

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Cytochrome P450 (P450) 2D6 is involved in the oxidation of a large fraction (~30%) of drugs used by humans and also catalyzes the O-demethylation of the model substrates 3- and 4-methoxyphenethylamine followed by subsequent ring hydroxylation to dopamine. Burst kinetics were not observed; rate-limiting step(s) must occur prior to product formation. Rates of reduction of ferric P450 2D6 were stimulated by 3- or 4-methoxyphenethylamine or the inhibitor quinidine; reduction is not the most rate-limiting step. The non-competitive intramolecular deuterium isotope effect, an estimate of the intrinsic isotope effect, for 4-methoxyphenethylamine O-demethylation was 8.6. Intermolecular non-competitive deuterium isotope effects of 3.1–3.8 were measured for \( k_{cat} \) and \( k_{cat}/K_m \) for both O-demethylation reactions, implicating at least partially rate-limiting C–H bond breaking. Simulation of steady-state kinetic data yielded a catalytic mechanism dominated by the rates of (i) \( \text{Fe}^{2+}/\text{O}_2 \) protonation (plus O–O bond scission) and (ii) C–H bond breaking, consistent with the appearance of the spectral intermediates in the steady state, attributed to iron–oxygen complexes. However, all the rates of individual steps (or rates of combined steps) are considerably higher than \( k_{cat} \) and the contributions of several steps must be considered in understanding rates of the P450 2D6 reactions.

P450 enzymes are found throughout nature and function primarily as monooxygenases (4, 5). Many P450s play catalytic roles critical to life (e.g. sterol hydroxylases in mammals) or, because of less restricted selectivity, provide organisms with the means of (i) generating secondary metabolites for defense (e.g. antibiotics) (6), (ii) provide organisms with the capability to use certain chemicals as carbon sources (e.g. several microorganisms) (7), or (iii) detoxicate chemicals encountered in the environment. The so-called drug-metabolizing P450s fit into this latter category, and much interest has developed because of the relevance in pharmaceutical development (8).

Currently considerable attention has been given to substrate docking in P450 structures and models as a means of understanding and predicting catalytic activities (9, 10). The juxtaposition of a substrate in the ground state can influence the rate-limiting steps in catalysis and, therefore, the catalytic selectivity. However, many of the details of catalysis may be relatively independent of ground state docking. Thus a need exists to examine many P450 reactions in as much detail as possible, in terms of providing an extensive description of exactly what steps are involved in influencing rates of P450 catalysis (11).

The matter of identifying the rate-limiting steps in P450 reactions is not a new one, and the subject has been reviewed several times (11–14). However, much of the early literature involved the use of relatively crude systems and limited methodology, and many of the key issues remain today. The literature often leaves a reader with the general impression that the reduction of P450 is the rate-limiting step (e.g. Refs. 12 and 15), but the evidence indicates that this is not the common case, at least with purified P450 systems (16). Some of the steps in the generally accepted catalytic cycle (Scheme 1) shown to be rate-limiting in certain instances include (2) introduction of the first electron into ferric P450 (16), (4) introduction of the “second” electron, as judged by the stimulation of reactions by the action of cytochrome \( b_5 \) in some cases (18, 19), (5) protonation of the fully reduced (\( \text{Fe}^{2+}/\text{O}_2 \) complex (20, 21), (6) C–H bond breaking (11, 22) or 1-electron oxidation (23, 24), (7) a step following product formation (25, 26), and (10, 11) competition with rates of abortive \( \text{O}_2 \) reduction (27).

P450 2D6 was the first human P450 involved in drug oxidations that was demonstrated to be under monogenic control (28). The genetic polymorphism has now been characterized extensively (29), and P450 2D6 is estimated to be involved in the oxidation of ~30% of the drugs used today (8, 30). This protein has been the subject of investigations in this laboratory for some time (31); studies with recombinant P450 2D6 expressed in Escherichia coli have been done on the role of the N-terminal region of the protein in interactions with the

1.14.14.1 (1); THF, tetrahydrofuran; HPLC, high performance liquid chromatography; MS, mass spectrometry. The conventions \( bV = \frac{1}{k_{cat}} \) and \( b(V/K) = \frac{1}{k_{cat}(K_m)} + \frac{1}{k_{cat}} \) are used in the designation of kinetic hydrogen isotope effects.
NADPH-P450 reductase, the mechanism of P450 2D6 reactions supported by oxygen surrogates, and the role of Asp-301 in substrate binding and protein folding (32–35). In the course of studies with the prototypic substrate bufuralol, we found that the rate of reduction of ferric P450 2D6 was enhanced by NADPH-P450 reductase, the mechanism of P450 2D6 reactions supported by oxygen surrogates, and the role of Asp-301 in substrate binding and protein folding (32–35). In the course of studies with the prototypic substrate bufuralol, we found that the rate of reduction of ferric P450 2D6 was enhanced by

The relatively rapid rates of oxidation of the methoxyphe-nyethylamines by P450 2D6 and the ability to employ kinetic hydrogen isotope effect analysis provided an opportunity to analyze the kinetics of these reactions in some detail. The step in which C–H bond cleavage occurs can be studied in detail in O-demethylation reactions due to (i) general considerations about using non-competitive intermolecular experiments to assess the rate-limiting contributions of C–H bond cleavage (3, 38); (ii) the ease of synthesis of the labeled substrates; (iii) the lack of an issue of prochirality of hydrogens; and (iv) the lack of complications of base-catalyzed proton abstraction encountered in N-dealkylation reactions (39). We analyzed several of the individual reaction steps in the P450 2D6-catalyzed O-demethylation of 3- and 4-methoxyphenethylamine and, as in earlier work with P450 1A2-catalyzed O-dealkylation of 4-alkoxyacylanilides (27), applied kinetic simulation analysis to the results. The work collectively implicates both the accumulation of an oxygenated iron complex and C–H bond breaking in contribution to the rate-limiting step(s) of the reactions. As in the case of the P450 1A2 reactions, these P450 2D6 reactions are probably sensitive to a set of transition state barriers of similar energies.

**EXPERIMENTAL PROCEDURES**

**Materials**

(+) Bufuralol/HCl was a gift of Hoffmann-La Roche. All commercially obtained chemicals were used without further purification, unless otherwise noted. THF was purchased as an anhydrous solvent and handled under argon or N2 when dry conditions were needed; all other solvents were ACS grade and used as received. Dopamine, 3-methoxyphenethylamine, 4-methoxyphenethylamine, and 4-hydroxyphenethylamine (p-tyramine) were purchased from Aldrich, and 3-hydroxyphenethylamine (m-tyramine) was from Sigma. These compounds were dissolved as free bases in (C6H5)2O and saturated with dry HCl gas. The precipitated HCl salts were recrystallized once or twice from absolute C2H5OH to yield white salts, which were dissolved in H2O and added to enzyme reactions (in the absence of organic solvents).

**Synthesis of Deuterated Methoxyphe-nyethylamines**

4-(2H) Methoxyphe-nyethylamine (d6)−4-Hydroxybenzyl cyanide (Aldrich, 4.26 g, 32 mmol) was mixed with d6-CH3I (Aldrich, 5.00 g, 35 mmol) and K2CO3 (4.42 g, 32 nmol) in 100 ml (CH3)2CO and heated with stirring under reflux for 40 h. The mixture was filtered, and the filtrate was concentrated to dryness in vacuo, dissolved in CHCl3, washed 3 times with 10% aqueous ROH, dried with Na2SO4, and (after filtration) evaporated to dryness in vacuo as follows: 4.68 g (98% yield); 1H NMR (9C-H), δ 8.60 (s, 2H, −CH3), 3.69 (s, 1H, −OCF3H), 6.80 and 7.18 (d, 2H each, aromatic).

Part of the above product (2.64 g, 18 mmol, unpurified) was dissolved in 20 ml of THF and added dropwise to a stirred solution of 18 ml of 1 M HCl in THF (under dry N2) in a 3-neck flask equipped with a condenser and drying tube (40). The reaction was stirred for an additional 90 min after all of the nitrile was added, and then 15 ml of concentrated HCl was carefully added. THF was removed in vacuo, and the residual aqueous phase was washed once with (C6H5)2O and made basic to litmus paper by the careful addition of 12 n NaOH. The product was extracted 3 times with CH2Cl2, and the combined organic fractions were dried with Na2SO4, filtered, and reduced in vacuo to give the HCl salt, which was recrystallized from absolute C6H5OH (yield 2.05 g, 60% yield): mp 211–215 ºC (uncorrected) (lit. 212–214 ºC (41)); m/z 154 (MH+), relative abundance 100, 137 (MH−−17, 20); 1H NMR (9C-H) δ 2.96 (dd, 2H, −CH2NH2), 3.28 (2H, PhCH2-CH2NH2), 3.70 (s, 1H, −OCF3H2), 7.04 (2H, 2H each, aromatic). The residue was treated with C2H3I (Cambridge Isotopes, Cambridge, MA) in methanol (10 ml), and the mixture was allowed to stand overnight under very mild reflux after the addition had been started at −78 ºC and allowed to come to room temperature (73% yield): 1H NMR (9C-H) δ 6.74 (d, 2H, −CH2NH2), 6.33 (s, 1H, −OH), 6.83 (m, 3H, aromatic), 7.21 (1H, aromatic α-H). The phenol was methylated with C6H5I as described for the 4-isomer and subsequently reduced with BH3 (see above) to yield the HCl salt, which was recrystallized from absolute C6H5OH (mp 209–212 ºC, uncorrected). 1H NMR and MS analysis indicated >99% atomic excess (MH+ m/z 155).

3-(3H) Methoxyphe-nyethylamine (d3)−3-Methoxbenzylecyanide (Aldrich) was demethylated by treatment with BBr3 in CH2Cl2 according to Ref. 42, with the exception that heating was done overnight under reflux after the reaction had been started at −78 ºC and allowed to come to room temperature (73% yield): 1H NMR (9C-H) δ 8.74 (s, 2H, −CH2NH2), 6.33 (s, 1H, −OH), 6.83 (m, 3H, aromatic), 7.21 (1H, aromatic α-H). The phenol was methylated with C6H5I as described for the 4-isomer and subsequently reduced with BH3 (see above) to yield the HCl salt, recrystallized from C6H5OH (mp 131.5–133.5 ºC); >99% atomic excess as indicated by MS (MH+ m/z 155) and 1H NMR: (9C-H) δ 8.24 (dd, 2H, −CH2NH2), 3.04 (dd, 2H, PhCH2-NH2), 6.82 (m, 3H, aromatic), 7.20 (1H, aromatic α-H).

**Expression and Purification of P450 2D6 in E. coli**

The cDNA sequence of full-length P450 2D6 construct DB 6 (32) was modified by PCR mutagenesis to insert a (His)6 sequence at the C terminus (33). In addition, a Met-374 to Val substitution was introduced into the original cDNA (32, 43) by site-directed mutagenesis to reflect the more common (wild type) P450 2D6 sequence (29, 44, 45).

**Expression (in E. coli) and purification were as described elsewhere (33). NADPH-P450 reductase (rat) was expressed in E. coli and purified as described (46).**

**Catalytic Assays**

Oxidation assays were generally conducted in 0.5-ml reaction volumes at 37 ºC for 5 min in 100 mM potassium phosphate buffer (pH 7.4) containing a NADPH-generating system (0.5 mM NADP+, 10 mM glucose 6-phosphate, and 1 unit of yeast glucose-6-phosphate dehydrogenase ml−1). Reactions contained P450 2D6 (200 pmol) reconstituted with NADPH-P450 reductase (500 pmol) and 30 µg of L-α-diuberyl-sn-

**SCHEME 2. Oxidations of phenethylamines (37).**

dissolved as free bases in (C6H5)2O and saturated with dry HCl gas. The precipitated HCl salts were recrystallized once or twice from absolute C6H5OH to yield white salts, which were dissolved in H2O and added to enzyme reactions (in the absence of organic solvents).

**Scheme 1. Generalized catalytic mechanism of P450.** See Refs. 11 and 17. This scheme is developed later in kinetic simulations. Note that k3 combines protonation and O−O bond scission; k8 combines hydrogen atom abstraction and rebound. Steps 8 and 9 are added in Scheme 4.
glycero-3-phosphocholine for 10 min at room temperature (prior to reactions). Mixtures were also supplemented with 1000 units of bovine liver catalase (previously diazoyl to remove thymol) and 20 μg of bovine erythrocyte superoxide dismutase, in order to destroy partially reduced oxygen species. Reaction mixtures were quenched with 50 μl of 68% HClO4 and kept on ice for 10 min (to precipitate protein and salts), followed by centrifugation at 3000 × g for 10 min. Aliquots (50–200 μl) of the recovered supernatants were injected into a Zorbax octadecylsilane HPLC column (3 μm, 6.2 × 80 mm, Mod-Cor, Chads Ford, PA), and eluted compounds were detected using a fluorescence detector (λ excitation 277 nm; λ emission 300 nm) (36). Chromatography was done at a flow rate of 2.5 ml min⁻¹ utilizing a 5 μl injection, 4.5 mm buffer containing CH₃CN (1.8%, v/v), with the gradient increasing to 45%, v/v, after holding for 4 min. Quantitation of the products was achieved using standard curves generated using 3-hydroxy-, 4-hydroxy-, and 3,4-dihydroxyphenethylamine (traces of the latter did not contribute appreciably and could be ignored in the analyses).

**Measurements of NADPH Oxidation and H₂O₂ and H₂O Production**

Measurements of NADPH oxidation were made using the reconstituted system described above (without catalase or superoxide dismutase) and with NADPH (200 μl) substituted for the NADPH-generating system. The decrease in A₄₁₀ was monitored (37 °C) in a modified Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Borton, CA). For the estimation of H₂O₂ reactions were done as described in the case of catalytic assays (no catalase or superoxide dismutase) and determined after 5 min with 0.83 ml of 3% Cl₂CCOCH₃ (w/v) (47). H₂O₂ was determined spectrophotometrically by reaction with ferroinophosphate sulfamate and KSCN as described (48). H₂O formation was determined by calculating the difference between total NADPH utilized and the sum of the product (derived from substrate) and H₂O₂ produced and then dividing by 2, because four electrons are required to reduce O₂ to H₂O (49). No correction was made for O₂ formation through degassing or dissolved in the solution. The decrease in absorbance at a flow rate of 2.5 ml min⁻¹ was followed by reaction with ferroinophosphate sulfamate and KSCN as described (48). H₂O formation was determined by calculating the difference between total NADPH utilized and the sum of the product (derived from substrate) and H₂O₂ produced and then dividing by 2, because four electrons are required to reduce O₂ to H₂O (49). No correction was made for O₂ formation through degassing or dissolved in the solution.

**Estimation of Kinetic Isotope Effects**

Deuterium isotope effects were determined by both non-competitive and competitive methods (2, 3). In the non-competitive experiments, P450 2D6 was incubated with either the unlabeled (d₅) or labeled (d₆) methoxyphenethylamine using a range of substrate concentrations (10–5000 μM) in the general reconstituted system (37 °C, 5 min), and aliquots were analyzed for product formation (phenols) by HPLC as described above. Kₘ and Vₘ₀ were first estimated by nonlinear regression analysis of the data at the lower substrate concentrations, in which inhibition by a product was not observed, using GraphPad Prism software (GraphPad, San Diego, CA).

The competitive isotope effect was determined for the O-demethylation of a-phenylphenethylamine (d₅-OCCH₃). HCHO was recovered from an incubation (5 min, 37 °C) as the 2,4-dinitrophenylhydrazone and analyzed by combined gas chromatography/MS (positive ion mode) as described in detail previously (27). The [H₃]formaldehyde hydrazone was monitored at m/z 211, and the [H₄]formaldehyde hydrazone was monitored at m/z 212. The ratio (2M+2)/(M + 1) (m/z 212/211, from integration of selected ion monitoring chromatograms), was used as an estimate of the intrinsic kinetic isotope effect of 9.6 ± 0.99. Thus, rate-limiting steps in methoxyphenethylamine oxidations catalyzed by P450 2D6 occur prior to product formation (steps 1–6 of Scheme 1).

**Measurement of P450 2D6 Reduction Rates**

P450 2D6 (7.5 nmol) was mixed with NADPH-P450 reductase (15 nmol) and freshly sonicated 1-a-dialylor-s-n-glycero-3-phosphocholine (250 μg). The reconstituted protein was supplemented with glucose oxidase (10 units ml⁻¹), diazoyl against 100 μM potassium phosphate buffer (pH 7.4), bovine liver catalase (450 units ml⁻¹, diazoyl), and the substrate of interest in appropriate cases and brought to a final volume of 6.9 ml with 100 μM potassium phosphate buffer (pH 7.4) in gas-tight tonometers. Anaerobic conditions were achieved with intermittent vacuum and argon/CO cycles and the subsequent addition of glucose to 0.10 m. Rates of P450 reduction were measured using stopped-flow spectrophotometry at 37 °C, essentially as described (48). Measurements of NADPH Oxidation and H₂O₂ and H₂O Production

**Kinetic Modeling**

Results of steady-state kinetic experiments were fit using the program DynaFit (50), which is based in part upon KINSIM and FTTSIM approaches (51–53) but has several advantages and has been applied to simulation of P450 kinetics described previously (27). The program was kindly provided by P. Kuzmic (Biokin Ltd., Pullman, WA) and run on a Macintosh G4 computer (Apple Computer, Cupertino, CA). The program can be used for reiterative fitting of rate and equilibrium constants. Some typical files are included as Supplemental Data.

**RESULTS**

**Lack of Burst of Product Formation in Oxidation of Methoxyphenethylamines—Quench reactions were done to determine whether a burst in product formation occurs. An observed burst would indicate that the product of the O-demethylation reaction (i.e. phenol) remains in the active site, and therefore its release limits the steady-state rate, or that some other step following production formation is rate-limiting (25, 26). The kinetic courses of phenol formation are shown in Fig. 1. The observed data points were fit to linear regression curves, passing through zero with no obvious indication of any burst (r = 0.99). Thus, rate-limiting steps in methoxyphenethylamine oxidations catalyzed by P450 2D6 occur prior to product formation (steps 1–6 of Scheme 1).

**Effects of Methoxyphenethylamines on Rates of Ferric P450 2D6 Reduction—**The rate of P450 2D6 reduction (step 2, Scheme 1) was much faster than steady-state turnover, even in the absence of substrate (Table I), as reported previously (33). In the earlier work, reduction was stimulated in the presence of bufuralol (33). Rates of P450 2D6 reduction were stimulated 4- and 15-fold in the presence of 3- and 4-methoxyphenethylamine, respectively. In all cases, the observed absorbance changes were fitted to double exponentials which afforded better fits than single exponentials.

In a separate experiment, a reduction rate of 1920 min⁻¹ was measured in the presence of the ligand quinidine, a competitive inhibitor (54).

**Kinetic Hydrogen Isotope Effects—**Preliminary studies with 3- and 4- (d₅) and (d₆) methoxyphenethylamines yielded significant non-competitive kinetic deuterium isotope effects in both cases, in the order of 3–4. A non-competitive intramolecular experiment was done with 4-[H₃]methoxyphenethylamine (d₆), with analysis of the formaldehyde 2,4-dinitrophenylhydrazone by gas chromatography/MS (results not shown). Analysis of the selected ion monitoring data, with correction for background in control experiments devoid of NADPH, yielded an estimate of the intrinsic isotope effect of 9.6 ± 0.99.

**FIG. 1. Lack of kinetic burst in O-demethylation of 3- and 4-methoxyphenethylamine.** The reactions were done at 23 °C with 1.0 (4-methoxy (4-OME), □) or 0.5 (3-methoxy (3-OME), ●), nmol of P450 2D6.

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TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_1$</th>
<th>$\Delta \lambda_{448}$</th>
<th>$k_2$</th>
<th>$\Delta \lambda_{448}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>140</td>
<td>$1.1 \times 10^{-2}$</td>
<td>17</td>
<td>$0.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>4-Methoxymethamphetamine</td>
<td>2220</td>
<td>$1.8 \times 10^{-2}$</td>
<td>95</td>
<td>$0.4 \times 10^{-2}$</td>
</tr>
<tr>
<td>3-Methoxymethamphetamine</td>
<td>520</td>
<td>$1.1 \times 10^{-2}$</td>
<td>55</td>
<td>$0.5 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

* First part of reaction.
* Second part of reaction.

Intermolecular non-competitive isotope effects were measured with $d_5$- and $d_4$-methoxy substrates, under conditions in which minimal depletion of the substrate was observed and the further oxidation of phenols to catechols was not a factor (37). With both 3- and 4-methoxymethamphetamine, inhibition was observed at high substrate concentrations (to ~75% of the optimal activity, see below). The behavior was subsequently attributed to formation of an inhibitor (see below). Analysis of the data obtained with substrate concentrations of <100–200 μM (i.e. hyperbolic portions of curves) yielded estimates of $\Delta V = 3.7$ and $D(V/K) = 3.8$ for the O-demethylation of 3-methoxymethamphetamine and $\Delta V = 3.1$ and $D(V/K) = 3.4$ for the O-demethylation of 4-methoxymethamphetamine.

Measurements of NADPH Oxidation and H$_2$O$_2$ Formation—Rates were measured under conditions used for the assays of product formation and are presented in Table II. Rates of uncoupling of NADPH oxidation to produce H$_2$O were calculated by difference. These values were used in subsequent fitting of kinetic constants.

Steady-state Spectra of P450 2D6 Reactions—The steady-state experiments were done without substrate and with the substrate buforalol and the inhibitor quinidine, in a mode in which ferric P450 2D6 was the reference solution (with ligand but no NADPH) (Fig. 2). The first difference spectrum observed after the addition of NADPH to the incubation shown in Fig. 2 had a broad $\lambda_{max}$ at 440 nm in the difference spectrum (versus P450 2D6 reduced by NADPH in the absence of substrate), similar to previously reported spectra attributed to a Fe$^{2+}$-O$_2$ substrate complex observed with rat liver microsomes (55) and P450 2B4 (56). The 440-nm band persisted through the first 5–10 min of reaction at 37 °C. Similar spectra (Fig. 3, trace 2) were observed for both methoxymethanamines prior to formation of the later 448-nm complexes.

Oxidation of Methoxymethanamines to Inhibitors—The inhibition of O-demethylation of methoxymethanamines at high substrate concentrations was observed in our earlier work with these compounds (37). This behavior was also observed here, particularly at millimolar substrate concentrations. The inhibitory patterns persisted despite further recrystallization of the hydrochloride salts and was more pronounced with the deuterated substrates, i.e. began to occur at lower substrate concentrations.

One possible explanation for the inhibitory patterns is that an inhibitor was being generated in a pathway that is competitive with O-demethylation. Primary amines are known to be oxidized to C-nitroso compounds that can bind tightly to and inhibit ferrous P450 (57) (Scheme 3). These inhibitor complexes had not been described for P450 2D6. Incubations yielded a spectral complex with a $\lambda_{max}$ at 448 nm in the difference spectrum (Fig. 3, traces 3 and 4), characteristic of such complexes (57). The development of the 448-nm peak was faster with $d_5$-methoxy substrate (than $d_5$ substrate or the phenol) with both the 3- and 4-substituted phenethanamines (Fig. 4).

Kinetic Simulations of Individual Rate Constants—Several initial comments are in order about the approach, which has been applied to P450 1A2 previously (27). A possible mechanism (based on Scheme 1) is written for the program DynaFit, with estimates of rate constants. The program simulates the curve for a plot of $v$ versus $S$ and, if an estimate of a rate constant is provided, can adjust that constant to achieve the best fit to the actual data. The results must fit limits of experimental values, when these can be obtained. In this and other kinetic work, we routinely use diffusion-limited rates for bi-omolecular reactions (6000 μM$^{-1}$ min$^{-1} = 10^8$ M$^{-1}$ s$^{-1}$) as an upper limit for rates (58). One boundary on the system is that the fitted values should be consistent with estimated rates of production of H$_2$O$_2$ and H$_2$O (Table II), when analyzed with the DynaFit program in separate trials (for plots of product versus time). Another boundary is that substitution of a substrate protium with a deuterium should yield a satisfactory fit with no adjustment to binding constants but only the rate constants related to C–H bond cleavage (27).

Initial simulation work was done with the mechanism outlined in Scheme 1 applied to the initial hyperbolic phase of the O-demethylation of 4-methoxymethanamine, an approach that had been applied previously with O-dealkylations catalyzed by P450 1A2 (27). The aspect was relatively straightforward and yielded a good fit with rates or constants close to those shown for steps 1–7, 10, and 11 (Scheme 1) in Table III. The ratio $k_{1.2}/k_{1} = 200$ μM approximates the $K_s$ (substrate binding) of 170 μM previously estimated for the type I spectral change (37). $k_7$ (reduction) was used directly from Table I (first part of reaction). In the first analyses, $k_{1.2}$ values of 600–6,000 min$^{-1}$ were used (for release of O$_2$ from the Fe$^{2+}$–O$_2$ complex). Logically this value should be in a low micromolar range, in the context of estimates of the $K_{D,O_2}$ for bacterial P450 2A5s (59). $k_7$ and $k_6$ were estimated by fitting of the plots to the data, and $k_{10}$ and $k_{11}$ were set to be compatible with both fits of $v$ versus $S$ and the measured rates of H$_2$O$_2$ and H$_2$O (Table II). Rates of production of H$_2$O$_2$ are measured directly (and superoxide would decompose to H$_2$O in the assay); the rates of H$_2$O formation are calculated from differences and inherently have more error.

The value of $k_7$ ("on" rate for product) was optimized at 800 min$^{-1}$ (Table III) and could not be raised appreciably without a loss of fit of the plot to the data points. There is some possible incongruity here, because spectral titrations with the product 4-hydroxyphenethylamine yield an apparent $K_d$ of 2400 μM, compared with $k_7/k_{7.7} = 0.11$ μM$^{-1}$ (37). This situation is reminiscent of the kinetic simulations of 4-alkoxyacylanilide O-dealkylation by P450 1A2 (27). One explanation (for both cases) is that the spectral titrations do not reflect the actual affinity of the product for the P450. This rationalization probably has more validity in the case of 4-hydroxyphenethylamine/P450 2D6 than of 4-hydroxyacylanilides/P450 1A2, because the type II binding spectrum may reflect a different substrate-ligand interaction than that relevant to the affinity of product binding during catalysis. However, a further problem is that the $K_s$ value for the oxidation of 4-hydroxyphenethylamine to dopamine was 1600 μM (37). The Michaelis constant $K_m$ may not reflect the inherent affinity, but the values tend to favor a relatively low affinity of 4-hydroxyphenethylamine for the P450 2D6-binding site. A more favored explanation for the relatively low value of the apparent "off" rate $k_7$, is that $k_7$ is a relatively slow step, followed by an equilibrium with only medium affinity. Such a system, with an added step, can be added to the mechanism and yields good fits that are not very sensitive to the actual affinity (Fig. 5).

A major problem in fitting a model to the data points of Fig. 5 was the inhibition that occurred at high substrate concentrations. This inhibition might have been dismissed as due to impurities in the substrate, i.e. amine free bases often develop color rapidly upon standing in air. However, the inhibitory patterns were seen with twice-recrystallized HCl salts of both.
3- and 4-methoxyphenethylamine, with both $d_0$ and $d_3$ substrates. Good fits could be easily obtained by simply adding a second, possibly allosteric, binding of substrate ($E_{SS}/H11001S_iE_{SS}$, where $E_{SS}$ is inactive). However, we have no independent evidence for such a second binding site, and the model will not explain the results obtained with deuterated substrates; the substrate concentration at which optimal activity was seen was shifted to a lower value by deuterium substitution (Fig. 5). This pattern is consistent with the conversion of methoxyphenethylamines to inhibitors, as well as the hydroxyphenethylamines. Although we did not isolate and characterize the inhibitory product, the production of a 448 nm complex was observed in reactions (Figs. 3 and 4), and the nature of this complex is probably that of a $C$-nitroso-$Fe^{2+}/H11001$ complex (57) (Scheme 3). By using the assumption that a product was generated with high affinity for ferrous P450 2D6 (57), some steps were added to the general scheme (Scheme 1) to include these phenomena (Scheme 4). For simplicity, the substrate $S$ is converted (by P450 2D6) to a second product $Q$ (catalyzed by the enzyme, P450 2D6 = $E$), which has high affinity for the reduced form of P450 (F). (The substrate-bound form was used here (FS), simply to avoid the need to introduce a substrate dissociation step for ferrous P450 2D6. Furthermore, the rapid reduction of ferric to ferrous P450 (see Table 1) indicates that this should not be an issue.)

The model involving steps 8 (inhibitor formation) and 9 (in-

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>NADPH oxidation$^a$</th>
<th>Product formation</th>
<th>$H_2O_2$ production$^a$</th>
<th>$H_2O$ formation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinidine</td>
<td>5</td>
<td>24 ± 1</td>
<td>4.6 ± 1.1</td>
<td>9.5</td>
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</tr>
<tr>
<td>Bufuralol</td>
<td>200</td>
<td>13 ± 4</td>
<td>&lt;0.001</td>
<td>5.9 ± 1.4</td>
<td>3.4</td>
</tr>
<tr>
<td>4-Methoxyphenethylamine ($d_0$)</td>
<td>300</td>
<td>50 ± 4</td>
<td>21</td>
<td>7.0 ± 0.1</td>
<td>11</td>
</tr>
<tr>
<td>4-Methoxyphenethylamine ($d_3$)</td>
<td>66 ± 3</td>
<td>2.8</td>
<td>8.6 ± 1.8</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxyphenethylamine</td>
<td>300</td>
<td>27 ± 1</td>
<td>10</td>
<td>8.4 ± 1.6</td>
<td>4</td>
</tr>
<tr>
<td>3-Methoxyphenethylamine ($d_0$)</td>
<td>500</td>
<td>42 ± 3</td>
<td>18</td>
<td>6.4 ± 0.2</td>
<td>9</td>
</tr>
<tr>
<td>3-Methoxyphenethylamine ($d_3$)</td>
<td>500</td>
<td>24 ± 2</td>
<td>3.0</td>
<td>15.2 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>3-Hydroxyphenethylamine</td>
<td>300</td>
<td>30 ± 1</td>
<td>2.5</td>
<td>7.0 ± 1.1</td>
<td>10</td>
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TABLE II

<table>
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<tr>
<th>Coupling efficiency for P450 2D6 reactions</th>
</tr>
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<tbody>
<tr>
<td>Addition</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Quinidine</td>
</tr>
<tr>
<td>Bufuralol</td>
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<tr>
<td>4-Methoxyphenethylamine ($d_0$)</td>
</tr>
<tr>
<td>4-Methoxyphenethylamine ($d_3$)</td>
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<td>3-Methoxyphenethylamine ($d_3$)</td>
</tr>
<tr>
<td>3-Hydroxyphenethylamine</td>
</tr>
</tbody>
</table>

$^a$ Means (± S.D.) of triplicate determinations.

$^b$ Calculated by difference: nmol of $H_2O = \frac{1}{2}$ (nmol NADPH oxidized-nmol $H_2O_2$-nmol product formed) (49).

$^c$ Ref. 34.


FIG. 2. Spectral complexes accumulating in steady-state turnover reactions catalyzed by P450 2D6. A, absolute spectra of ferric P450 2D6 (2.0 μM, in presence of 4.0 μM NADPH-P450 reductase), to which 10 μM quinidine and then an NADPH-generating system were subsequently added. B, difference spectra (+NADPH-generating system versus none) for similar incubations versus ferric state, under conditions used in A ($t = 2–5$ min, at $37^\circ C$).

FIG. 3. Conversion of 3-methoxyphenethylamine to an inhibitor complex. Both the reference and sample cuvettes contained 1.0 μM P450 2D6, 2.0 μM NADPH-P450 reductase, 45 μM L-α-dilauroyl-sn-glycero-3-phosphocholine, and 100 mM potassium phosphate buffer (1.0 ml volume) (all at $37^\circ C$). A base line was recorded, and 500 μM 3-[2H_3]methoxyphenethylamine was added to the sample cuvette (trace 1); then 50 μl of a preincubated mixture of 67 mM glucose 6-phosphate, 3.3 mM NADP$^+$, and 13 μg of yeast glucose-6-phosphate dehydrogenase ml$^{-1}$ was added to both cuvettes. The indicated spectra were recorded after 2.5 min (trace 2), 10 min (trace 3), and 20 min (trace 4).
Inhibitor binding (Scheme 4) could be used to fit the O-demethylation of both 3- and 4-methoxyphenethylamine (Fig. 5). The steps described by \( k_a \) and \( k_a \) indicate slow production of the inhibitor but very high affinity. Attempts to lower the affinity (raise \( k_a \)) and raise the rate of formation (\( k_a \)) yielded a drop in the maximal rate of product formation.

Fitting of the model to the \( d_3 \) data points was done with the concept that affinity constants should not be altered and only steps associated with C–H bond breaking chemistry and compensations should be altered. Good fits for both the 3- and 4-methoxyphenethylamine \( d_3 \) data sets could be handled by decreasing \( k_a \) (productive O-demethylation) and increasing \( k_a \) (inhibitor formation) (Fig. 5). The decreases in \( k_a \) are consistent with the observed \( \Delta V \) and \( \Delta (V/K) \) effects at the lower substrate concentrations (Fig. 5). The increases in \( k_a \) in these simple models are proposed to reflect partial "metabolic switching" to alternate reactions (60) because the decreases in hydroxyphenethylamine formation were not compensated for in \( \text{H}_2\text{O}_2 \) production, and \( \text{H}_2\text{O} \) production has uncertainty in this setting because of the inability of direct measurement.

The models for 3-methoxyphenethylamine O-demethylation could be developed with very similar constants as for 4-methoxyphenethylamine, with some adjustment for the lower rate of \( k_2 \) (Table I and Fig. 5).
DISCUSSION

In previous work (27), we systematically analyzed kinetic aspects of the catalytic cycle of human P450 1A2 and some mutants, utilizing kinetic hydrogen isotope effects and simulations. Similar approaches were applied to P450 2D6 and the oxidation of methoxyphenethylamines. The oxidation of hydroxyphenethylamines may have some physiological relevance in the formation of dopamine (36), although the O-demethylation reactions should only be considered models. However, these models are of significance in that they enable the application of kinetic hydrogen isotope approaches, and the rates of these demethylation reactions are among the highest reported for P450 2D6, an enzyme involved in the oxidation of ~30% of the drugs used today.

Of the steps shown in Scheme 1, few are amenable to direct measurement. In step 2, the reduction of ferric P450 2D6 was measured using stopped-flow techniques (Table I). The rate of reduction is relatively fast in the absence of substrate and is further enhanced in the presence of methoxyphenethylamines. Another step amenable to experimental measurement is step 1, substrate binding, in which $K_d$ can be estimated using spectral titrations (37). We assume that the “on” rate ($k_1$) is fast, as generally treated in kinetic analysis (58, 61), and that $K_d$ is dominated by the off rate ($k_1^{-1}$).

Beyond steps 1 and 2 of Scheme 1 our analysis of individual rate constants is rather indirect. In the simulation analysis, we have arbitrarily set the reduction rates $k_4 = k_5$. This assumption may or may not be valid; only a situation with $k_4 < k_5$ will affect the major conclusions we have reached. Several arguments for thermodynamic ease of reduction step 4 relative to step 2 have been presented (11, 62, 63). The kinetic situation may differ, however. One of the bases of the rate-limiting contribution of step 4 in P450 reactions has been the stimulatory effect of cytochrome $b_5$ (with facilitation of step 4 through electron transport (18, 19, 64, 65)), although to our knowledge no stimulation of P450 2D6 reactions by cytochrome $b_5$ has been observed (65). Step 7 (presented as a product off rate in Scheme 1) is apparently fast enough not to produce burst kinetics (Fig. 1), but the simulations indicated that $k_7$ was much less than predicted by spectral estimates of the product $K_d$ (37). Although this step ($k_7$) should not be considered rate-limiting per se, it appears to be slow enough to contribute to the apparent $K_m$. To expand on this, simulations show that the $K_d$ of the reaction product can modify the apparent $K_m$ for the substrate. Step 10 can be approximated by direct measurement of $H_2O_2$ formation, and step 11 ($H_2O$ production) can be estimated from differences; however, these measured rates should not be considered to be $k_{10}$ and $k_{11}$, as shown by the fitting analyses (Table III). These parameters can affect both the $k_{cat}$ and apparent $K_m$ of the overall O-demethylation reactions, as shown previously for P450 1A2 (27). This discussion of competition of productive catalysis with abortive oxygen reduction ($k_{4}$ versus $k_{4'}$ of Scheme 1) has a parallel in discussions of “unmasking” of kinetic isotope effects by Higgins et al. (66); i.e. step 11 is viewed as unmasking the kinetic hydrogen isotope effect on step 6. Thus, the kinetic isotope effect can be viewed as a manifestation of the competition of steps 6 and 11. In a general sense, this view also sets the rate of step 6 as not fast enough to fully compete with an alternate step, which is also a form of rate limitation, although not exactly in the sense of stopping a reaction with no alternative “vents.”

What is rate-limiting in the overall O-demethylation reactions considered here? Our results implicate primarily steps 4 (entry of second electron) and 5 (O–O bond cleavage). Step 6, the C–H bond breaking step, clearly contributes as manifested in the $^{18}V$ and $^{1}V(K)$ values of ~3.5. There is some uncertainty in these values because the estimates were restricted to the hyperbolic phase of the reactions; nevertheless, the effect of deuterium substitution on both $k_{cat}$ and $k_{cat}/K_m$ is clearly seen (Fig. 5). Evidence for this view comes from the DynaFit simulations (Table III), with kinetic isotope effects of 6.0 and 2.5 estimated on $k_6$ in the respective O-demethylations of 3- and 4-methoxyphenethylamine (Fig. 5 and Table III). The estimated intrinsic kinetic deuterium isotope effect (9.6) is clearly attenuated in the non-competitive experiments (Fig. 5). The other major kinetic contributor to the mechanism appears to be step 5 (Scheme 1). The accumulation of a spectral complex(es) in the reaction steady-state supports this view (Figs. 2 and 3). Further evidence for this conclusion comes from the DynaFit simulations (Table III). The accumulated intermediate has a difference spectrum that resembles that attributed to the FeO$_2^{2+}$-substrate species in previous work (55, 56, 67). The appearance of the complex was dependent upon the presence of a substrate or the inhibitor quinidine (Figs. 2 and 3).2 However, the electronic identity of this complex is unknown, and the possibility cannot be ruled out that this is a mixture of complexes. Hypervalent iron (oxygen) complexes have somewhat similar spectra (69), and exact assignments of many of the optical spectra in the literature are tenuous in the absence of additional evidence.

A difficulty of dealing with intermediate steps in P450 catalysis is the complexity and uncertainty regarding the intermediate Fe–O complexes (70–72). We have chosen a relatively simplified model for these reactions, although it may not be inclusive of all possibilities. Some limits can be imposed on the kinetic possibilities in this model by the $v$ versus $S$ plots and also the experimentally determined rates of $H_2O_2$ (and, at least in the case of $d_0$ substrates, estimated rates of $H_2O$ production). Although step 10 yields $H_2O_2$, other steps in the mechanism may contribute to $H_2O_2$ production (e.g. collapse of the FeO$_2^{2+}$ intermediate to $O_2$ and non-enzymatic dismutation). In addition, we did not separate 1-electron reduction and protonation within step 4 of the minimal model (Scheme 1).

In comparison with other kinetic modeling work done on P450s in this laboratory (27), some difficulty was experienced in deriving a model to explain the inhibition of O-demethylation at high substrate concentrations. Fits of the $d_0$ data (for O-demethylation of either 3- or 4-methoxyphenethylamine) could be achieved by simply introducing the step $ES + S = ESS$ and floating a $K_d$ value ($E = P450$ 2D6, $S =$ methoxyphenethylamine) (results not shown). Indeed, the fitting of non-hyperbolic P450 catalytic results to multiple substrate models based on multiple $k_{cat}$ and $K_m$ values has been popular (73–76). However, these models are relatively easy to fit to complex data but often are fraught with the issues of (i) lack of limitations on the systems used (i.e. limited data sets) and particularly (ii) limited explanation of parameters (i.e. what $k_{cat}$ and $K_m$ really represent). As mentioned previously, the addition of an $ES + S = ESS$ (inactive) step has no experimental basis in this case and is inconsistent with other experimental information reported here (e.g. changes in inhibitory patterns due to deuterium substitution), and a more complex model was deemed to be more appropriate (Schemes 1 and 4; Table III). It should be pointed out that some combinations of rate constants in the

2 Quinidine is a competitive inhibitor of P450 2D6 (54) and did not appear to be a substrate (68). We carried out HPLC/MS assays of incubations of quinidine with reconstituted recombinant P450 2D6 and could find no evidence for any oxidation (detection limit < 0.001 nmol of product formed min$^{-1}$ (nmol P450 2D6)$^{-1}$. The presence of quinidine enhanced the rate of reduction of ferric P450 2D6 (to 1920 min$^{-1}$) (cf. Table I). However, in steady-state assays quinidine lowered the rate of NADPH oxidation and had no significant effect on the rate of $H_2O_2$ production (suggesting decreased $H_2O$ formation) (Table II).
kinetic models even began to yield sigmoidal kinetics in the absence of multiple substrate contexts, and further consideration of the complexity of P450 reactions showing non-hyperbolic kinetics is in order (77). Despite limitations in the proposed kinetic mechanism (26, 27) (Schemes I and 4; Table III), we are of the opinion that there is considerable value in applying stepwise kinetic models in P450 analysis.

One issue that should be considered further is $k_{cat}$, an off rate (Scheme 1). As emphasized earlier, plots of $v$ versus $S$ did not fit for the $O$-demethylation reactions unless $k_{cat}$ was assigned a value much lower than that predicted by the estimated $K_{m}$ for the product. The same problem was observed with P450 1A2 reactions in earlier work (27). The rates used here are still relatively high (Table III), and burst kinetics were not observed (Fig. 1). In the cases of oxidation of ethanol and acetaldehyde by P450 2E1, burst kinetics were clearly observed, and the only viable interpretation is that the rate-determining step occurs after product formation (25, 26). 3 We showed that neither P450 2E1 nor P450 1A2 appeared to have strong product affinity, and this seems to be the case with these phenolic products of 2E1 nor P450 1A2 appeared to have strong product affinity, and the work presented here appears to be the first reported catalysis of O-demethylation reactions unless $k_{cat}$ did not fit.

In conclusion, we have examined several aspects of the catalysis of O-demethylation of 3- and 4-methoxyphenethylamine by recombinant P450 2D6 using direct assays, kinetic hydrogen isotope effects, steady-state optical spectra, and kinetic simulation. Although much of the available literature on P450 2D6 emphasizes virtual substrate docking as a determinant of catalysis (e.g. Refs. 10 and 81), our studies with these model substrates lead to a paradigm of P450 2D6 catalysis in which the rate of C–H bond breaking is a major contributor but not the only contributor. The ease of bond breaking can be related in part to proximity of substrate docking (23). Evidence was also obtained from spectral accumulation and kinetic fitting that high-valent iron intermediates accumulate, implicating other contributions to rate determination. Effects of changes in other steps of catalysis also influenced the fitting of the models to the data. Some aspects of the kinetic analysis may be more general to other mammalian P450s, e.g. rate-limiting C–H bond breaking and accumulation of a high-valent FeO complex.

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Oxidation of Methoxyphenethylamines by Cytochrome P450 2D6: ANALYSIS OF RATE-LIMITING STEPS
F. Peter Guengerich, Grover P. Miller, Imad H. Hanna, Hideaki Sato and Martha V. Martin

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