Investigation of the Mechanisms Underlying the Differential Effects of the K262R Mutation of P450 2B6 on Catalytic Activity

Namandé N. Bumpus and Paul F. Hollenberg

Department of Pharmacology, University of Michigan, Ann Arbor, Michigan

Received May 5, 2008; accepted July 11, 2008

ABSTRACT

Human P450 2B6 is a polymorphic enzyme involved in the oxidative metabolism of a number of clinically relevant substrates. The lysine 262-to-arginine mutant of cytochrome P450 2B6 (P450 2B6.4) has been shown to have differential effects on P450 2B6 catalytic activity. We reported previously that the mutant enzyme was unable to metabolize 17-α-ethynylestradiol (17EE) or become inactivated by 17EE or efavirenz, which are inactivators of the wild-type enzyme. Studies were performed to elucidate the mechanism by which this mutation affects P450 2B6 catalytic activity. Studies using phenyldiazene to investigate differences between the active site topologies of the wild-type and mutant enzymes revealed only minor differences. Likewise, K_s values for the binding of both benzphetamine and efavirenz were comparable between the two enzymes. Using the alternate oxidant tert-butyl hydroperoxide, the mutant enzyme was inactivated by both 17EE and efavirenz. The stoichiometry of 17EE and efavirenz metabolism by P450s 2B6 and 2B6.4 revealed that the mutant enzyme was more uncoupled, producing hydrogen peroxide as the primary product. Interestingly, the addition of cytochrome b_5 improved the coupling of the mutant, resulting in increased catalytic activity. In the presence of cytochrome b_5 the variant readily metabolized 17EE and was inactivated by both 17EE and efavirenz. It is therefore proposed that the oxyferrous or iron-peroxo intermediate formed by the wild-type enzyme in the presence of 17EE and efavirenz may be less stable than the same intermediates formed by the wild-type enzyme.

The cytochromes P450 (P450s) are a superfamily of heme-containing monooxygenases that catalyze the oxidative metabolism of a number of endogenous and exogenous compounds, including clinically relevant drugs, pesticides, and carcinogens. The P450 catalytic cycle consists of a number of steps, including: substrate binding to ferric P450; reduction as a result of the transfer of an electron from NADPH via NADPH-cytochrome P450 reductase (reductase); binding of molecular oxygen to ferrous P450, leading to the formation of oxyferrous P450; transfer of a second electron to oxyferrous P450 from NADPH via reductase, or in some instances cytochrome b_5; formation of the oxygenating species; and subsequent oxidation of the substrate followed by product release. In addition, hydrogen peroxide can be formed via the decomposition of the oxyferrous complex or by autooxidation of the two-electron reduced P450 (Oprian et al., 1983). This phenomenon is referred to as "uncoupling."

Human P450 2B6 plays a major role in the metabolism of a growing list of compounds, including bupropion, an antidepressant and smoking cessation aid; efavirenz, a non-nucleoside HIV-1 reverse transcriptase inhibitor; and cyclophosphamide, a chemotherapeutic prodrug that requires metabolic activation (Roy et al., 1999; Faucette et al., 2000; Hesse et al., 2000; Ward et al., 2003). Certain substrates of P450 2B6, such as efavirenz, are also mechanism-based inactivators of the enzyme. Mechanism-based inactivation occurs when a substrate, in the process of metabolism, is converted to a reactive intermediate that binds covalently to the active site of the P450, rendering it inactive. A number of single-nucleotide polymorphisms have been found in the P450 2B6 gene (Lang et al., 2001). Recent studies in patients have demonstrated that some of these mutations can have significant effects on clinical outcomes (Tsuchiya et al., 2004; Wang et al., 2006).

This study was supported in part by grant CA16954 from the National Cancer Institute, National Institutes of Health (to P.F.H.); National Institutes of Health grant T32-GM007767 and a predoctoral fellowship in pharmacology/toxicology from the PhRMA Foundation (to N.N.B.).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.048637.

ABBREVIATIONS: P450, cytochrome P450; P450 2B6.4, P450 2B6 Lys-262-Arg mutant; BSA, bovine serum albumin; 17EE, 17-α-ethynylestradiol; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; rBHP, tert-butyl hydroperoxide; HPLC, high-performance liquid chromatography; reductase, NADPH-cytochrome P450 reductase.
ever, studies investigating the mechanisms underlying these effects are lacking. To address this issue, we have used recombinant P450 2B6 and a mutant P450 2B6 K262R (2B6.4), which corresponds to the P450 2B6*4 variant allele observed in humans (785A>G, exon 5), to investigate the functional consequences of this amino acid mutation. This mutant is of particular interest because it is present in a number of P450 2B6 variant alleles and therefore has a high mutation frequency.

We demonstrated previously that efavirenz and 17EE (Fig. 1), which both inactivate P450 2B6 in the reconstituted system, do not inactivate P450 2B6.4 (Bumpus et al., 2005, 2006). Furthermore, in those studies, the mutant enzyme was not able to metabolize 17EE, a substrate readily metabolized by the wild-type enzyme. In this study, we have systematically investigated some of the aspects of P450 catalytic function that could potentially be altered by the K262R mutation. Therefore, we conducted studies to elucidate whether differences in the catalytic activities of P450 2B6 and P450 2B6.4 are related to: 1) active site topology, 2) substrate binding, 3) interaction with reductase, and 4) reaction coupling. Our approach included the use of phenyldiazene to probe the active site of the P450, and the use of an alternate oxidant to support catalytic activity in the absence of reducing equivalents from NADPH. The results presented here suggest that there may be some differences in the active site topologies of the two enzymes, although the binding constants derived from spectral binding studies were similar. It is noteworthy that reaction stoichiometry experiments revealed that the reactions catalyzed by the mutant were more uncoupled than the reactions catalyzed by the wild-type enzyme. The addition of cytochrome b₅ improved the coupling of P450 2B6.4 and facilitated inactivation of the enzyme by both compounds.

Materials and Methods

Materials. Benzphetamine, BSA, 17EE, catalase and NADPH were purchased from Sigma (St. Louis, MO). Efavirenz was purchased from Toronto Research Chemicals (Toronto, ON, Canada). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Invitrogen (Carlsbad, CA). Phenyldiazene was purchased from Research Organics (Cleveland, OH). The P450 2B6 plasmid was a generous gift from Dr. James Halpert (University of Texas Medical Branch, Galveston, TX). All other chemicals were of the highest grade commercially available.

Site-Directed Mutagenesis, Expression, and Purification of P450s and Reductase. Construction of the P450 2B6.4 mutant was performed as described by Bumpus et al. (2005). P450 2B6, P450 2B6.4, and NADPH-P450 reductase were expressed in Escherichia coli Topp 3 cells and purified according to published protocols (Hanna et al., 1998, 2000; Scott et al., 2001). Cytochrome b₅ was purified from liver microsomes of phenobarbital-treated Long-Evans rats.

N-Phenytoprotoporphyrin IX Regioisomer Formation. The procedures used in these studies was adapted from published protocols (Swanson et al., 1991; Tuck et al., 1992). The phenyldiazene stock used in these experiments was prepared by adding 2.5 µl of phenyldiazene to 200 µl of 1N KOH. For the myoglobin experiments, 5 nmol of myoglobin in 100 mM KPi, pH 7.4, was placed into a 1-ml cuvette, and the absorbance spectrum from 400 to 500 nm was recorded. Then, 3 µl of the phenyldiazene stock was added to the cuvette and the absorbance spectrum was once again determined. A peak was observed at 430 nm, which is characteristic of a myoglobin phenyl-iron complex. Once the peak reached a maximum (approximately 10 min), the protein was denatured by adding the contents of the cuvette to 5 ml of 5% sulfuric acid (aq). After sitting for 2 h, the sample was extracted twice with an equal volume of methylene chloride. The extract was then dried down under a stream of nitrogen. The sample was then reconstituted in 150 µl of solvent A (40% water, 59.5% methanol, 0.5% acetic acid). For the P450 experiments, 2 nmol of P450 in 100 mM KPi, pH 7.4, was placed into a 1-ml cuvette, and the absorbance from 400 to 500 nm was measured. Then, 1.5 µl of the phenyldiazene stock solution was added to the cuvette, and peak formation at 478 nm was monitored. After the peak formation reached a maximum (approximately 10 min), 3 µl of potassium ferricyanide (50 µM) was added to the cuvette, and the contents of the cuvette were mixed and allowed to sit for 3 min. This was repeated twice to induce migration of the phenyl group from the iron to the porphyrin nitrogens. The sample was then denatured and extracted with methylene chloride as described above for myoglobin. After being dried under nitrogen, the N-phenytoprotoporphyrins were reconstituted in 150 µl of solvent A (40% water, 59.5% methanol, 0.5% acetic acid). The samples were analyzed by HPLC liquid chromatography-mass spectrometry using a Phenomenex (Torrance, CA) phenyl-hexyl column under isocratic conditions with 70% A and 30% B (99.5% methanol, 0.5% acetic acid). The area under the curve was determined for each of the four resulting regioisomers. These data were then expressed as a percentage of the total sum of the areas under the curve of all four peaks.

Spectral Binding. Spectral binding experiments were performed by titrating 1 µM P450 2B6 and P450 2B6.4 with either benzphetamine (dissolved in water) or efavirenz (dissolved in ethanol) at room temperature. Samples were brought to a total volume of 1 ml using 100 mM KPi₅, pH 7.4, and placed into a cuvette. The reference cuvette also contained 1 µM P450s in 100 mM KPi₅, pH 7.4. Vehicle solvent was added to the reference cuvette immediately after the titration of either benzphetamine or efavirenz into the sample cuvette. UV visible spectra were recorded from 350 to 500 nm after the addition of each aliquot of the ligand to the sample cuvette and an equal volume of the vehicle solvent to the reference. The absorbance differences between the maximum and minimum absorbencies observed in the difference spectrum after each addition were recorded and plotted against the concentrations of benzphetamine or efavirenz used to prepare the solution cuvette. The difference spectra for benzphetamine and efavirenz binding were approximated by plotting the inverse of the absorbance changes between 390 and 420 nm.
(type I) versus the inverse of the concentrations of either benzphetamine or reductase, and the inverse of the changes between 436 and 416 nm versus the inverse of the efavirenz concentrations.

Alternate Oxidant Studies. The alternate oxidant tert-butyl hydroperoxide (tBHP) was used to support P450 catalytic activity in place of NADPH, reductase, and molecular oxygen. P450s 2B6 or 2B6.4 were placed in 50 mM KP, pH 7.4, and diluted to a final volume of 200 μL. After a 5-min preincubation of the P450 with tBHP and inactivator, an aliquot (12 μL) of this primary mixture was transferred into 990 μL of assay mix that contained 100 μM 7-EFC and 40 μg of BSA/ml in 50 mM potassium phosphate buffer, pH 7.4. The assays were performed as described previously (Sridar et al., 2005). The concentration of tBHP (2.5 mM) was used in experiments to test for inactivation was determined to be optimal by measuring 7-EFC O-deethylation activity at concentrations of tBHP ranging from 0 to 5 mM. The presence of 2.5 mM tBHP resulted in maximum formation of the 7-EFC O-deethylated product, with no measurable inactivation of the enzyme.

Reaction Stoichiometry. P450 2B6 or P450 2B6.4 (65 pmol) was incubated with reductase at a 1:2 M ratio of P450/reductase for 45 min at 4°C. In the experiments in the presence of cytochrome b₅, samples were reconstituted in a 1:2:1 M ratio of P450/reductase/cytochrome b₅. P450 and reductase were incubated together on ice for 5 min before the addition of cytochrome b₅. The sample was brought to a total volume of 1 mL using 100 mM potassium phosphate buffer, pH 7.4, and placed into a cuvette. The sample was allowed to sit at room temperature for 5 min before the addition of NADPH to a final concentration of 200 μM. NADPH consumption was measured continuously, in both the presence and the absence of substrate (10 μM 17EE or efavirenz), by monitoring the absorbance at 418 nm (data not shown). After oxidation using ferricyanide, the samples were analyzed by liquid chromatography-mass spectrometry, and all four N-phenylprotoporphyrin IX regioisomers were observed. The elution times of the resulting regioisomers were compared with the standards produced from the incubation of phenylazidyl with myoglobin (data not shown). The major product formed by the wild-type enzyme was Nα, which accounted for 46 ± 2% of the total regioismer formation. Nα, Nβ, and Nδ were also detected and accounted for 10 ± 0.7, 7 ± 0.5, and 37 ± 1%, respectively (Fig. 2). Interestingly, there were some differences observed between the mutant and the wild-type enzyme. The reaction of phenylazidyl with P450 2B6.4 also resulted in the formation of all four N-phenylprotoporphyrin IX regioisomers (Fig. 3). However, Nα represented only 37 ± 1% of the overall formation, whereas Nα, Nβ, and Nδ constituted 15 ± 0.5, 11 ± 3, and 38 ± 4%, respectively. These data suggest that there may be differences in the active site topologies of the two enzymes.

Spectral Binding of Benzphetamine and Efavirenz to P450 2B6 and P450 2B6.4. Spectrophotometric titrations were performed to investigate whether these two substrates of P450 2B6 and P450 2B6.4 showed differences in binding affinity to the two enzymes. The dissociation constants (Kₘ) were determined from the titration curves. Benzphetamine was chosen because it produces a prominent type I spectral change in P450 2B6. Efavirenz caused a type II spectral change that is characteristic of a nitrogen atom coordinating to the heme. Both enzymes showed similar affinities for both of the substrates. The Kₘ values for benzphetamine binding to P450 2B6 and 2B6.4 were 18 ± 0.7 μM and 17 ± 0.4 μM, respectively. The efavirenz spectral dissociation constants were also similar between the two enzymes, with a value of 85 ± 2.3 μM for the wild-type enzyme and 123 ± 3.1 μM for the variant enzyme. The maximum ΔA values were similar in all cases. The Kₘ values for 17EE binding could not be determined because 17EE does not induce measurable spectral shifts. These data on the binding of benzphetamine and efavirenz suggest that P450 2B6 and P450 2B6.4 are able to bind substrates in a similar manner. Therefore, the differences in catalysis may not be directly related to substrate binding.

Results

P450 2B6 and P450 2B6.4 Active Site Topology. Phenylazidyl forms a σ-bonded complex with the heme iron of the P450, resulting in the formation of a phenyl-iron complex. Oxidation of the iron facilitates the migration of the phenyl group to an available pyrrole nitrogen belonging to ring A, B, C, or D. The ratio of formation of the resulting N-protoporphyrin IX regioisomers, denoted Nα, Nβ, Nδ, and Nγ, allows for inferences to be made regarding the accessibility of each of the four pyrrole rings (Swanson et al., 1991; Tuck et al., 1992; Yamaguchi et al., 2004). Phenylazidyl was added to P450s 2B6 and 2B6.4, and the formation of the phenyl-iron complex was determined spectrally by monitoring the peak formation at 478 nm and the concomitant decrease at 418 nm (data not shown). After oxidation using ferricyanide, the samples were analyzed by liquid chromatography-mass spectrometry, and all four N-phenylprotoporphyrin IX regioisomers were observed. The elution times of the resulting regioisomers were compared with the standards produced from the incubation of phenylazidyl with myoglobin (data not shown). The major product formed by the wild-type enzyme was Nα, which accounted for 46 ± 2% of the total regioismer formation. Nα, Nβ, and Nδ were also detected and accounted for 10 ± 0.7, 7 ± 0.5, and 37 ± 1%, respectively (Fig. 2). Interestingly, there were some differences observed between the mutant and the wild-type enzyme. The reaction of phenylazidyl with P450 2B6.4 also resulted in the formation of all four N-phenylprotoporphyrin IX regioisomers (Fig. 3). However, Nα represented only 37 ± 1% of the overall formation, whereas Nα, Nβ, and Nδ constituted 15 ± 0.5, 11 ± 3, and 38 ± 4%, respectively. These data suggest that there may be differences in the active site topologies of the two enzymes.
Inactivation of P450s by 17EE and Efavirenz Using an Alternate Oxidant. We used tert-butyl hydroperoxide as an activated oxygen surrogate to investigate whether the wild-type and/or mutant enzymes could become inactivated by 17EE or efavirenz in a reductase and electron-free system. tBHP produces an active iron-oxygen species with the P450 that can support P450 catalytic activity in the absence of molecular oxygen and reducing equivalents from NADPH (White and Coon, 1980). Because peroxides by themselves have the ability to inactivate P450s, we tested a range of tBHP concentrations to ensure that the inactivation we were measuring was indeed due to the compounds being investigated. Ultimately, the concentration that we chose exhibited no measurable inhibitory effect on the activity of the enzyme in incubations ranging from 0 to 20 min. Furthermore, the values reported here for inactivation of both enzymes in the presence of 17EE and efavirenz are expressed as a percentage of the activity detected in the presence of tBHP alone. Therefore, the activity in the presence of the peroxide alone was considered to be 100% and was comparable with activity that would be expected using the NADPH-requiring reductase system. Both P450 2B6 and P450 2B6.4 were inactivated by 17EE and efavirenz when tBHP was used as an oxidant. P450 2B6 7-EFC O-deethylation activity remaining was 52 ± 2% when the concentration of efavirenz was 50 μM and 32 ± 1% when incubated with 80 μM efavirenz (Table 1). P450 2B6 was also inactivated by 17EE in the presence of tBHP in a concentration-dependent manner (Table 1). It is noteworthy that tBHP was also able to support the inactivation of P450 2B6.4 by both compounds, although the mutant enzyme does seem to be less susceptible to inactivation by 17EE compared with the wild-type enzyme (Table 1). Thus, in a system that is not dependent upon reductase, NADPH, and oxygen, the variant enzyme behaved in a manner similar to that of the wild-type enzyme. These data suggest that the ability of P450 2B6.4 to interact with reductase may be compromised.

Determination of the Apparent $K_d$ of Reductase Binding to P450s 2B6 and 2B6.4. To determine whether

---

**Fig. 2.** Formation of P450 2B6 N-phenylprotoporphyrin IX regioisomers. P450 2B6 was incubated with phenyldiazene and the phenyl-iron complex formation was observed spectrally as described under Materials and Methods. Oxidation caused migration of the phenyl to the porphyrin nitrogens. The individual peaks represent migration of the phenyl to pyrrole rings A ($N_A$), B ($N_B$), C ($N_C$), or D ($N_D$). Sample treatment and HPLC separation of the regioisomers were performed as described under Materials and Methods. The inset shows the heme structure with labeled pyrrole rings A, B, C, and D. The chromatograms are representative of three separate experiments.
the lack of inactivation of the mutant enzyme by 17EE and efavirenz in the reconstituted system was the result of impaired interaction with reductase, complex formation of the P450s with reductase was measured spectrophotometrically. The binding of reductase to P450s results in a low to high spin shift in the heme iron, characterized spectrally by a decrease in the absorbance at 418 nm and an increase in the absorbance at 385 nm (French et al., 1980). The apparent $K_d$ for the interaction of the reductase with P450 2B6.4 (918 nM) was almost 4-fold greater than the value obtained from experiments with the wild-type enzyme (240 nM). This difference does not seem to be marked enough to solely account for the differences in catalytic activity we observed between the two enzymes, because we routinely used reductase concentrations in excess of P450. To gain a more complete understanding of the catalytic activities of the two enzymes, the reaction stoichiometry for the metabolism of 17EE and efavirenz by P450 2B6 and P450 2B6.4 was determined.

**Reaction Stoichiometry.** As shown in Table 2, NADPH consumption was not increased by the presence of substrate when measured for either of the enzymes. During the metabolism of 17EE and efavirenz by P450 2B6, hydrogen peroxide was formed at a rate similar to the rate of product formation. In contrast, the majority of NADPH consumed by P450 2B6.4 resulted in the formation of hydrogen peroxide (Table 2). These data indicate that the metabolic reactions of P450 2B6.4 with 17EE and efavirenz are more uncoupled than the metabolism of these compounds by P450 2B6.

Several studies have demonstrated that cytochrome $b_5$ can increase the coupling of P450 catalyzed reactions, including those involving P450 2B enzymes (Gruenke et al., 1995; Perret and Pompon, 1998). With this in mind, we measured NADPH consumption, hydrogen peroxide formation, and product formation in the presence of cytochrome $b_5$. Reconstitution of P450 2B6.4 with cytochrome $b_5$ as well as reductase dramatically improved the coupling of both reactions (Table 3). It is noteworthy that cytochrome $b_5$ had only a minimal effect on the coupling of the wild-type reactions (Table 3). Furthermore, P450 2B6.4-mediated 17EE product formation, as measured by substrate depletion, was observed.
when cytochrome \( b_5 \) was present in the reconstitution mixture (Table 3). In light of these findings, we went on to test whether inactivation of P450 2B6.4 by efavirenz and 17EE could occur in the presence of cytochrome \( b_5 \).

**TABLE 2**

<table>
<thead>
<tr>
<th>P450</th>
<th>Inactivator</th>
<th>Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 ( \mu )M Inactivator</td>
<td>80 ( \mu )M Inactivator</td>
</tr>
<tr>
<td>2B6</td>
<td>Efavirenz</td>
<td>52 ± 2 32 ± 1</td>
</tr>
<tr>
<td>2B6.4</td>
<td>Efavirenz</td>
<td>66 ± 3 48 ± 1</td>
</tr>
<tr>
<td>2B6</td>
<td>17EE</td>
<td>33 ± 1 21 ± 2</td>
</tr>
<tr>
<td>2B6.4</td>
<td>17EE</td>
<td>81 ± 4 70 ± 2</td>
</tr>
</tbody>
</table>

**Discussion**

We have demonstrated previously that the mutation of lysine 262 of the P450 2B6 protein to arginine can result in...
marked changes in catalytic activity (Bumpus et al., 2005; Bumpus et al., 2006). Our findings that P450 2B6.4 was not inactivated by efavirenz and 17EE, known inactivators of the wild-type enzyme, were the most intriguing (Bumpus et al., 2005). Additional studies performed in our lab have demonstrated that differences in catalytic activity between the two enzymes are not limited to efavirenz and 17EE because phencyclidine, a well characterized inactivator of wild-type P450 2B6, also does not inactivate P450 2B6.4 (Shebley and Hollenberg, 2007).

Previous studies using a number of P450s have demonstrated that phencyclidine is a useful tool for gaining information about the topology of the P450 active site (Dierks et al., 1998; Schrag and Wienkers, 2000; Yamaguchi et al., 2004). In the case of certain P450 isoforms, including bacterial P450s and P450 3A4, the information gained using phencyclidine has been consistent with X-ray crystallography data (Ortiz de Montellano, 1995; Yamaguchi et al., 2004). In the present study, we used phencyclidine to investigate whether the K262R mutation leads to significant changes in active site topology. Although the overall profiles for the formation of the regioisomers were similar between the two enzymes, there was a difference in the migration of the phenyl group to the nitrogen of the pyrrole ring C. In the experiments with the wild-type enzyme, the NC regioisomer accounted for 46% of the total formation. However, in the case of the mutant, NC formation accounted for only 37% of the total. Although this difference is small, it suggests that the active site topologies of the two enzymes differ to some extent. Subsequent spectral binding studies using benzphetamine and efavirenz as substrates revealed that the binding of these two substrates was similar in the two enzymes, suggesting that the K262R mutation does not significantly affect binding.

According to the P450 2B4 crystal structure, the only structure of a P450 2B family member currently available, residue 262 is in the G/H loop (Scott et al., 2003, 2004). Although this region is not near the active site, it could potentially play a role in the interaction with reductase. To test whether the K262R mutation alters the ability of the enzyme to interact with reductase, we performed experiments to measure the ability of each P450 to associate with reductase. These studies suggested that the mutant may have a somewhat lower affinity for reductase; however, the difference between the mutant and wild-type enzymes in reductase binding did not seem profound enough to account for the marked differences in catalytic activity. In addition, we used tBHP as an oxidant to determine whether the mutant enzyme would be catalytically similar to the wild-type in the absence of the requirement to interact with reductase. It is noteworthy that in the presence of alternate oxidants, the mutant was readily inactivated by 17EE and efavirenz, suggesting that electron transfer to P450 2B6.4 may be compromised during these reactions when using the reconstituted system.

To gain a comprehensive understanding of a particular reaction, it is necessary to determine the stoichiometry of the reaction. One way to do this is by measuring the change in absorbance over time as a function of substrate concentration and reaction rate. This data can be plotted to show the relationship between absorbance change and substrate concentration. The slope of the line can be used to calculate the reaction rate, and the y-intercept can be used to determine the initial absorbance.

**Fig. 4. Inactivation of P450 2B6.4 by efavirenz in the presence of cytochrome b₅.** The time- and concentration-dependent inactivation of P450 2B6.4 by efavirenz in the presence of cytochrome b₅ was measured by determining the 7-EFC O-deethylation activity. After initiation of reaction by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 15, and 20 min. The concentrations of efavirenz were 0 µM (■), 10 µM (▲), 20 µM (●), 40 µM (○), and 50 µM (□). The data show the means and standard deviations from four separate experiments done in duplicate. In some cases, the standard deviations were less than the size of the symbols. The inset shows the double reciprocal plot of the rates of inactivation as a function of the efavirenz concentrations.
reaction. Investigation of the stoichiometry for metabolism of 17EE and efavirenz by the two enzymes indicated that the mutant appeared to be more uncoupled. Coupling can be defined by the percentage of electrons from NADPH used toward the formation of monooxygenated metabolites. Therefore, uncoupling refers to a decrease in monooxygenated metabolite formation and a concomitant increase in nonproductive metabolite product formation. This can occur as a result of the autooxidation of the oxyferrous P450 to superoxide, which is ultimately converted to hydrogen peroxide, as well as from the release of hydrogen peroxide from the peroxy-iron intermediate. Furthermore, “excess” water can be produced from the 4-electron reduction of oxygen by two molecules of NADPH. Reaction stoichiometry relates NADPH and oxygen consumption with hydrogen peroxide and metabolite formation. It is noteworthy that the rate of NADPH consumption by each of the enzymes was similar in both the presence and absence of substrate. In the absence of substrate, the major product formed by the wild-type enzyme was water, whereas hydrogen peroxide formation was favored by the mutant enzyme. This suggests that P450s 2B6 and 2B6.4 are highly uncoupled enzymes and that in the absence of substrate they may function as NADPH oxidases and proceed through the catalytic cycle, resulting in the formation of the shunted products hydrogen peroxide and water. In an attempt to improve the coupling of the P450 2B6.4 reactions, cytochrome $b_5$ was added to the reconstitution mixture. The presence of cytochrome $b_5$ improved the coupling of the mutant enzyme, and the hydrogen peroxide levels were closer to those observed for the reactions catalyzed by the wild-type enzyme. However, the rate of water formation by both enzymes was similar in both the presence and absence of cytochrome $b_5$, suggesting that cytochrome $b_5$ preferentially affected the shunt pathway leading to $H_2O_2$ formation. Furthermore, the addition of cytochrome $b_5$ to the reconstitution mixture resulted in the inactivation of P450 2B6.4 by efavirenz and 17EE and the ability of the enzyme to metabolize 17EE to generate a number of products.

The precise mechanism by which cytochrome $b_5$ acts upon certain P450s remains unknown, although it has been shown to increase, inhibit, or have no effect on P450 activity depending upon the P450 isoform and the substrate being investigated (Schenkman and Jansson, 2003). Two primary hypotheses explain the stimulatory effect of cytochrome $b_5$ on some P450-mediated reactions. The first is that reduced cytochrome $b_5$ donates the second electron in the catalytic cycle to the P450 (Yamazaki et al., 2001, 2002; Zhang et al., 2005). This is supported by studies showing electron transfer from cytochrome $b_5$ to P450 as well as the observation that “uncoupling” is decreased in the presence of the $b_5$ protein (Perret and Pompon, 1998). In this instance, the presence of cytochrome $b_5$ results in a decrease in hydrogen peroxide formation and a subsequent increase in product formation, possibly by stabilizing the oxyferrous P450 complex, leading to a decrease in release of superoxide (Perret and Pompon, 1998). It is noteworthy that studies performed by Zhang et al.

---

**Fig. 5.** Inactivation of P450 2B6.4 by 17EE in the presence of cytochrome $b_5$. The time- and concentration-dependent inactivation of P450 2B6.4 by 17EE was measured by determining the 7-EFC O-deethylation activity. After initiation of the reaction by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 15, and 20 min. The concentrations of efavirenz were 0 µM (■), 40 µM (▲), 80 µM (●), 120 µM (□), and 160 µM (◆). The data show the means and standard deviations from four separate experiments done in duplicate. In some cases the standard deviations were less than the size of the symbols. The inset shows the double reciprocal plot of the rates of inactivation as a function of the 17EE concentrations.
(2003), under single-turnover conditions, demonstrate that cytochrome b₅ and reductase reduce oxyferrous P450 (in this case P450 2B4) at a similar rate, although catalysis occurs faster in the presence of cytochrome b₅ (Zhang et al., 2003). To explain this phenomenon, the authors hypothesize that the conformation of the oxyferrous P450 may be different in the presence of cytochrome b₅ and reductase, leading to more rapid catalysis although the rate of reduction is the same. The second hypothesis is that cytochrome b₅ physically interacts with the P450 causing a conformational change that facilitates interaction with the substrate or reductase. This notion is supported by studies in which the apo-cytochrome b₅, which cannot be reduced and thereby donate the second electron, was able to stimulate P450-catalyzed reactions (Yamazaki et al., 1996). In our studies, cytochrome b₅ improved the coupling of the mutant enzyme, facilitating metabolism of 17EE by the mutant and inactivation by both 17EE and efavirenz. Furthermore, we performed studies using both apo-cytochrome b₅ and Mn-cytochrome b₅, which cannot act as electron donors, and saw no improvement in the catalytic activity of P450 2B6.4 (data not shown). With these data in mind, we propose that cytochrome b₅ may act in our system by stabilizing the oxyferrous P450 2B6.4 in the presence of 17EE and efavirenz, as evidenced by a decrease in hydrogen peroxide formation and increase in product formation. The fact that the rate of water formation, which occurs later in the cycle, was not affected by cytochrome b₅ lends more support to this notion. However, we cannot rule out the possibility that oxyferrous P450 2B6.4 may exist in a different conformation in the presence of 17EE and efavirenz compared with the wild type. Although the exact molecular mechanism underlying the differences between these two enzymes remains to be elucidated, the studies reported here have provided evidence that the stability and/or conformation of the oxyferrous intermediate may play a role. In addition, it is possible that multiple mechanisms may be involved, because the presence of cytochrome b₅ did not result in P450 2B6.4-mediated metabolism of 17EE comparable with that of the wild type. Further studies to elucidate the precise mechanism by which cytochrome b₅ increases the catalytic activity of P450 2B6.4 could potentially include measuring the formation and stability of the oxyferrous complex of P450 2B6.4 versus P450 2B6.

In summary, we have investigated the effects of the K262R mutation on active site topology, substrate binding, interaction with reductase and reaction stoichiometry. The inactivation of P450 2B6.4 by efavirenz and 17EE showed an absolute requirement for cytochrome b₅. In the presence of cytochrome b₅, the reactions catalyzed by the mutant enzyme exhibited improved coupling. These studies provide evidence that the differences in the catalytic properties of P450 2B6 and P450 2B6.4 are related to uncoupling of P450 2B6.4-mediated metabolism.

**Fig. 6.** Metabolism of 17EE by P450 2B6.4 in the presence of cytochrome b₅. Metabolites A₁, A₂, and C are monohydroxylated metabolites, although the exact identities have not yet been determined. Metabolite D corresponds to 2-hydroxy-17EE, metabolite E corresponds to estrone. The parent compound, 17EE, is labeled. We previously showed that in the absence of cytochrome b₅, P450 2B6.4 does not readily metabolize 17EE (Bumpus et al., 2005).
Acknowledgments

We thank Hisia-lien Lin for expression and purification of reductase and Dr. Kate Noon for thoughtful discussions and suggestions regarding the phenyldiazene experiments.

References


Address correspondence to: Dr. Paul F. Hollenberg, Department of Pharmacology, The University of Michigan, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0632. E-mail: phollen@umich.edu