Diversity in Mechanisms of Substrate Oxidation by Cytochrome P450 2D6

LACK OF AN ALLOSTERIC ROLE OF NADPH-CYTOCHROME P450 REDUCTASE IN CATALYTIC REGIOSELECTIVITY*

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Cytochrome P450 (P450) 2D6 was first identified as the polymorphic human debrisoquine hydroxylase and subsequently shown to catalyze the oxidation of a variety of drugs containing a basic nitrogen. Differences in the regioselectivity of oxidation products formed in systems containing NADPH-P450 reductase/NADPH and the model oxidant cumene hydroperoxide have been proposed to arise at least in part to be due to an allosteric influence of the reductase on P450 2D6 (Modi, S., Gilham, D. E., Sutcliffe, M. J., Lian, L.-Y., Primrose, W. U., Wolf, C. R., and Roberts, G. C. K. (1997) Biochemistry 36, 4461–4470). We examined the differences in the formation of oxidation products of N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine, metoprolol, and bufuralol between reductase-, cumene hydroperoxide-, and iodosylbenzene-supported systems. Catalytic regioselectivity was not influenced by the presence of the reductase in any of the systems supported by model oxidants, ruling out allosteric influences. The presence of the reductase had little effect on the affinity of P450 2D6 for any of these three substrates. The addition of the reaction remnants of the model oxidants (cumyl alcohol and iodosobenzene) to the reductase-supported system did not affect reaction patterns, arguing against steric influences of these products on catalytic regioselectivity. Label from H2O to H218O was quantitatively incorporated into 1'-hydroxybufuralol in the iodosobenzene- but not in the reductase- or cumene hydroperoxide-supported reactions. We conclude that the P450 2D6 systems utilizing NADPH-P450 reductase, cumene hydroperoxide, and iodosobenzene use similar but distinct chemical mechanisms. These differences are the basis for the variable product distributions, not an allosteric influence of the reductase.

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P4501 enzymes (also termed “heme-thiolate protein P450”; Ref. 1) are involved in the oxidations of many organic chemicals (2–4). The P450 enzymes are found in nearly all life forms, but there has been particular interest in the mammalian P450 enzymes that dominate the metabolism of drugs (5). P450s constitute an important target in pharmacogenomic efforts because variation among individual humans can have a major influence on the efficacy of drugs (6).

P450 2D6 was first identified as the polymorphic enzyme involved in debrisoquine hydroxylation (7) and sparteine oxidation (8). This enzyme is involved in the metabolism of approximately one third of the drugs used today (5). P450 2D6 polymorphism is relatively well understood today (9–11), and our own efforts have been directed to better understanding the biochemical basis of P450 2D6 activity. In our early research with purified P450 2D6 (12), the observation was made that most of the substrates of P450 2D6 contain a basic nitrogen atom, which is located ~5 Å away from the site of oxidation (13, 14). This concept was developed with more detailed pharmacophore and homology models for P450 2D6 (15–18).

A problem in modeling of P450 2D6 arose with the report of Modi et al. (19) that differences were observed between MPTP oxidations catalyzed by P450 2D6 supported with the usual NADPH/NADPH-P450 reductase/O2 system and with the model oxidant (“oxygen surrogate”) CuOOH. The authors interpreted the differences in product distribution as evidence for an allosteric role of NADPH-P450 reductase in orienting the substrate, and some evidence for this view was obtained with NMR relaxation studies of MPTP bound to ferric P450 2D6 (19).

We investigated the issue of possible allosterism imposed by NADPH-P450 reductase, in the context of our general interest in the cooperativity of other P450s, particularly P450 3A4 (20–22). We find that some product profiles differ for P450 2D6 oxidations of the substrates MPTP, metoprolol, and bufuralol supported by NADPH-P450 reductase and the oxygen surrogates CuOOH and PhIO, as in the case of some other P450s (23–27). These differences were found not to be due to any effect of NADPH-P450 reductase or to steric interaction of the P450 2D6 substrates with reaction remnants of the oxygen surrogates, but they are attributable to inherent chemical differences in the systems as revealed with H218O studies in the case of PhIO. Another conclusion regarding the pharmacophore models is that they have limited ability to predict sites of

1 The abbreviations used are: P450, microsomal cytochrome P450; MPTP, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PTP, 4-phenyl-1,2,3,6-tetrahydropyridine; PTP-OH, 4-1-methyl-1,2,3,6-tetrahydropyridin-4-yl)phenol; CuOOH, cumene hydroperoxide; PhIO, iodosylbenzene; PhI, iodo benzene; MS, mass spectrometry; HPLC, high performance liquid chromatography.
oxidation for a typical drug substrate, bufuralol, where oxidation by P450 2D6 was shown to generate multiple products.

EXPERIMENTAL PROCEDURES

**Caution!**—MPTP is a potent neurotoxin and should be handled with gloves and use of other appropriate precautions. CuOOH can be explosive (86).

**Chemicals**—Bufuralol-HCl and 1'-hydroxybufuralol were generous gifts of Hoffman-LaRoche (Nutley, NJ). Metoprolol-HCl. *Escherichia coli* superoxide dismutase, and 1,2-diarylur-sn-glycero-3-phosphocholine were purchased from Sigma. Quinidine-HSO₄, PTP, and iodosylbenzene diacetate were purchased from Aldrich. Metoprolol oxidation products (O-demethylmetoprolol and α-hydroxymetoprolol) were gifts from Astra Hässle AB (Mölndal, Sweden). MPTP was a gift of N. Catagnoli, Jr. (Virginia Polytechnic University, Blacksburg, VA). Bovine liver catalase (Sigma) was dialyzed prior to use to remove the preservative thymol. CuOOH was obtained from Aldrich and purified by extraction with alkali as described (28) prior to use. PhIO was prepared by the alkaline hydrolysis of the diacetate (29) and stored at −20 °C.

MPTP-OH was synthesized by the condensation of phenol with 1-methyl-1-piperidone in CH₃CO₂H/HCl solution as described (30): 1H NMR (δ) 1.15 (d, 3H, CH₃), 2.98 (d, 2H, CH₂-N), 3.35 (m, 2H, CH₂, CH₂-N), 5.97 (t, 1H, CH₂CN), 6.71 (d, 2H, Ar), 7.23 (d, 2H, Ar), 9.40 (s, 1H, OH); MS m/z 190.1 (MH⁺).

**Enzymes**—The cDNA sequence of P450 2D6 (DB6) (31) was modified by polymerase chain reaction to incorporate a C-terminal His₆ peptide (32). The modified protein was expressed in *E. coli* (MV1304 strain) in the presence of 1.0 mg of chloramphenicol liter⁻¹ (35). Expression P450 2D6 was purified by Ni²⁺-immobilized metal affinity chromatography as described (32, 36). NADPH-P450 reductase was expressed in *E. coli* (TOPP 3 strain) containing the plasmid pOR263 (37) and purified as described elsewhere (36, 38).

**Oxidation Assays**—Standard oxidation reactions with bufuralol, MPTP, and metoprolol were conducted in 0.5-ml final volumes of 0.10 M potassium phosphate buffer (pH 7.4) containing P450 2D6 (0.25 nmol), NADPH-P450 reductase (0.5 nmol), and freshly sonicated L-α-dipalmityl phosphatidyl ethanolamine (20 μg, respectively) (40). Incubations were carried out for 10 min at 37 °C and were started by the addition of an NADPH-generating system (1.0 mm NADP⁺, 10 mm glucose 6-phosphate, and 1 unit of yeast glucose-6-phosphate dehydrogenase ml⁻¹) (39). In order to prevent heme destruction due to the generation of reactive oxygen species during catalysis, the reaction mixtures were supplemented with catalase and *E. coli* superoxide dismutase (1000 units and 20 μg, respectively) (40). Incubations were carried out for 10 min at 37 °C and were quenched by the addition of 50 μl of 60% HClO₄. Bufuralol and metoprolol reductase reaction mixtures were centrifuged (3000 × g, 10 min) to sediment the precipitated proteins and salts, and aliquots of the supernatants were directly injected onto an HPLC system for analysis.

**CuOOH- and PhIO-supported P450 2D6 Oxidation of MPTP, Metoprolol, and Bufuralol**—Incubations were carried out essentially as described above except for the exclusion of the NADPH-generating system, catalase, and superoxide dismutase. NADPH-P450 reductase was included in some oxidation reactions to determine if it had any allosteric effects on P450 2D6 during catalysis. Reactions were carried out at 37 °C for 5–10 min and were initiated by the addition of methanolic solutions of CuOOH or PhIO (final concentrations of 0.5 and 0.25 mM, respectively, and 1% CH₃OH, v/v). (In contrast to many other P450s the presence of 1.0 mg of chloramphenicol liter⁻¹ (35) was found to be optimal for bufuralol 1'-hydroxylation (39). In order to prevent heme destruction due to the generation of reactive oxygen species during catalysis, the reaction mixtures were supplemented with catalase and *E. coli* superoxide dismutase (1000 units and 20 μg, respectively) (40). Incubations were carried out for 10 min at 37 °C and were quenched by the addition of 50 μl of 60% HClO₄. Bufuralol and metoprolol reductase reaction mixtures were centrifuged (3000 × g, 10 min) to sediment the precipitated proteins and salts, and aliquots of the supernatants were directly injected onto an HPLC system for analysis.

**HPLC Analysis**—Metoprolol oxidation reactions were analyzed on a Whatman SCX column (10 μm, 4.6 × 250 mm, Whatman, Hillsboro, OR) as described elsewhere (45). Products were identified by comparison of tₚ to those obtained with authentic standards.

Buparalol oxidation mixtures were separated on a C₁₈ column (5 μm, 4.6 × 250 mm, ODS-AQ, YMC) using a linear gradient beginning with 50% Buffer A (20 mM NaClO₄ (pH 2.5)/CH₃CN, 9:1, v/v) and 50% Buffer B (20 mM NaClO₄ (pH 2.5)/CH₃CN, 3:2, v/v), changing to 100% Buffer B over 8 min. Elution was continued for an additional 5 min while holding at 100% Buffer B, followed by a 4-min recovery period to the initial conditions. The flow rate was 2.0 ml min⁻¹, and products were detected by UV measurements (λ₂₅₄).

**MPTP oxidation reactions were analyzed on an Agilent 1100 instrument (Agilent, Santa Clara, CA) using a Dionex (USA) column (5 μm, 4.6 × 250 mm, Whatman, Hillsboro, OR) as described elsewhere (45).**

**Spectroscopy and Ligand Binding Assays**—Absorbance spectra were recorded using an Aminco DW2/2/OLIS instrument (On-Line Instrument Systems, Bogart, GA). The interaction of substrates with P450 2D6 was examined by perturbation of the heme Soret spectra. P450 2D6 (2.0 μM) was included in a 1.0-ml cuvette containing 45 μM 1,2-diarylur-sn-glycero-3-phosphocholine and 10.0 m potassium phosphate buffer (pH 7.4) (123 °C). NADPH-P450 reductase was included at a concentration of 4.0 μM when indicated (a 2:1 ratio of reductase to P450 2D6 was found to be optimal in previous work (Ref. 32), and this ratio was also used in the catalytic assays (see above)). A baseline was established from 350 to 500 nm, and sequential additions (2–10 μl) of concentrated aqueous solutions of MPTP, metoprolol, or bufuralol (HCl salts) were made, recording spectra each time. Kᵥ values were estimated by fitting plots of Δλmax versus t with hyperbolic plots (using Graphpad Prism software, Graphpad, San Diego, CA).

**MS—HPLC/electrospray MS studies on the determination of the structure of 3',4'-dehydrobufuralol** were performed using a Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan-MAT, Sunnyvale, CA) operating in the positive ion mode with an electrospray needle voltage of 4.5 kV. N₂ was used as the sheath gas (70 p.s.i.) to assist nebulization and as the auxiliary gas (10 p.s.i.) to assist with

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2 The cDNA we expressed in previous work (31) had been obtained with a change (to Met) at codon 374 that appears to be the result of a cloning artifact (33, 34). The change of M374V was made, and the resulting sequence is that generally agreed to be the most common allele (9).

3 P450 2D6 also catalyzes the N-deisopropyl formation of metoprolol (44), but we did not analyze these products with the HPLC system we used.
desolvation. The stainless steel capillary was heated to 200 °C, and the electrospray ionization interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. The tube lens and the heated capillary were operated at 75 and 20 V, respectively, and the electron multiplier was set at 1900 V. HPLC conditions (Zorbax Rx-C8 octylsilane, 2.1/H11003/150 mm, Mac-Mod, Chadds Ford, PA) were as follows: flow rate 0.2 ml min−1; Solvent A, 20 mM NH4CH3CO2/CH3CN (95:5, v/v); Solvent B: 20 mM NH4CH3CO2/CH3CN (5:95, v/v); t0 min, 100% A; t12.5 min, 70% A; t20 min, 10% A; t22.5 min, 10% A; t25 min, 100% A; t30 min, 100% A.

MS studies for measurement of 18O incorporation into 1-hydroxybufuralol were performed using same mass spectrometer, source, and ion mode described above. The HPLC-isolated oxidation products were concentrated to dryness under a stream of N2, dissolved in 100 l CH3OH containing 1% CH3CO2H (v/v), and directly infused into the electrospray ionization source at a flow rate of 0.2 ml min−1. The optimized instrument parameters were as follow: electrospray needle voltage 4 kV, sheath gas (N2) 40 p.s.i., capillary temperature 200 °C, capillary voltage 20 V, tube lens 80 V, and electron multiplier voltage 1400 V.

RESULTS

Oxidation of MPTP by P450 2D6—Initial experiments on the possible allosteric effect of NADPH-P450 reductase on P450 2D6 were done with MPTP because of previous reports of differences in the product profiles obtained using oxidation systems supported with NADPH-P450 reductase and CuOOH (19). Oxidation of MPTP resulting in the formation of both the N-demethylated product PTP and the ring-hydroxylated product MPTP-OH (Fig. 1) when supported by NADPH/NADPH-P450 reductase, CuOOH, or a mixture of the two (without NADPH). Only PTP was obtained when the oxidation reaction was supported by PhIO (0.3 mM, results not shown). Supplementation of PhIO-supported P450 2D6 MPTP oxidation reactions with PhI (remnant of PhIO cleavage; Refs. 24 and 46) to concentrations as high as 0.2 mM did not alter the regioselectivity of MPTP oxidation (results not shown). The addition of NADPH-P450 reductase did not change the product profile (i.e.
Oxidation of Metoprolol by P450 2D6—Because the work with MPTP did not show major differences in the distribution of products from the NADPH-P450 reductase- and CuOOH-supported reactions, we examined the distribution of the multiple products of oxidation of the classic P450 2D6 substrate metoprolol (47). Oxidation of metoprolol yielded two distinct products (O-demethylmetoprolol and α-hydroxymetoprolol).
when the reaction was supported with the usual NADPH/ NADPH-P450 reductase system (Fig. 2A). Only one product, O-demethylmetoprolol, was formed when the oxidation reaction was supported by the oxygen surrogates CuOOH (Fig. 2B) or PhIO (results not shown). In neither case did the inclusion of NADPH-P450 reductase in CuOOH- or PhIO-supported reactions significantly influence the composition of the oxidation products (Fig. 2C and other results not shown).

Characterization of Bufuralol Oxidation Products—Another classic P450 2D6 substrate is bufuralol, which was utilized in the original purifications of P450 2D6 from human liver (12, 48, 49). The literature on P450 2D6 generally suggests that 1'-hydroxybufuralol is the only product (12, 49, 50), which may be the result of the use of fluorescence detection or lack of separation of the reaction products. Bufuralol oxidation reaction mixtures were subjected to HPLC under gradient elution conditions, and both UV (A260) and fluorescence (F230/230) signals were monitored. The identity of the 1'-hydroxy product was verified by comparison of its mobility to that of an authentic standard. The identities of 6- and 4-hydroxybufuralol were indirectly verified by comparing their mobility and UV spectra to the P450 1A2 bufuralol oxidation products previously identified by MS and NMR (51).

Another significant product (peak 5 of Ref. 51), which exhibited mobility similar to that of bufuralol, was yet unidentified (52). The product in question exhibited more intense fluorescence than bufuralol, suggesting the generation of a compound with greater bond conjugation. Relatively greater amounts of this product were obtained when P450 2D6 oxidation reactions were supported with CuOOH as compared with the usual NADPH/NADPH-P450 reductase system (see below). HPLC/MS analysis of the unidentified product indicated an apparent MH+ ion at m/z 260, 2 mass units less than the parent compound bufuralol (m/z 262, MH+)(Fig. 3B).4 The UV spectrum of the unidentified product was different than that of the parent compound (Fig. 3B) and identical to that of the undefined M-5 peak described previously (51). The decrease of the MH+ ion by 2 atomic mass units is consistent with only either of two stable products, the 1'-x,6-olefin or an oxidation of the carbinol present in the substrate, barring any unusual rearrangement of the benzofuran ring system. The 1H NMR spectrum (Fig. 3C) was similar to one of the unidentified components in the M-5 peak of the earlier work (51) and is a major basis for assigning the 1'-x,6-olefin structure (Fig. 4). Key differences with the bufuralol spectrum (51) were the absence of the H-2' (δ 1.3) and particularly the H-1' (δ 2.9) signals from the ethyl group. The 1' carbinol was not oxidized to a ketone because the H-2' protons are still present at δ 3.30 and 3.44. If a 1'-ketone were present, an upfield shift would have been expected. The 1' carbinol multiplet appears at δ 5.50, and subsequent experiments with another sample of this product indicated that the carbinol OH proton was overlapped. The multiplet at δ 6.76 is assigned to the two olefinic protons and the δ 6.93 multiplet to the H-1' olefinic proton; the apparent Jtrans coupling constant of 12 Hz was useful in the assignment. The remaining downfield protons are singlets (H-3, δ 7.10), doublets (H-4, 6, δ 7.11, 7.15), or multiplets (H-5, δ 7.37), displaced downfield because of the increased conjugation with the aromatic system.

Treatment of the product with NaBH4 under typical mild conditions did not change the HPLC tR. If the carbinol (1') had been oxidized to the ketone, bufuralol would have been the product.

1'-Hydroxybufuralol did not yield the Δ1',6'-desaturated product (i.e. due to dehydration) under the enzyme reaction conditions. Thus, the olefin appears to be a direct oxidation product, as in the case of other desaturation reactions catalyzed by P450s (4, 24, 54–56).

Oxidation Products of Bufuralol Formed in Different P450 2D6 Systems—The demonstration of multiple products of bufuralol oxidation by P450 2D6 (Fig. 4) provided an opportunity for further comparisons of the NADPH-P450 reductase- and

4 Very recently Hiroi et al. (53) reported a P450 2D6 bufuralol product with similar HPLC mobility and m/z 260 (MS) but did not define the site of unsaturation by other spectral methods. Comparison of our chromatograms and spectra with Drs. Hiroi and Funae indicate that Δ1',6'-dehydrobufuralol is also the product isolated in their work.
The bufuralol used here was a racemic mixture, and the contributions of the individual enantiomers to the individual products have not been ascertained. P450 2D6 is known to convert both enantiomers to 1-hydroxybufuralol (12). The situation is not as clear with the other products. The (S)\(^-\) (→) enantiomer has been suggested to be the source of the 4- and 6-hydroxy products on the basis of studies with liver microsomes (57).

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Fig. 8. Lack of effect of products of oxygen surrogates on profiles of P450 2D6-generated bufuralol oxidation products in NADPH-P450 2D6-supported reactions. A. Increasing concentrations of cumyl alcohol (0, 100, 200, 300, 500 \(\mu M\), in order noted with arrow) were present. B, Increasing amounts of PhI (0, 50, 100, 200, 300 \(\mu M\), in order noted with arrow) were present. Chromatograms are offset 0.5 min and products are identified.
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oxygen surrogate-supported systems. The profiles obtained with the NADPH-reductase-, CuOOH-, and PhIO-supported systems were very different (Fig. 5). All of the products formed in the reductase-supported system were also formed with CuOOH, but the ratios were different, with relatively less 4- and 6-hydroxybufuralol and more \(\Delta^{1,2}\) dehydrobufuralol (Fig. 5B). PhIO yielded mainly 1-hydroxybufuralol and trace 6-hydroxybufuralol (Fig. 5C).

The addition of NADPH-P450 reductase to the CuOOH-supported reaction did not change the profile of bufuralol products (Fig. 6A). In a similar manner, the addition of NADPH-P450 reductase to the PhIO-supported reaction did not change the profile of bufuralol products (Fig. 6B).

Spectral Estimation of Dissociation Constants with P450 2D6—One approach to examination of the effect of NADPH-P450 reductase on P450 2D6/substrate interaction involves the measurement of spectral dissociation constants. The binding of MPTP, metoprol, or bufuralol to P450 2D6 resulted in the formation of a typical “type I” difference spectrum (21, 22), with a conversion of low spin to high spin iron associated with the partial displacement of the distal \(\text{H}_2\text{O}\) ligand (Fig. 7, A and B).

When such titrations were done with MPTP, the plots were nearly superimposable (\(K_r = 16.5 \pm 0.5 \mu M, r^2 = 0.998\) without reductase, \(22 \pm 1 \mu M\) with reductase) (Fig. 7C). The data obtained over a wide substrate range fit well to a plot for a single hyperbola (\(r^2 = 0.986\) and 0.991).

Titrations with metoprol (0–750 \(\mu M\)) also fit single hyperbolas, with only a slight difference due to the presence of the reductase (\(K_r = 13.6 \pm 0.2 \mu M, r^2 = 0.999\) with reductase) (data not shown). With bufuralol, a \(K_r = 8.1 \pm 0.1 \mu M\) (\(r^2 = 0.999\)) was estimated in the absence of reductase and a \(K_r = 6.7 \pm 0.1 \mu M\) (\(r^2 = 0.999\)) was estimated in the presence of the reductase. The addition of cumyl alcohol (isopropylbenzyl alcohol, 500 \(\mu M\)), the major degradation product resulting from heterolytic cleavage of CuOOH (64, 65), elicited only a slight change in the spectrum. The subsequent titration of this mixture yielded a \(K_r = 10.5 \pm 0.1 \mu M\) (\(r^2 = 0.999\)) for bufuralol, only slightly different than for P450 2D6 devoid of cumyl alcohol.

**DISCUSSION**

P450 2D6 has been of historical interest in that this is the first monooxygenase involved in drug metabolism that was demonstrated to be under monogenic control (7). Another reason for biochemical interest in this particular P450 has been the apparent ligand selectivity relative to many less specific microso-
The ability to produce P450 2D6 and site-directed mutants in heterologous expression systems has allowed exploration of several issues, including the role of the N terminus in catalytic selectivity (31, 32) and the effects of amino acid substitution at Asp301 (32, 70).

The history of model building for P450 2D6 began at the time of purification of the enzyme, when the observation was made that many of the known substrates of P450 2D6 contained a basic nitrogen atom (12, 13). Inspection of these ligands indicated that the basic nitrogen could be placed 5–7 Å away from the site of oxidation (13). The pharmacophore model was further developed for substrates (15–17) and, by our own group in collaborations, for inhibitors (14). Subsequently Asp301 has been considered to provide the putative negative charge bonding to the basic nitrogen, on the combined basis of work with homology modeling and site-directed mutagenesis (70–74).

The proposal that NADPH-P450 reductase plays an allosteric role in P450 2D6 specificity (74) poses a challenge to modeling efforts, in that the prediction of the juxtaposition of ligand in a ternary complex (P450 2D6/NADPH-P450 reductase/substrate) would be difficult to predict, if the structure is influenced by the interactions of all of the three components. The general concept that an accessory electron transfer protein plays an allosteric role with a P450 should be considered. The current literature on P450 3A4 indicates that many small molecules, including substrates, can modulate catalytic function through interactions that are still not well understood (21, 75). Evidence has also been presented that apo-cytochrome b5 (devoid of heme) can influence rates and selectivity of several P450s (76–79). However, the only report of a role for NADPH-P450 reductase binding in influencing catalytic selectivity is that of Modi et al. (19), which relies on (i) the differences in product distribution for the P450 2D6 oxidation of MPTP between the NADPH-P450 reductase- and CuOOH-supported systems and (ii) the differences in the proximity of sites of MPTP atoms to the ferric iron atom of P450 2D6 as measured by NMR relaxation methods. The interpretation of the latter observation requires caveats about relevance to the structure of P450 2D6 in its catalytically relevant form (FeO3 or possibly FeOOH).

In our own work we did not see a major difference between the patterns of MPTP oxidation products formed in the NADPH-P450 reductase- and CuOOH-supported systems and, in contrast to Modi et al. (19), a difference was observed when PhIO was used to support the reaction, but the addition of the reductase had no influence on the product profile. We did observe major differences in the CuOOH- and reductase-supported reaction product profiles for oxidation of metoprolol and bufuralol, two classic P450 2D6 substrates (Figs. 2 and 5). Neither the CuOOH nor the PhIO reaction was influenced by the presence of NADPH-P450 reductase with either substrate (Figs. 2 and 6).
In this work we routinely added enzyme systems to scavenge reduced oxygen species generated from uncoupled reactions and restricted the time of enzyme incubations to 10 min. Whether these methods led to the differences with the results of Modi et al. (19) is not clear, because the incubation times had not been indicated, except for 10 h in one case. However, our lack of differences between the reductase-coupled and the hydroperoxide-dependent MPTP oxidation systems would not be explained. The pH was 8.0 in the work of Modi et al. (19), instead of the 7.4 routinely used here. The conclusions derived from the NMR measurements are not without interest but require some caveats in interpretation. Relatively weak affinity of MPTP to P450 2D6 ($K_d = 0.1 \text{ mM}$) (19) would cause its floppy binding to the enzyme and thus this substrate would not be tightly fixed in the active site. In general, regio- and stereospecificities of membrane-bound microsomal P450s that are involved in drug (often small and flexible structures) oxidation may not be simply interpreted and rationalized just by physicochemical methods, including paramagnetic relaxation techniques. In particular, the latter method requires numerical structure and physical assumptions for calculation. The situation for membrane-bound microsomal P450s may be different from those of steroid-related P450s in that some of the latter P450s have distinct less flexible substrates (e.g., steroids, camphor) that would be properly fixed in the active site and thus regio- and stereospecificities could be more clearly explained by physicochemical methods.

Spectral titrations of the binding of MPTP to P450 2D6 were nearly superimposable in the presence and absence of the reductase (Fig. 7), with $K_s = 22-29 \mu M$. These results are in contrast to those reported by Modi et al. (19), in which $K_s$ values of 110–149 $\mu M$ were obtained using optical and NMR methods in the absence of reductase. In the presence of reductase, their data were fit to hyperbolic equations with $K_s = 149$ $\mu M$ (for a mode with the N-methyl group near the iron atom) and $K_s = 25$ $\mu M$ (for a mode with the C9 hydrogen (para H of pheryl ring) closest to the iron). The relationship of results of Modi et al. (19) to our own (Fig. 7) is unclear. We did not find evidence for a lower affinity component in either set of optical spectra. It is possible that the higher pH (8.0) used by Modi et al. (19) (or the 4 °C temperature) affected their results, but we have restricted our own analysis to work at pH 7.4. In the present work we saw only small, if any, quantitative differences in the affinity of P450 2D6 for MPTP, metoprolol, or bufuralol due to the presence of NADPH-P450 reductase. Further, 500 $\mu M$ cumyl alcohol only increased the $K_s$ for bufuralol by ~25% (see above).

Differences in product profiles of P450 reactions supported by NADPH-P450 reductase and oxygen surrogates are not unusual and have been reviewed by Ortiz de Montellano (23, 24). In some of the early literature (23, 25, 26), the differences can probably be attributed to the presence of multiple P450 enzymes in microsomal preparations or to secondary oxidations of initial products (24, 80). However, the differences remain obscure in many cases. As discussed above, the differences between these P450 2D6 reactions cannot be attributed to an allosteric effect of the reductase (Figs. 2 and 6). One possibility is a steric effect of the remnant of the oxygen surrogate in the active site (24, 58–60). However, no evidence for this hypothesis was obtained in experiments in which these compounds (cumyl alcohol, PhI) were added to NADPH-P450 reductase-supported bufuralol oxidation reactions in concentrations as high as the oxygen surrogates used in the reactions (Fig. 8). Our preferred hypothesis for the differences is one based on differences in the chemistry of the reactions supported by the oxygen surrogates. CuOOH has long been known to undergo both homolytic and heterolytic cleavage by P450s, yielding a variety of oxidants with distinct properties (23, 24, 64, 65, 67, 68) (Fig. 9A). Evidence for a different chemical mechanism in the P450 reaction comes from the H$_2^{18}$O incorporation experiments with bufuralol (see above). A scheme to explain the exchange of oxygen from H$_2^{18}$O with the Fe-O complex comes from Ortíz de Montellano (23) (Fig. 9B), in which two potential oxidants are present, FeO$_2^{3+}$ and Fe(OH)P450H. The possibility is presented that the putative FeO$_2^{3+}$ (generated by the reductase) is involved in multiple oxidations but the complex with the P450 attached has a more restricted scope, e.g., N-demethylation of MPTP or (primarily) 1'-hydroxylation of bufuralol. Other potential high valent iron intermediates (e.g., FeOOH and FeOOH$_2$; Refs. 81 and 82) that could be generated in the reductase-supported system are not shown but can also be considered to add to the complexity of differences and to the versatility of the reductase-supported system. The scheme shown in Fig. 9 may be oversimplified, however, in that different amino acids in the region of the heme distal ligand would be expected to exert varying influence in the different systems, i.e., protonation of FeO$_2^{3+}$ in the reductase-supported reaction and O-O cleavage in that and the CuOOH-supported reactions.

We conclude that the basis of differences in the products of P450 2D6 reactions supported by NADPH-P450 reductase and the oxygen surrogates is neither an allosteric influence of the reductase nor steric crowding by remnants of the oxygen surrogates. The most probable explanation is inherent differences in the chemistry of catalysis (Fig. 9). The presence of the reductase has not been demonstrated to perturb the regioselectivity of catalysis of P450 2D6 or any other P450 to date. However, the reliability of pharmacophore and homology models of P450 2D6 in predicting substrates and regioselectivity is nevertheless attenuated by the plethora of products generated from a single substrate (Fig. 4 and Ref. 71) and recent reports of P450 2D6 binding and oxidation of steroids (53, 83–85) and drugs devoid of a basic nitrogen atom (e.g., spirosulfonamide).  

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Diversity in Mechanisms of Substrate Oxidation by Cytochrome P450 2D6: LACK OF AN ALLOSTERIC ROLE OF NADPH-CYTOCHROME P450 REDUCTASE IN CATALYTIC REGIOSELECTIVITY
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