

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

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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 mutant 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 decom-

position 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). How-

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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; *t*BHP, *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-Phenylprotoporphyrin 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 μ l of phenyldiazene to 200 μ l of 1N KOH. For the myoglobin experiments, 5 nmol of myoglobin in 100 mM KP_i , pH 7.4, was placed into a 1-ml cuvette, and the absorbance spectrum from 400 to 500 nm was recorded. Then, 3 μ l 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. For the P450 experiments, 2 nmol of P450 in 100 mM KP_i , pH 7.4, was placed into a 1-ml cuvette, and the absorbance from 400 to 500 nm was measured. Then, 1.5 μ l 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 μ l of potassium ferricyanide (50 μ M) 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*-phenylprotoporphyrins were reconstituted in 150 μ l 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 μ M 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 KP_i , pH 7.4, and placed into a cuvette. The reference cuvette also contained 1 μ M P450s in 100 mM KP_i , 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 added using Prism software (GraphPad Software, San Diego, CA). Spectral binding studies to determine the apparent K_d of reductase binding to P450 were performed in a similar fashion by titrating 1 μ M P450 with reductase (0–8 μ M) as described previously by French et al. (1980). The K_s for benzphetamine or efavirenz binding and the apparent K_d values for reductase binding were approximated by plotting the inverse of the absorbance changes between 390 and 420 nm

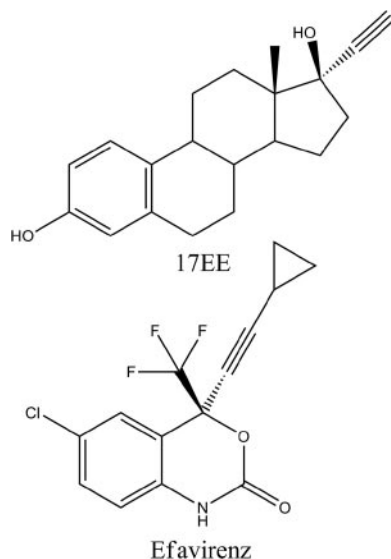


Fig. 1. Chemical structures of efavirenz and 17EE.

(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 (*t*BHP) 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 KPi, pH 7.4, and diluted to a final volume of 200 μ l. After a 5-min preincubation of the P450 with *t*BHP and inactivator, an aliquot (12 μ l) of this primary mixture was transferred into 990 μ l of assay mixture that contained 100 μ M 7-EFC and 40 μ g 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 *t*BHP (2.5 mM) used in experiments to test for inactivation was determined to be optimal by measuring 7-EFC *O*-deethylation activity at concentrations of *t*BHP ranging from 0 to 5 mM. The presence of 2.5 mM *t*BHP 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 *b*₅, samples were reconstituted in a 1:2:1 M ratio of P450:reductase/cytochrome *b*₅. P450 and reductase were incubated together on ice for 5 min before the addition of cytochrome *b*₅. 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 3 min before the addition of NADPH to a final concentration of 200 μ M. NADPH consumption was measured continuously, in both the presence and the absence of substrate (10 μ M 17EE or efavirenz), by monitoring the absorbance at 340 nm over 4 min. The concentration of NADPH was determined using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹ (Gorsky et al., 1984). To measure product formation, 700 μ l of the sample was removed and the reaction was quenched by the addition of 300 μ l of acetonitrile. Because the metabolism of both efavirenz and 17EE leads to the formation of multiple products, substrate depletion was used to quantify product formation. For this reason, substrate concentrations were used in which depletion could be readily determined. The samples were analyzed by HPLC as described previously (Kent et al., 2002; Bumpus et al., 2006). The remaining 300 μ l of the sample was used to determine the amount of hydrogen peroxide formed using the ferrithiocyanate method (Hildebrandt et al., 1978). Excess water formed as a result of the four-electron reduction of oxygen by two units of NADPH was estimated using the following equation: $H_2O = (NADPH - (\text{Product} + H_2O_2))/2$.

Inactivation of P450 2B6.4 in the Presence of Cytochrome *b*₅. The purified P450 was reconstituted with reductase and cytochrome *b*₅ as described above for 45 min at 4°C. The primary reaction mixtures contained 1 μ M P450, 2 μ M reductase, 1 μ M cytochrome *b*₅, 110 U of catalase, and 0–50 μ M efavirenz or 0–160 μ M 17EE in 50 mM potassium phosphate buffer, pH 7.4. The primary reaction mixtures were then incubated for 10 min at 30°C before the addition of NADPH to a final concentration of 1.2 mM. After the initiation of the reaction by the addition of NADPH, 12- μ l aliquots were removed from the primary reaction mixtures at the times indicated (5, 10, 15, and 20 min) and transferred to 990 μ l of the secondary reaction mixtures, which contained 100 μ M 7-EFC, 1 mM NADPH, and 40 μ g BSA/ml in 50 mM potassium phosphate buffer, pH 7.4. The secondary reaction mixtures were incubated for 10 min at 30°C, and then quenched by the addition of 334 μ l of acetonitrile. The amount of 7-hydroxy-4-(trifluoromethyl) coumarin formed was measured at room temperature using an excitation wavelength of 410 nm and an emission wavelength of 510 nm on a RF-5310 Spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Wood Dale, IL).

17EE Metabolism. P450 2B6.4 was reconstituted together with reductase and cytochrome *b*₅ as described above. The primary reaction mixture contained 1 μ M P450, 2 μ M reductase, 1 μ M cytochrome *b*₅, 200 μ g/ml ascorbate, 110 U of catalase, 40 μ M 17EE, and

50 mM potassium phosphate buffer, pH 7.4. The metabolites were resolved by reversed-phase HPLC according to a published protocol (Kent et al., 2002).

Results

P450 2B6 and P450 2B6.4 Active Site Topology. Phenyldiazene 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 N_A, N_B, N_C, and N_D, allow 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). Phenyldiazene 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 phenyldiazene with myoglobin (data not shown). The major product formed by the wild-type enzyme was N_C, which accounted for 46 \pm 2% of the total regioisomer formation. N_A, N_B, and N_D were also detected and accounted for 10 \pm 0.7, 7 \pm 0.5, and 37 \pm 1%, respectively (Fig. 2). Interestingly, there were some differences observed between the mutant and the wild-type enzyme. The reaction of phenyldiazene with P450 2B6.4 also resulted in the formation of all four *N*-phenylprotoporphyrin IX regioisomers (Fig. 3). However, N_C represented only 37 \pm 1% of the overall formation, whereas N_A, N_B, and N_D constituted 15 \pm 0.5, 11 \pm 3, and 38 \pm 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 (*K*_s) 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 *K*_s values for benzphetamine binding to P450s 2B6 and 2B6.4 were 18 \pm 0.7 μ M and 17 \pm 0.4 μ M, respectively. The efavirenz spectral dissociation constants were also similar between the two enzymes, with a value of 85 \pm 2.3 μ M for the wild-type enzyme and 123 \pm 3.1 μ M for the variant enzyme. The maximum ΔA values were similar in all cases. The *K*_s 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.

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. *t*BHP 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 *t*BHP 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 *t*BHP 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 *t*BHP was used as an oxidant. P450 2B6 7-EFC *O*-deethylation activity remaining was $52 \pm 2\%$ when the concentration of efavirenz was $50 \mu\text{M}$ and $32 \pm 1\%$ when incubated with $80 \mu\text{M}$ efavirenz (Table 1). P450 2B6 was also inactivated by 17EE in the presence of *t*BHP in a concentration-dependent manner (Table 1). It is noteworthy that *t*BHP 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

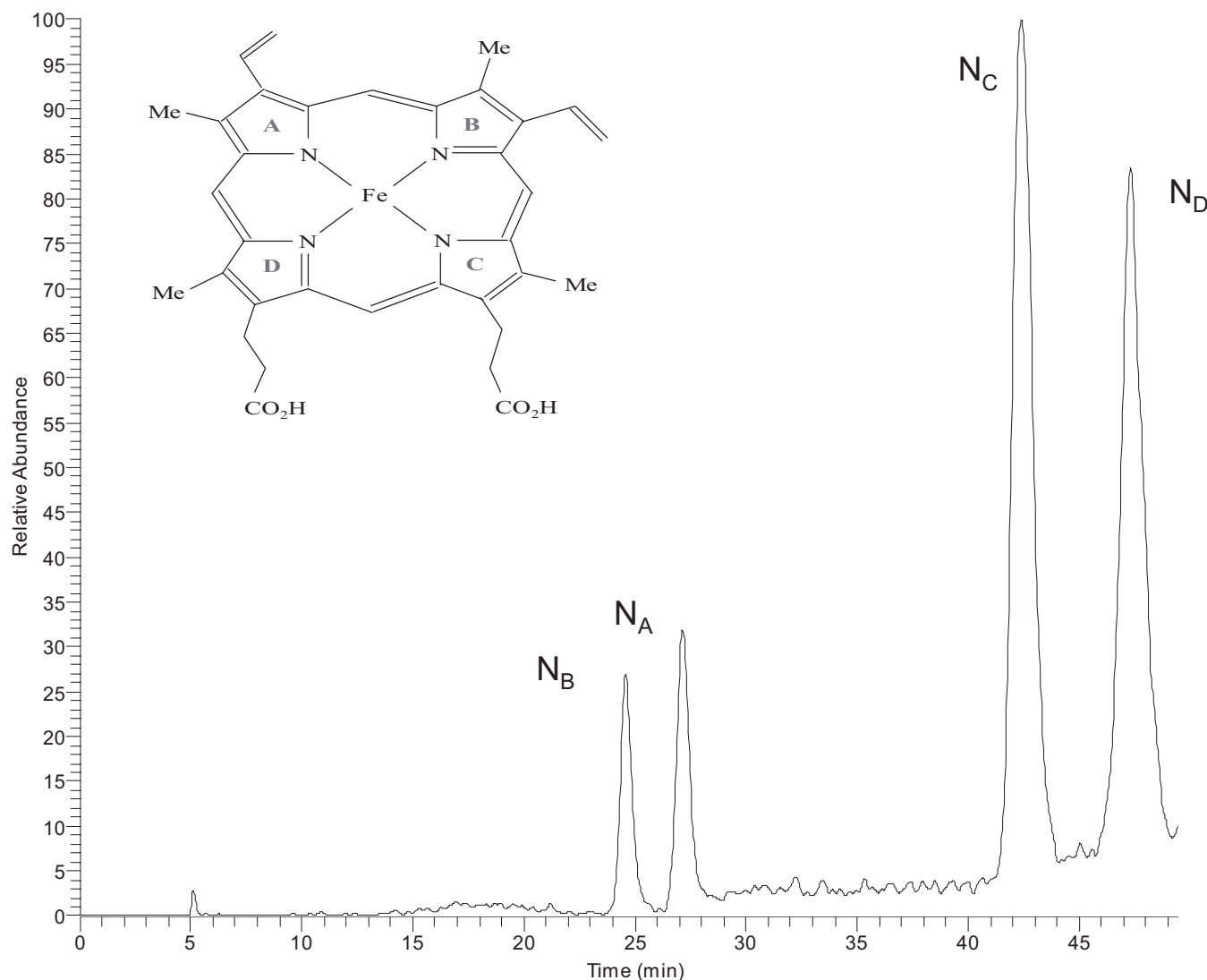


Fig. 2. Formation of P450 2B6 *N*-phenylprotoporphyrin IX regioisomers. P450 2B6 was incubated with phenyldiazene and the phenyl-iron complex formation was observed spectrally as described under *Materials and Methods*. Oxidation caused migration of the phenyl to the porphyrin nitrogens. The individual 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

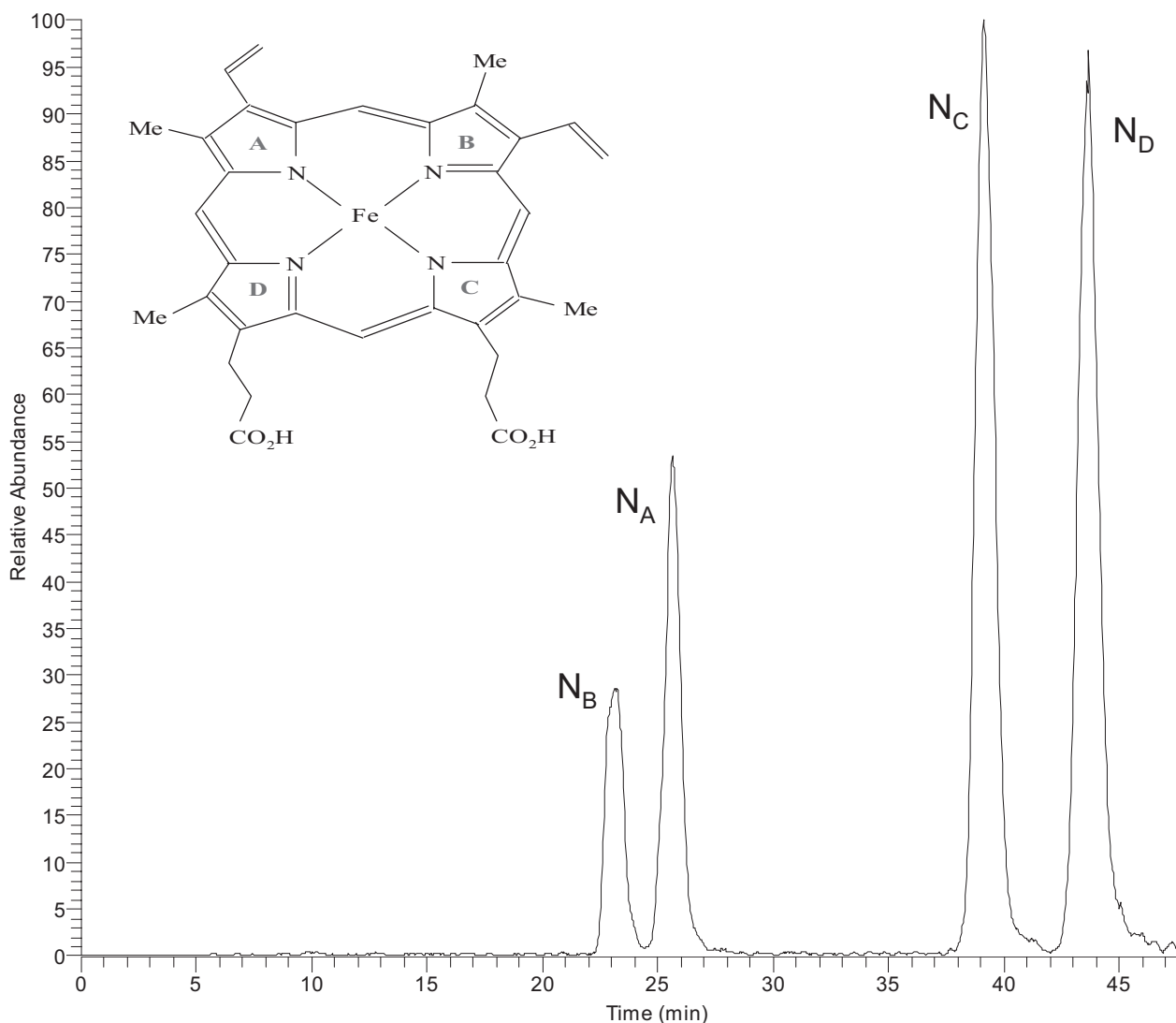


Fig. 3. Formation of P450 2B6.4 *N*-phenylprotoporphyrin IX regioisomers. P450 2B6.4 was incubated with phenyldiazene and the phenyl-iron complex formation was observed spectrally as described under *Materials and Methods*. Oxidation caused migration of the phenyl to the porphyrin nitrogens. The individual 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.

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 .

Inactivation of P450 2B6.4 by 17EE and Efavirenz in the Presence of Cytochrome b_5 . We previously reported that P450 2B6.4 is not inactivated by 17EE and efavirenz when reconstituted with reductase alone (Bumpus et al., 2005, 2006). Inactivation of the mutant enzyme by 17EE and efavirenz in the presence of cytochrome b_5 was measured using the 7-EFC *O*-deethylation assay. P450 2B6.4 was inactivated by efavirenz (Fig. 4) and 17EE (Fig. 5) in a time- and concentration-dependent manner, and the inactivation exhibited an absolute requirement for NADPH. The activity loss followed pseudo-first-order kinetics. Linear regression analysis was performed, and the kinetic constants for the efavirenz-mediated inactivation of the mutant enzyme were determined from the insets in Figs. 4 and 5. The K_i values for inactivation of P450 2B6.4 by efavirenz and 17EE were 30

and 113 μM , respectively. We have previously observed, for the wild-type 2B6, K_i values of 20 μM for efavirenz-mediated inactivation (Bumpus et al., 2006) and 10 μM for inactivation by 17EE (data not shown).

Metabolism of 17EE by P450 2B6.4 Requires Cytochrome b_5 . Studies were conducted to determine which 17EE metabolites were formed during metabolism by P450 2B6.4 in the presence of cytochrome b_5 . 17EE was incubated with P450 2B6.4 that was reconstituted with reductase and cytochrome b_5 in the presence or absence of NADPH. The metabolites were analyzed using reversed-phase HPLC as shown in Fig. 6. P450 2B6.4 metabolized 17EE to give a number of major metabolites, denoted C, D, and E, as well as minor metabolites A₁, A₂, and B. Metabolites D and E have been identified as estrone and 2-hydroxy-17EE, respectively. To date, the structures of metabolites A₁, A₂, B, and C have yet to be elucidated; however, LC/MS mass-to-charge ratios of 528, 451, 451, and 456 have been determined for these metabolites, respectively. Metabolite C is only present under conditions in which inactivation is observed, suggesting that this metabolite may play a critical role in the inactivation of the P450 by 17EE (Kent et al., 2002). Overall metabolite formation by the mutant enzyme was approximately 10-fold lower than what we previously observed for the wild-type enzyme. The ratios of formation for each of the metabolites were similar between the two enzymes, although metabolite B was not present in the profiles for the wild-type enzyme observed in our previous studies (Kent et al., 2002; Bumpus et al., 2005).

Discussion

We have demonstrated previously that the mutation of lysine 262 of the P450 2B6 protein to arginine can result in

TABLE 1

Inactivation of P450 2B6 and P450 2B6.4 using *tert*-butylhydroperoxide to support the reaction.

P450s were incubated with *t*BHP and the inactivators indicated as described under *Materials and Methods*. Activity remaining was determined using the 7EFC *O*-deethylation assay. The data are presented as percent activity remaining as compared to a control sample incubated with *t*BHP in the absence of efavirenz or 17EE.

P450	Inactivator	Activity Remaining	
		50 μM Inactivator	80 μM Inactivator
		%	%
2B6	Efavirenz	52 \pm 2	32 \pm 1
2B6.4	Efavirenz	66 \pm 3	48 \pm 1
2B6	17EE	33 \pm 1	21 \pm 2
2B6.4	17EE	81 \pm 4	70 \pm 2

TABLE 2

Stoichiometry for the metabolism of 17EE and efavirenz by P450 2B6 and P450 2B6.4.

P450s were reconstituted with reductase as described under *Materials and Methods*. NADPH oxidation was measured spectrally by monitoring absorbance at 340 nm. Portions of the sample were then used to determine hydrogen peroxide and product formation. Product formation was measured by substrate depletion.

P450 and Substrate	NADPH Oxidation	H ₂ O ₂ Formed	Product Formed	H ₂ O Formed
	nmol/nmol P450/min			
2B6				
No substrate	15.7 \pm 0.5	2.2 \pm 0.1	0	6.8
Efavirenz	16.2 \pm 0.9	6.3 \pm 0.1	6.8 \pm 0.2	1.6
17EE	15.1 \pm 0.6	7.8 \pm 0.3	7.3 \pm 0.4	0
2B6.4				
No substrate	14.7 \pm 0.6	7.2 \pm 0.3	0	3.8
Efavirenz	16.3 \pm 0.5	10.1 \pm 0.4	3.8 \pm 0.1	1.2
17EE	16.9 \pm 0.4	12.6 \pm 0.6	0.78 \pm 0.2	1.8

TABLE 3

Improvement in P450 2B6.4 reaction coupling upon reconstitution with cytochrome b_5

P450s were reconstituted with reductase and cytochrome b_5 and the assays were performed as described under *Materials and Methods*. NADPH oxidation was measured for 4 min then the sample was used to determine both hydrogen peroxide formation and product formation.

P450 and Substrate	NADPH Oxidation	H ₂ O ₂ Formed	Product Formed	H ₂ O Formed
	nmol/nmol P450/min			
2B6				
No substrate	14.3 \pm 0.4	1.8 \pm 0.2	0	6.3
Efavirenz	15.8 \pm 0.7	5.6 \pm 0.3	9.6 \pm 0.6	0.6
17EE	15.0 \pm 0.7	6.5 \pm 0.1	8.2 \pm 0.4	0.2
2B6.4				
No substrate	13.1 \pm 0.2	5.1 \pm 0.1	0	4.0
Efavirenz	15.4 \pm 0.5	5.2 \pm 0.2	7.7 \pm 0.2	1.3
17EE	15.0 \pm 0.3	6.9 \pm 0.1	5.3 \pm 0.3	1.4

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 phenylhydrazine, 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 phenylhydrazine 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 phenylhydrazine has been consistent with X-ray crystallography data (Ortiz de Montellano, 1995; Yamaguchi et al., 2004). In the present study, we used phenylhydrazine 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 N_C regioisomer accounted for 46% of the total formation. However, in the case of the mutant, N_C 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 *t*BHP 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

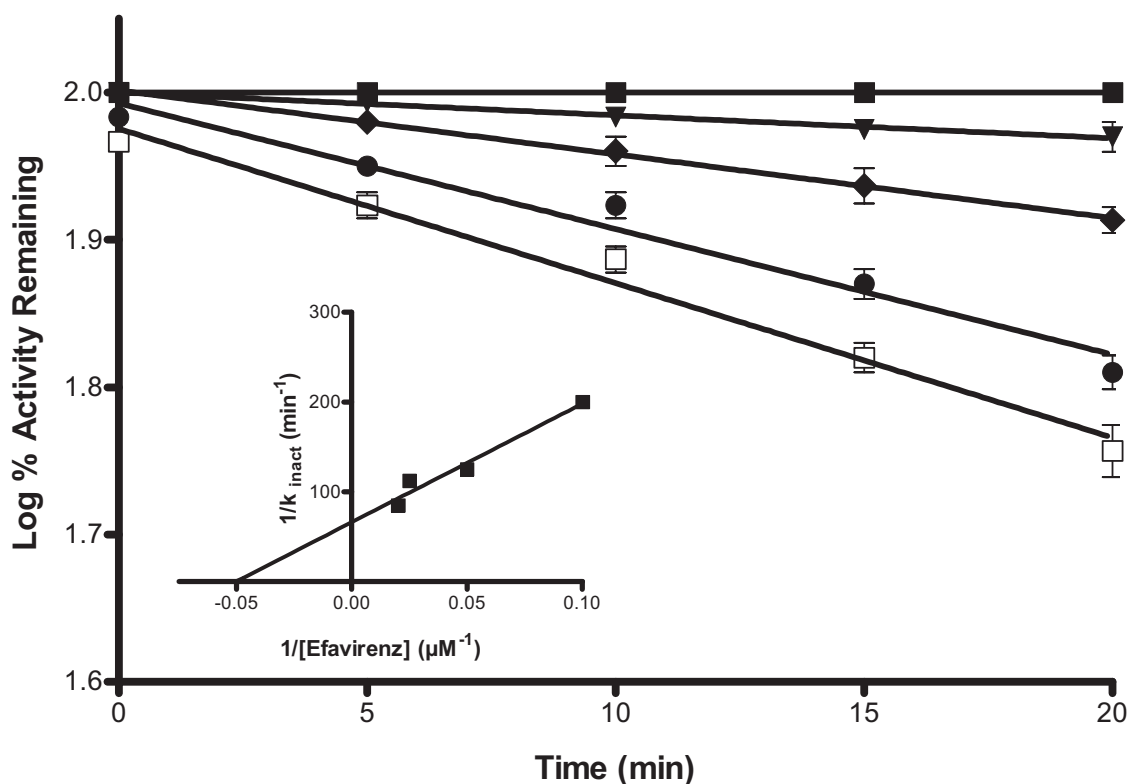


Fig. 4. Inactivation of P450 2B6.4 by efavirenz in the presence of cytochrome *b₅*. The time- and concentration-dependent inactivation of P450 2B6.4 by efavirenz in the presence of cytochrome *b₅* was measured by determining the 7-EFC *O*-deethylation activity. After initiation of reaction by the addition 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 peroxo-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.

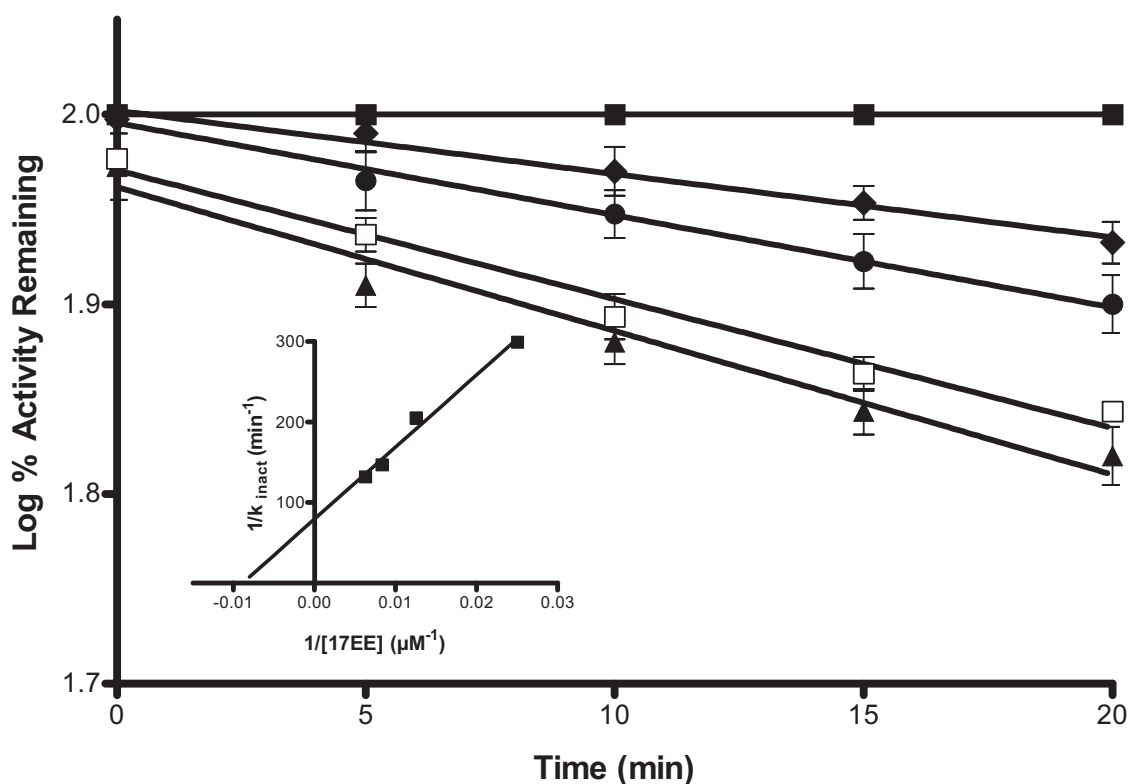


Fig. 5. Inactivation of P450 2B6.4 by 17EE in the presence of cytochrome b_5 . The time- and concentration-dependent inactivation of P450 2B6.4 by 17EE was measured by determining the 7-EFC *O*-deethylation activity. After initiation of the reaction by the addition of NADPH, aliquots were removed from the primary reaction mixture at 0, 5, 10, 15, and 20 min. The concentrations of efavirenz were 0 μ M (■), 40 μ M (◆), 80 μ M (●), 120 μ M (□), and 160 μ M (▲). 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 b_5 and reductase reduce oxyferrous P450 (in this case P450 2B4) at a similar rate, although catalysis occurs faster in the presence of cytochrome b_5 (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 b_5 and reductase, leading to more rapid catalysis although the rate of reduction is the same. The second hypothesis is that cytochrome b_5 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 b_5 , 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 b_5 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 b_5 and Mn-cytochrome b_5 , 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 b_5 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 b_5

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 b_5 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 b_5 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 b_5 . In the presence of cytochrome b_5 , 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.

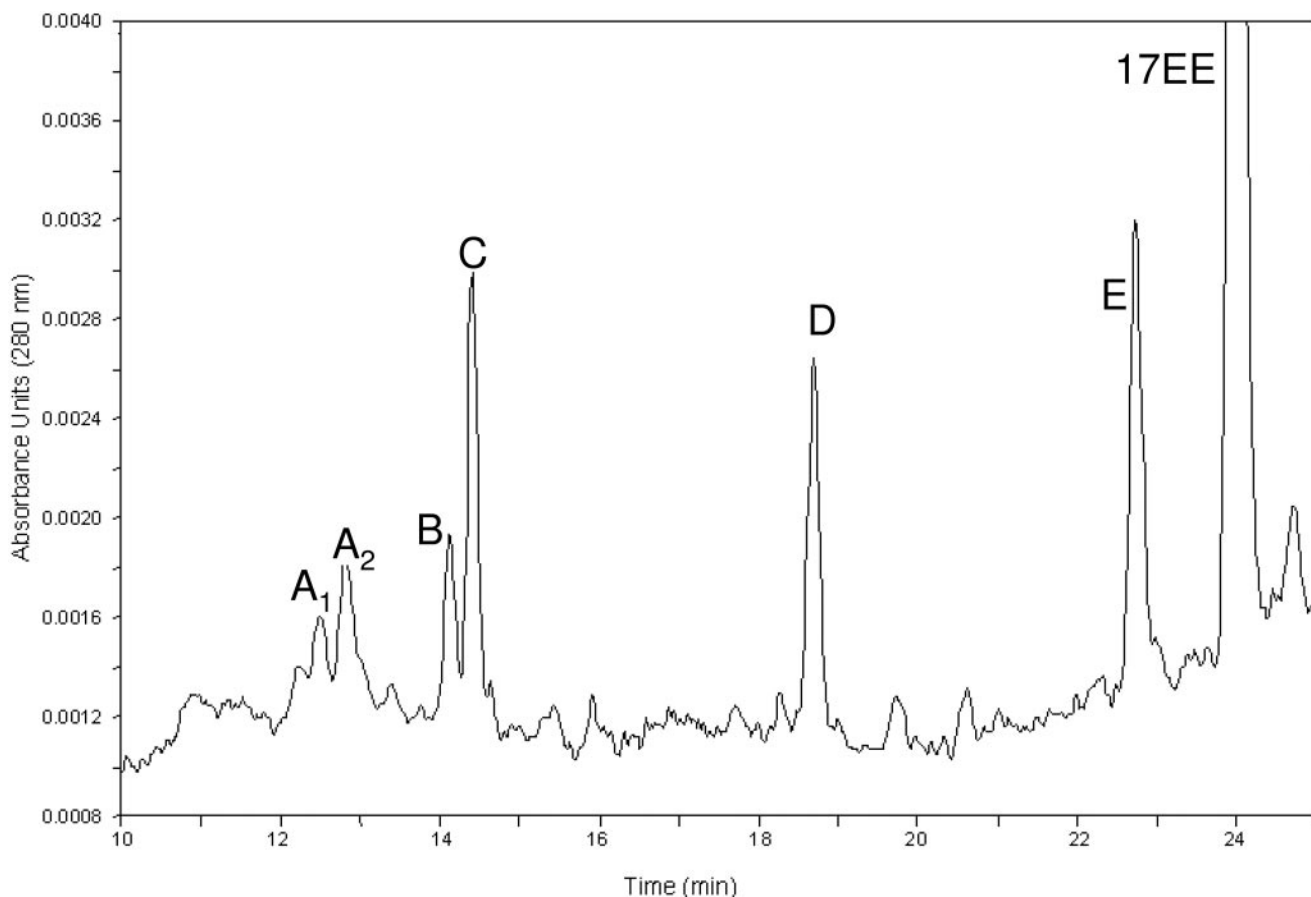


Fig. 6. Metabolism of 17EE by P450 2B6.4 in the presence of cytochrome b_5 . Metabolites A₁, A₂, and C are monohydroxylated metabolites, although the exact identities have not yet been determined. Metabolite D corresponds to 2-hydroxy-17EE; metabolite E corresponds to estrone. The parent compound, 17EE, is labeled. We previously showed that in the absence of cytochrome b_5 , P450 2B6.4 does not readily metabolize 17EE (Bumpus et al., 2005).

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