Oxidation of Methoxyphenethylamines by Cytochrome P450 2D6

ANALYSIS OF RATE-LIMITING STEPS*\[5\]

F. Peter Guengerich‡, Grover P. Miller§, Imad H. Hanna¶, Hideaki Sato, and Martha V. Martin
From the Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

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 \( K_m \) 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\[1\] 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...
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 ferrous P450 2D6 was enhanced by bufuralol and was not the rate-limiting step in the overall 1'-hydroxylation reaction (33). Hiroi et al. (36) had reported the oxidation of 4-hydroxyphenethylamine to 3,4-dihydroxyphenethylamine (dopamine) by P450 2D6; we subsequently found that the rates of O-demethylation of 3- and 4-methoxyphenethylamines were among the fastest of the reactions catalyzed by P450 2D6 (37). Analysis of the binding of these compounds to P450 2D6 provided evidence for the alteration of the substrate pKₐ by P450 2D6 (37).

The relatively rapid rates of oxidation of the methoxyphenethylamines 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**

(z)-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 (C₅H₅)₂O and saturated with dry HCl gas. The precipitated HCl salts were recrystallized once or twice from absolute C₂H₂O to yield white salts, which were dissolved in H₂O and added to enzyme reactions (in the absence of organic solvents).

**Synthesis of Deuterated Methoxyphenethylamines**

4-²H₂[Met]hoxophenethylamine (d₂)-4-Hydroxybenzyl cyanide (Aldrich, 4.26 g, 32 mmol) was mixed with d₄-CH₃I (Aldrich, 5.00 g, 35 mmol) and K₂CO₃ (4.42 g, 32 mmol) in 100 ml of (C₅H₅)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 KOH, and (after filtration) evaporated to dryness in vacuo as follows: 4.68 g (98% yield); [H] NMR (C⁶D₆), δ 6.36 (s, 2H, –CH₂–), 3.69 (s, 1H, –OCF₂H₂), 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 (C₂H₅)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, and (after filtration) evaporated to dryness in vacuo as follows: 2.05 g (60% yield): mp 211–215°C (uncorrected) (lit. 212–214°C (41)); m/z 154 (M⁺, relative abundance 100), 137 (M⁺–17, 20); [H] NMR (C⁶D₆), δ 2.96 (dd, 2H, CH₂–), 3.28 (dd, 2H, PhCH₂–), 3.70 (s, 1H, –OCF₂H₂), 7.04 (d, 2H, aromatic H ortho to CH₂–), 7.20 (d, 2H, aromatic H meta to CH₂–).

4-²H₃[Met]hoxophenethylamine (d₃)-4-Hydroxybenzyl cyanide was treated with [H]I, (Cambridge Isotopes, Cambridge, MA) in the manner described above and then reduced with BH₃, yielding the HCl salt, which was recrystallized from absolute C₂H₂O–HCl (yield 2.05 g, 60% yield): mp 211–215°C (uncorrected) (lit. 212–214°C (41)); m/z 154 (M⁺, relative abundance 100), 137 (M⁺–17, 20); [H] NMR (C⁶D₆), δ 2.96 (dd, 2H, CH₂–), 3.28 (dd, 2H, PhCH₂–), 3.70 (s, 1H, –OCF₂H₂), 7.04 (d, 2H, aromatic H ortho to CH₂–), 7.20 (d, 2H, aromatic H meta to CH₂–).

3-Methoxybenzylcyanide (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°C and allowed to come to room temperature (73% yield): [H] NMR (C⁶D₆), δ 3.74 (s, 2H, –CH₂–), 6.33 (s, 1H, –OH), 6.83 (m, 3H, aromatic), 7.21 (t, 1H, aromatic o-H). The phenol was methylated with C⁶H₄I as described for the 4-isomer and subsequently reduced with BH₃ (see above) to yield the HCl salt, recrystallized from C₂H₂O–HCl (mp 131.5–133.5°C); >99% atomic excess as indicated by MS (M⁺+m/z 155) and [H] NMR: (C⁶D₆), δ 2.84 (dd, 2H, –CH₂–), 3.04 (dd, 2H, PhCH₂–), 6.82 (m, 3H, aromatic), 7.20 (t, 1H, aromatic o-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 His₆ 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°C for 5 min in 100 mM potassium phosphate buffer (pH 7.4) containing an 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-diethylamino-n-
glicyero-3-phosphocholine for 10 min at room temperature (prior to reactions). Mixtures were also supplemented with 1000 units of bovine liver catalase (previously dialyzed 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% (v/v) 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 onto a Zorbax octadecylsilane HPLC column (3 μm, 6.2 × 80 mm, Mod-Cod, Chadds 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 utilizing gradient 0–4.5% CH3CN 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 Production

Measurements of NADPH oxidation were made using the reconstituted system described above (without catalase or superoxide dismutase) and with NADPH (200 μM) substituted for the NADPH-generating system. The decrease in A440 was monitored (37 °C) in a modified Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Bo- gart, GA). For the estimation of H2O2 reactions, reactions were done as described in the case of catalytic assays (no catalase or superoxide dismutase) and terminated after 5 min with 0.83 ml of 3% Cl2COOH (w/v) (47). H2O2 was determined spectrophotometrically by reaction with ferroammonium sulfate 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 assay correction was made for O2* which was not measured and assumed to be converted to H2O under the acidic assay conditions.

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 (d0) or labeled (d2) methoxyphenethylamine using a range of substrate concentrations (10–500 μM) in the general reconstituted system (37 °C, 5 min), and aliquots were analyzed for product formation (phenols) by HPLC as described above. Kmax and Vmax 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 4-[2H2]methoxyphenethylamine (d2-P450) and 4-[2H2]methoxyphenethylamine (d0-P450) in a modified Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA) at 37 °C. The decrease in absorbance at 440 nm was measured in the presence of the ligand quinidine, a competitive inhibitor (54).

Kinetic Hydrogen Isotope Effects—Preliminary studies with 3- and 4-[d0] and 3- and 4-[d2] methoxyphenethylamines yielded significant non-competitive kinetic deuterium isotope effects in both cases, in the range of 3–4. A non-competitive intramolecular experiment was done with 3-[dH]methoxyphenethylamine (d0-P450), 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 ± 1.0.

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 iterative fitting of rate and equilibrium constants. Some typical files are included as Supplemental Data.

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.

Rate-limiting Steps in P450 2D6 Catalysis

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-[d0] and 3- and 4-[d2] methoxyphenethylamines yielded significant non-competitive kinetic deuterium isotope effects in both cases, in the range of 3–4. A non-competitive intramolecular experiment was done with 3-[dH]methoxyphenethylamine (d0-P450), 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 ± 1.0.

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 butafurulol (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 bet- ter fits than single exponentials.)
Measurement of reduction rates of ferric P450 2D6

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_1$</th>
<th>$\Delta \lambda_{457}$</th>
<th>$k_2$</th>
<th>$\Delta \lambda_{448}$</th>
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<tr>
<td>None</td>
<td>$1.40 \times 10^2$</td>
<td>17</td>
<td>$0.8 \times 10^2$</td>
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<tr>
<td>4-Methoxyphenethylamine</td>
<td>$2.22 \times 10^2$</td>
<td>95</td>
<td>$0.4 \times 10^2$</td>
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<tr>
<td>3-Methoxyphenethylamine</td>
<td>$5.20 \times 10^2$</td>
<td>55</td>
<td>$0.5 \times 10^2$</td>
<td></td>
</tr>
</tbody>
</table>

$\Delta \lambda_{457}$ and $\Delta \lambda_{448}$ are maxima in the difference spectrum (versus ferric P450 2D6) reduced by NADPH in the absence of substrate.

$\Delta \lambda_{457}$ was the inhibition that occurred at high substrate concentrations (55). The 448-nm peak was faster with $d_6$-methoxy substrate (than $d_6$ substrate or the phenol) with both the 3- and 4-substituted phenethylamines (Scheme 4).

Kinetik 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 biomolecular reactions ($6000 \text{M}^{-1} \text{min}^{-1} = 10^8 \text{M}^{-1} \text{s}^{-1}$) 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 $\text{H}_2\text{O}_2$ and $\text{H}_2\text{O}$ (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 $\text{C–H}$ 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-methoxyphenethylamine, 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 rate constants close to those shown for steps 1–7, 10, and 11 (Scheme 1) in Table III. The ratio $k_{-1} / k_{1} = 200 \mu\text{M}$ approximates the $K_s$ (substrate binding) of 170 $\mu\text{M}$ previously estimated for the type I spectral change (37). $k_{-1} / k_{2}$ (reduction) was used directly from Table I (first part of reaction). In the first analyses, $k_{-1} / k_{2}$ values of 600–6000 min$^{-1}$ were used (for release of $\text{O}_2$ from the $\text{Fe}^{2+}–\text{O}_2$ complex). Logically this value should be in a low micromolar range, in the context of estimates of the $K_{\text{d},\text{O}_2}$ for bacterial P450s (59). $k_5$ 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 $\text{H}_2\text{O}_2$ and $\text{H}_2\text{O}$ (Table II). Rates of production of $\text{H}_2\text{O}_2$ are measured directly (and superoxide would decompose to $\text{H}_2\text{O}_2$ in the assay); the rates of $\text{H}_2\text{O}$ 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 inconstancy here, because spectral titrations with the product 4-hydroxyphenethylamine yield an apparent $K_d$ of 2400 $\mu\text{M}$, compared with $k_{-1} / k_{1} = 0.11 \mu\text{M}$ (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 $K_{n}$ value for the oxidation of 4-hydroxyphenethylamine to dopamine was 1600 $\mu\text{M}$ (37). The Michaelis constant $K_{n}$ 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 \(d_3\) sub-
strates. Good fits could be easily obtained by simply adding a
second, possibly allosteric, binding of substrate (\(E_{SS}/H11001S_{Si}E_{SS}\),
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 methoxypheneth-
ylamines 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\(\text{II}\)/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 II

<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>
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<tr>
<td></td>
<td>(\mu M)</td>
<td>(n mol \text{ min}^{-1} (n mol \text{ P450})^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>5</td>
<td>24 (\pm) 1</td>
<td>4.6 (\pm) 1.1</td>
<td>9.5</td>
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<tr>
<td>Bufuralol</td>
<td>200</td>
<td>13 (\pm) 4</td>
<td>&lt;0.001</td>
<td>5.9 (\pm) 1.4</td>
<td>3.4</td>
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<tr>
<td>4-Methoxyphenethylamine ((d_0))</td>
<td>200</td>
<td>50 (\pm) 4</td>
<td>21</td>
<td>7.0 (\pm) 0.1</td>
<td>11</td>
</tr>
<tr>
<td>4-Methoxyphenethylamine ((d_3))</td>
<td>200</td>
<td>66 (\pm) 3</td>
<td>2.8</td>
<td>8.6 (\pm) 1.8</td>
<td>27</td>
</tr>
<tr>
<td>4-Hydroxyphenethylamine</td>
<td>300</td>
<td>27 (\pm) 1</td>
<td>10</td>
<td>8.4 (\pm) 1.6</td>
<td>4</td>
</tr>
<tr>
<td>3-Methoxyphenethylamine ((d_0))</td>
<td>500</td>
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<td>18</td>
<td>6.4 (\pm) 0.2</td>
<td>9</td>
</tr>
<tr>
<td>3-Methoxyphenethylamine ((d_3))</td>
<td>500</td>
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<td>3.0</td>
<td>15.2 (\pm) 0.7</td>
<td>3</td>
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<td>30 (\pm) 1</td>
<td>2.5</td>
<td>7.0 (\pm) 1.1</td>
<td>10</td>
</tr>
</tbody>
</table>

None, reductase only

\(^a\) Means (\(\pm\) 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.

\(^d\) \(d_3\) \([2H_3]\)methoxyphenethylamine.

FIG. 2. Spectral complexes accumulating in steady-state turnover reactions catalyzed by P450 2D6. A, absolute spectra of ferric P450 2D6 (2.0 \(\mu M\), in presence of 4.0 \(\mu M\) NADPH-P450 reductase), to which 10 \(\mu M\) 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 °C).

FIG. 3. Conversion of 3-methoxyphenethylamine to an inhibitor complex. Both the reference and sample cuvettes contained 1.0 \(\mu M\) P450 2D6, 2.0 \(\mu M\) NADPH-P450 reductase, 45 \(\mu M\) L-\(\alpha\)-dilauroyl-snglycerol-3-phosphocholine, and 100 \(\mu M\) potassium phosphate buffer (1.0 ml volume) (all at 37 °C). A base line was recorded, and 500 \(\mu M\) \(3-[2H_3]\)methoxyphenethylamine was added to the sample cuvette (trace 1); then 50 \(\mu l\) of a preincubated mixture of 67 mM glucose 6-phosphate, 3.3 \(\mu M\) NADP\(^+\), and 13 \(\mu g\) 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).
ethylamine (incubation (with the NADPH-generating system) with 4-methoxyphenethylamine (d1) and 4-[2H3]methoxyphenethylamine (d2). B, the inset shows ΔA_{630-400} ε at individual time points for intermediate spectra recorded during the incubations (d0, C; d3, D).

**FIG. 4.** Conversion of 4-methoxyphenethylamine to an inhibitor complex: comparison of d0 and d3 substrates. The design of the experiments was as in Fig. 3, except that the concentrations of P450 2D6 and the reductase were 2.0 and 4.0 μM, respectively. A, a base-line spectrum is shown. Also shown are the spectra recorded after 14 min of incubation (with the NADPH-generating system) with 4-methoxyphenethylamine (d1) and 4-[2H3]methoxyphenethylamine (d2). The inset shows ΔA_{630-400} ε at individual time points for intermediate spectra recorded during the incubations (d0, C; d3, D).

**TABLE III.**

<table>
<thead>
<tr>
<th>Rate constants used for modeling oxidations of methoxyphenethylamines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>4-Methoxyphenethylamine</td>
</tr>
<tr>
<td>k₃ = 6000; k₋₃ = 1,200,000</td>
</tr>
<tr>
<td>k₄ = 2200</td>
</tr>
<tr>
<td>k₅ = 600; k₋₅ = 1,200,000</td>
</tr>
<tr>
<td>k₆ = 2200</td>
</tr>
<tr>
<td>k₇ = 170</td>
</tr>
<tr>
<td>k₈ = 800; k₋₈ = 600</td>
</tr>
<tr>
<td>k₉ = 0.7</td>
</tr>
<tr>
<td>k₁₀ = 6000; k₋₉ = 0.00001</td>
</tr>
<tr>
<td>k₁₁ = 50</td>
</tr>
<tr>
<td>k₁₂ = 65</td>
</tr>
</tbody>
</table>

*See Schemes 1 and 4. First order reactions are expressed as min⁻¹ and second order reactions are expressed as min⁻¹ μM⁻¹.*

**FIG. 5.** Kinetic simulation of results of O-demethylation of 4-methoxyphenethylamine (A) (expanded in B) and 3-methoxyphenethylamine (C) (expanded in D). See Schemes 1 and 3 for equations and Table III for rate constants (d₀, O). For d₁, 4-methoxyphenethylamine (O), the same rate constants were used except that k₈ = 70 and k₉ = 77 min⁻¹; for d₃, 3-methoxyphenethylamine (O) k₈ = 34 and k₉ = 86 min⁻¹.

**SCHEME 4.** Additions to kinetic model (Scheme 1) used for simulations. S, substrate (methoxyphenethylamine); Q, inhibitory product; FS, ferrous P450 2D6 (substrate complex).

![Scheme 3](Image)

**Scheme 3.** Proposed oxidation of methoxyphenethylamines to C-nitroso inhibitors that bind ferrous P450 2D6 (57).
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 methoxyphenethamines. The oxidation of hydroxyphenethamines 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 ferrie 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 methoxyphenethamines. 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 (\( k_7 \)) is fast, as generally treated in kinetic analysis (58, 61), and that \( K_d \) is dominated by the off rate (\( k_{-7} \)).

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 \( k_7 = k_2 \). This assumption may or may not be valid; only a situation with \( k_7 \ll k_2 \) 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 \( k_2 \) 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 \( \text{H}_2\text{O}_2 \) formation, and step 11 (\( \text{H}_2\text{O} \) 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 \( k_{\text{cat}} \) 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 (\( k_7 \) versus \( k_{11} \) 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 (\( \text{O} \)-\( \text{O} \) bond cleavage). Step 6, the \( \text{C} \)-\( \text{H} \) bond breaking step, clearly contributes as manifested in the \( ^{13}\text{C} \) and \( ^{13}\text{N} \text{V} \) 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 \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) 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 \( k_4 \) in the respective \( O \)-demethyllations 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 \( \text{FeO}_2^{2-} \)-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). 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 \( \text{Fe}^2+ \)-oxygen 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 \( \text{H}_2\text{O}_2 \) (and, at least in the case of \( d_0 \) substrates, estimated rates of \( \text{H}_2\text{O} \) production). Although step 10 yields \( \text{H}_2\text{O}_2 \), other steps in the mechanism may contribute to \( \text{H}_2\text{O}_2 \) production (e.g. collapse of the \( \text{FeO}_2^{2-} \) intermediate to \( \text{O}^2- \) 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 \( ES + S \rightleftharpoons ESS \) and floating a \( K_d \) value (\( E = \text{P450 2D6, S = methoxyphenethylamine} \) (results not shown). Indeed, the fitting of non-hyperbolic P450 catalytic results to multiple substrate models based on multiple \( k_{\text{cat}} \) 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 \( k_{\text{cat}} \) and \( K_m \) really represent). As mentioned previously, the addition of an \( ES + S \rightleftharpoons ESS \) (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

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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 \( \text{H}_2\text{O}_2 \) production (suggesting decreased \( \text{H}_2\text{O} \) 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 1 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 $k_a$, an off rate (Scheme 1). As emphasized earlier, plots of $v$ versus $S$ did not fit for the $O$-demethylation reactions unless $k_a$ was assigned a value much lower than that predicted by the estimated $K_r$ 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). 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 the P450 2D6 reactions (37). As mentioned previously in this report, the kinetic patterns and fits could be explained by a somewhat slow step following product formation but not intrinsically coupled to product binding/release. We raise the possibility that a P450 relaxation step may be involved here and be fairly general in returning P450 to the ground state for substrate binding. Another precedent for this phenomenon may be found in work with bacterial P450 101; Hoffman and co-workers (78) reported spectroscopic evidence for two P450-product complexes. The kinetic contribution of the relaxation rate in the P450 101 reaction is unknown. In the P450 2D6 reactions described here, the step cannot be highly rate-limiting, however (Fig. 1).

A general issue is the contribution of the rates of the C–H bond breaking steps in catalysis, as demonstrated in the $D^V$ and $D^V(V/K)$ results (Fig. 5). In early work in the field, the observed intermolecular isotope effects were generally small, and the general consensus was that other steps controlled rates of P450 reactions (14, 79, 80). However, we have found notable examples of significant non-competitive isotope effects with human P450s 2E1 (25, 26), 1A2 (27), and 2D6 (this work). Searches for kinetic hydrogen isotope effects are complicated by the lack of ability to apply the approach in situations such as epoxidation and aromatic hydroxylation; even with alkane hydroxylation and heteroaromatic dealkylation reactions there are often complications of difficulty of synthesis, pro-chirality, and the complexity of interpretation of $N$-dealkylation reaction isotope effects due to base catalysis of deprotonation (39). $O$-Demethylation reactions have advantages in this regard, and the work presented here appears to be the first reported study of kinetic hydrogen isotope effects with P450 2D6. We hypothesize that C–H bond breaking, or an equivalent step involving the FeO$^-$ entity (or another high valent oxidant), is a rate-limiting step in many P450 reactions. Recent studies with rabbit and human P450 1A2 have yielded non-competitive deuterium isotope effects of $9–10$ for the $O$-demethylation of 4-substituted anisoles. We realize that caution is needed in extrapolating work largely based on $O$-dealkylation to other types of reactions, but further examination of kinetic hydrogen isotope effects is in order.

In conclusion, we have examined several aspects of the catalysis of $O$-demethylation of 3- and 4-methoxyphenethyamine 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.

Acknowledgments—We thank C. J. Rizzó and Y. Nishimura for their input into some of the initial experiments involving kinetic hydrogen isotope effects; J. A. Krauser for acquiring some of the NMR and mass spectra; and K. Trieler for assistance in preparation of the manuscript.

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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

doi: 10.1074/jbc.M205146200 originally published online July 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205146200

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