The Functional Role of Threonine-205 in the Mechanism-Based Inactivation of P450 2B1 by Two Ethynyl Substrates: The Importance of the F Helix in Catalysis

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ABBREVIATIONS: P450, cytochrome P450; 2EN, 2-ethynylnaphthalene; 2NA, 2-naphthylacetic acid; EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; 17EE, 17α-ethynylestradiol; DLPC, dilauroyl-L-α-phosphatidylcholine; SRS, substrate recognition site; WT, wild-type P450 2B1 expressed in E. coli; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TMS, trimethylsilane; TIC, total ion chromatogram; GC-MS, gas chromatography and mass spectrometry.
ABSTRACT

We have previously demonstrated that substituting Val for Thr-205 in P450 2B1 abolishes the 16β-hydroxylation of testosterone and markedly decreases the ability of 2-ethynlnaphthalene (2EN) and 17α-ethynylestradiol (17EE) to inactivate P450 2B1. The role of Thr-205 has been further investigated by measuring the kinetics of the mechanism-based inactivation of the 7-ethoxy-(trifluoromethyl)coumarin deethylation activity of 2B1 by 2EN and 17EE in wild type (WT) and mutant P450s. In general, the kinetics of the inactivation of the Ser- and Ala-mutants was not significantly altered compared to WT. In contrast, the efficiency of the inactivation of the Val-mutant decreased by ~6-fold and ~30-fold for 2EN and 17EE, respectively. HPLC analysis and SDS gel electrophoresis demonstrated the covalent binding of radiolabeled 2EN- and 17EE-reactive intermediates to the WT apoprotein, but not the Val-mutant. The Val-mutant was able to metabolize 2EN to 2-naphthylacetic acid except the initial rate was slower than the WT. HPLC analysis of the 17EE incubation mixtures revealed three major metabolites and showed a correlation between the efficiency of inactivation and the generation of one of the major metabolites (C). Metabolite C was generated by the WT, Ser- and Ala-mutants. Metabolite C may be formed by the oxidation of the ethynyl group and this reactive intermediate contributes to the inactivation of P450 2B1 by 17EE. The site specific mutation of one residue, Thr-205 to Val, is sufficient to alter the profile of products formed during 17EE metabolism such that very low levels of metabolite C are formed and inactivation is essentially abolished.
The cytochrome P450 (P450) superfamily of enzymes is a group of heme proteins that catalyzes the metabolism of an extensive series of compounds including drugs, chemical carcinogens, fatty acids, pesticides, and steroids (Porter and Coon, 1991; Nelson et al., 1996). The critical residues involved in substrate binding and catalysis by P450 2B1 have been extensively studied using site-directed mutagenesis, susceptibility to inhibition by mechanism-based inactivators, and homology modeling based on the crystal structures of bacterial P450s and P450 2C5 (Dai et al., 1998; Domanski and Halpert 2001; Kent et al., 2001; Spatzenegger et al., 2001; Wang and Halpert 2002).

Recently, we have substituted Val, Ser or Ala for the Thr-205 in the bacterially expressed wild type P450 2B1 (WT) in an attempt to elucidate the importance of this residue in the catalytic mechanism of P450 2B1 for the metabolism of several substrates. Our results revealed that the 16β-hydroxylation activity for testosterone was dramatically suppressed in Ala- and Val-mutants and that the susceptibility to inactivation by 2-ethynynaphthalene (2EN) and 17α-ethynylestradiol (17EE) was markedly decreased in the Val-mutant (Lin et al., 2003a). The mechanism for the inactivation of P450 2B1 by 2EN and 17EE has been well characterized (Roberts et al., 1993; Kent et al., 2002). The results of these studies suggested that the site of covalent modification responsible for inactivation was in the peptide corresponding to residues 290-314 for 2EN-inactivation and residues 347-376 for 17EE-inactivation. Moreover, the susceptibility to inactivation varied among the members of the P450 2B subfamily. For example, 2EN inactivated P450s 2B1 and 2B4, but not P450 2B5, while 17EE inactivated P450s 2B1 and 2B6, but not P450s 2B2 and 2B4 (Roberts et al., 1994; Strobel et al., 1999; Kent et al., 2002).
F helix residues in several P450s: 2A, 2B, 2D, 2E, 3A, 11A, and 27A have been suggested to be part of substrate recognition site (SRS-2) as proposed by Gotoh (1992) and to play a role in the regio- and stereo-selectivity for product formation (Lindberg and Negishi, 1989; Iwasaki et al., 1990; Domanski and Halpert, 2001; Pikuleva et al., 2001; Xue et al., 2001; Kirton et al, 2002; Guengerich et al, 2003; Lin et al., 2003a; Spatzenegger et al., 2003). The key F helix residues were reported to be: Leu-209 in 2A4; Phe-209 in 2A5; Thr-205, Phe-206 and Leu-209 in 2B1; Glu-216 in 2D6; Leu-209 in 2E1; Asn-206 and Leu-210 in 3A4; Phe-202 in 11A1; and Phe-207, Ile-211 and Phe-215 in 27A1. Analyses of several P450 crystal structures have suggested that: (1) the F and G helices form both sides of the substrate access channel and the ceiling of the active site; (2) the residues lining the interior of the substrate access channel could play important roles in determining the orientation of the substrate as it enters the active site and influencing substrate and product selectivity; and (3) in the P450BM3-substrate complex, the F and G helices and the loop between them exhibit a rocking motion with the I helix as a fulcrum in the substrate-docking region to position the substrate for catalysis (Graham-Lorence et al., 1995; Hasemann et al., 1995; Williams et al., 2000; Haines et al., 2001). Taken together, it appears that F helix residues are prime targets for study in order to gain more information about the relationship between structure and function in mammalian P450s. Therefore, we have characterized the molecular mechanism of P450 2B1 inactivation by two ethynyl substrates, 2EN and 17EE, in WT 2B1 and three site specific mutants of 2B1 in which Ser, Ala or Val was substituted for Thr-205 in the F helix. The following three parameters were examined: (1) the kinetics of the inactivation of the 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) deethylation activity
by 2EN and 17EE; (2) the covalent binding of radiolabeled 2EN and 17EE to the inactivated apoprotein; and (3) the metabolism of 2EN and 17EE by the WT and mutant P450s. Our results showed that the Val-mutant displayed the most dramatic changes with respect to the three parameters studied. All of the P450s were able to metabolize 2EN to 2-naphthylacetic acid (2NA) to about the same level except for the Val-mutant, which exhibited a ~40% slower initial rate of metabolism. HPLC analysis demonstrated that all P450s were able to metabolize 17EE but they exhibited different metabolite profiles. The Val-mutant, which was resistant to inactivation by 17EE, generated extremely low levels of one of the major metabolites. Docking studies of 2EN and 17EE in the putative active site of P450 2B1 provided additional insights that were useful in interpreting these experimental results.
Materials and Methods

Materials. NADPH, L-α-dilauroyl-phosphatidyl choline (DLPC), testosterone, 2-naphtylacetic acid (2NA), 17α-ethynylestradiol (17EE), and catalase were from Sigma-Aldrich Chemical Co. (St. Louis, MO). 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) was from Molecular Probes (Eugene, OR). 2-ethynylnaphthalene (2EN) and [3H]-2EN were prepared as described previously (Roberts et al., 1993). [3H]-17EE was obtained from Amersham Biosciences (Piscataway, NJ). 2-OH-17EE was a generous gift from Dr. William Slikker (Department of Health and Human Services, Food and Drug Administration, AR). All other chemicals and solvents used were the highest purity available from commercial sources.

Purification of Enzyme. The site specific mutations of Thr-205 to Ser, Ala or Val in P450 2B1 were performed as previously described (Lin et al., 2003a). All P450s and reductase were expressed in \textit{Escherichia coli} and purified according to previous published procedures (Hanna et al., 1998). Rat P450 2B1 was purified from the livers of male Long Evans rats and rabbit P450 2B4 was purified from the livers of male New Zealand rabbits (Saito and Strobel, 1981).

EFC Deethylation Activity. To assess catalytic activity, purified P450 and reductase were reconstituted with DLPC at 22 °C for 30 min. The primary reaction mixture contained 300 pmol P450, 300 pmol reductase, 100 µg DLPC, 100 units of catalase and 2EN (up to 5 µM) or 17EE (up to 200 µM) in 300 µl of 100 mM potassium phosphate
buffer (pH 7.7). After incubation of the primary reaction mixture with 2EN or 17EE with or without 1 mM NADPH at 30 °C for the time indicated (up to 18 min), a 10 µl of aliquot was removed and added to 1 ml of a secondary reaction mixture containing 0.1 mM EFC and 0.2 mM NADPH. The formation of the product 7-hydroxy-(trifluoromethyl)coumarin was determined by fluorescence measurement as described previously (Lin et al., 2003a).

**HPLC Analysis of the Reconstituted System.** Control (-NADPH) and inactivated (+NADPH) samples containing 150 pmol of WT or the Val-mutant 2B1 incubated with 10 µM [³H]-2EN or [³H]-17EE were dialyzed extensively to remove non-covalently bound radioactivity. Following dialysis, the samples were analyzed by HPLC on a C4 protein and peptide column (4.6 x 250 mm, 300 Å; VYDAC, Hesperia, CA) using a linear gradient from 35% B to 80% B over 45 min with a flow rate of 1ml/min as previously described (Lin et al., 2002). The solvent system consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.05% trifluoroacetic acid in acetonitrile). The eluate was monitored at 220 nm for protein and 405 nm for heme. Fractions were collected and the radioactivity was determined by liquid scintillation counting.

**SDS-PAGE Analysis of the reconstituted System.** The reconstitution conditions were the same as those described for the primary reaction mixtures of the EFC deethylation assays. Control (-NADPH) and inactivated (+NADPH) samples containing 25 pmol of WT or the Val-mutant that had been incubated with 10 µM [³H]-17EE were resolved on a 10% polyacrylamide gel to investigate the relationship between the amount of
inactivation and the covalent binding of 17EE to the apoprotein. The Ala-mutant was also included for comparison to the WT and Val-mutant enzymes. The gel was treated with EN3HANCE autoradiography enhancer (Perkin Elmer Inc., Boston, MA), dried on a piece of 3-mm chromatography paper and exposed to Kodak BioMax MS film (Eastman Kodak, Rochester, NY) at -80 °C for 2 weeks.

**Metabolism of 2EN.** Each reaction mixture contained 250 pmol of P450 and 500 pmol of reductase in the reconstituted system with 50 µM 2EN and 1 mM NADPH in 250 µl of 100 mM potassium phosphate buffer (pH 7.7). After incubating for the indicated time, 40 µl of 30% phosphoric acid was added, the metabolites were extracted with 4 ml of ethyl acetate and the organic layer was dried under N₂. Quantitative analysis of the product was carried out on a Varian Microsorb-MV C18 column (5 µm, 100 Å, 4.6 x 250 mm; Walnut Creek, CA) according to previously described method (Roberts et al., 1993). The dried samples were redissolved in 150 µl of 37% solvent B (solvent A, 0.1% TFA and solvent B, 95% acetonitrile/0.1%TFA) and subjected to HPLC analysis using isocratic conditions of 37% B for 20 min followed by a linear gradient to 90% B over 5 min at a flow rate of 1.0 ml/min. The amount of 2NA product in the reaction mixture was determined at 220 nm by comparison to an authentic standard.

**Metabolism of 17EE.** Each reaction mixture contained 0.5 nmol of P450 and 1 nmol of reductase in the reconstituted system with 100 µM 17EE and 1 mM NADPH in 500 µl of 100 mM potassium phosphate buffer (pH 7.7). After 30 min, the samples were extracted with methylene chloride, dried under N₂ and redissolved in 150 µl of 50% solvent B
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(solvent A, 0.1% acetic acid in H₂O and solvent B, 49.9% CH₃OH/50% CH₃CN/0.1% acetic acid). The metabolites were separated on a C18 column by using a linear gradient from 30% B to 50% B over 3 min, to 60% B within the next 12 min, and then to 95% B for an additional 10 min at flow rate of 1.2 ml/min. The absorbance was monitored at 280 nm as previously described (Kent et al., 2002).

Identification of Metabolite C from 17EE Metabolism. Samples of metabolite C from the metabolism of 17EE by WT, Ser- and Ala-mutants in the reconstituted system were collected and analyzed by GC-MS at the Michigan State University Mass Spectrometry Facility. The procedure was essentially as previously described (Kent et al., 2002).

Docking of 2EN and 17EE in the 2B1 active site. The substrates 2EN and 17EE were docked into the putative active site of P450 2B1 using the Autodock 3.0 program on a Silicon Graphics Indigo platform (Morris et al., 1996). The homology model of P450 2B1 was constructed as previously described (Lin et al., 2003b). The coordinates for the substrate were built with CS Chem3D Pro software (Cambridge Software Corp., Cambridge, MA) and low energy conformers of 2EN and 17EE were obtained by energy minimization using the MOPAC PM3 potential function. The Autodock Tools software was used to add polar hydrogen atoms, assign partial charges, set up docking parameters, and analyze docked conformations. To explore all possible conformations, 2EN and 17EE were docked to the active site as flexible ligands. Initially, the substrates were randomly placed in the active site and the docking simulation was carried out using a
Lamarckian genetic algorithm search method. A cluster analysis with a tolerance of 0.5 Å was performed at the end of docking after a typical run of 100 cycles. Prior to docking 2EN and 17EE to P450 2B1, the docking process was tested on P450cam using camphor as the substrate and compared to the available crystal structure. Coordinates of P450cam and camphor were obtained from the Protein Data Bank (PDB ID 2CPP). The coordinates of camphor were split from those of P450cam and then docked to the active site of P450cam using the Autodock 3.0 program as described above. The results showed that camphor docked in the active site of P450cam was in virtually the same conformation as revealed in the crystal structure, verifying the reliability of the docking procedure.
Results

Kinetic Values for Inactivation. The inactivation of the EFC deethylation activity of WT and mutant P450s by the two ethynyl substrates was measured using four concentrations of inactivators (0.5 to 5 µM for 2EN and 5 to 200 µM for 17EE) and using four time points (2, 4, 8, and 18 min) at each concentration. The kinetic values: $K_i$, $k_i$ and $t_{1/2}$ were calculated essentially as previously described (Roberts et al., 1993; Kent et al., 2002) and are shown in Table 1. In general, the kinetic values for the inactivation of the Ser- and Ala-mutants by 2EN or 17EE were not dramatically different from those for the WT enzyme. If the efficiency of inactivation is expressed as $k_i/K_i$, then the mutation of Thr-205 to Val resulted in an approximately 6-fold and 30-fold decrease in the efficiency of inactivation by 2EN and 17EE, respectively (Roberts et al., 1998). The extents of inactivation as a function of the concentration of the inactivator following incubation for 8 min are shown in Fig. 1 to highlight the differences with the Val-mutant. Fig. 2 shows the time-dependent inactivations of the various P450s by either 5 µM 2EN (A) or 50 µM 17EE (B) in the P450 reconstituted system. With both inactivators, substitution of Val for Thr resulted in a P450 which was much more resistant to inactivation than the WT or the Ser- or Ala-mutants.

Covalent Binding of Radiolabeled 2EN or 17EE to the P450 Apoprotein. HPLC separation of control (-NADPH) and inactivated samples (+NADPH) incubated with radiolabeled 2EN or 17EE showed that essentially all of the radioactivity was associated with the P450 apoprotein eluting at ~40 min for 2EN (Fig. 3A) and 17EE (Fig. 3B). In
all the HPLC experiments, the apoprotein of the Val mutant eluted two ml later than the WT, indicating the Thr to Val conversion at position 205 may have changed the conformation of the mutant resulting in tighter binding to the reverse-phase column and slightly longer retention times of the apoprotein. We do not believe this is due to a different protein modification. Essentially no radioactivity was associated with heme, which eluted at ~11 min or reductase, which eluted at ~23 min (data not shown). Although some radioactivity was bound to the Val-mutant apoprotein after inactivation, the WT had ~6 and ~10 times more radioactivity associated with it than the Val-mutant after inactivation by 2EN and 17EE, respectively. Negligible amounts of radioactivity were associated with the apoproteins in any of the control samples. The covalent binding of a reactive intermediate of 17EE to the apoprotein was also demonstrated by SDS-PAGE followed by autoradiography. As shown in Fig. 4, covalent binding of $[^3]$H]-17EE to the WT protein was observed in the inactivated sample from WT, but not from the Val-mutant. Most of the radioactivity was associated with the P450 apoprotein; however, a small amount of radioactivity was also associated with reductase. With longer exposures, trace amounts of radioactivity were also seen with some high-molecular weight bands having MW > 110 kDa in the inactivated sample of WT (data not shown). Western blot analysis revealed that these bands were recognized by an anti-P450 2B1 antibody (data not shown), suggesting that 17EE-inactivation may also lead to the formation of aggregates of P450 2B1. The Ala-mutant, which can be effectively inactivated by 17EE, was included for comparison. It can be seen in Fig. 4 that the covalent binding of 17EE to the Ala-mutant apoprotein was similar to that of the WT. Our results from the HPLC and SDS-PAGE
studies with radiolabeled inactivators clearly demonstrate that there is significant covalent binding to the apoprotein and that the binding correlates with the extent of inactivation of the P450s.

**Metabolism of 2EN and 17EE.** The marked decrease in the level of covalent binding to the apoprotein of the Val-mutant coupled with the decreased susceptibility of this mutant to inactivation by both inactivators when incubated with NADPH could be due to the inability of this mutant P450 to metabolize the inactivators or it could be due to the substituted residue affecting the stability and/or the binding orientation of the reactive intermediate towards the target residue in active site. Therefore, the ability of the WT protein and the mutant P450s to metabolize 2EN and 17EE was characterized.

The metabolism of 2EN to form 2NA by the WT and Val-mutant was determined at various time points and the results are shown in Fig. 5. Although the Val-mutant initially exhibited a ~40% slower rate of product formation compared to WT, total product formation reached a level comparable that seen with the WT by 30 min of incubation. The Ser- and Ala-mutants also produced about the same amount of 2NA during a 30 min incubation with 2EN (data not shown). The slower initial rate of 2EN metabolism may in part contribute to the reduced susceptibility to 2EN-inactivation by the Val-mutant.

The HPLC separation profiles for 17EE and its metabolites formed by various P450 isoforms are displayed in Fig. 6. After incubation of the WT 2B1 enzyme with 17EE in the reconstituted system, approximately the same levels of all three major metabolites (B, C and D) were generated. However, with the Val-mutant only two of the
major metabolites (B and D) were observed and the generation of metabolite C was severely impaired. Metabolite D (2-OH-17EE) and metabolite B (unknown) have previously been shown not to be responsible for the inactivation of the P450 2B subfamily of enzymes by 17EE (Kent et al., 2002). In order to determine whether the extent of inactivation of the P450s correlated with the formation of metabolite C, the metabolism of 17EE by the Ser- and Ala-mutants was further characterized. Surprisingly, these two mutants generated metabolite C as the predominant metabolite, whereas metabolites B and D were diminished compared to the WT. To assure that this predominant metabolite had the same retention time as metabolite C from WT, the metabolites formed by the Ala- or Ser-mutant were co-injected together with those from the WT. The intensity of the metabolite profiles was altered as expected, with metabolites B and D remaining at about same level whereas metabolite C was elevated markedly (data not shown). The total major metabolites of the Val-, Ser-, and Ala-mutants were ~75% of WT. Because all the P450s have the ability to metabolize 17EE, the efficiency of inactivation may, therefore, depend on the formation of metabolite C. A previous study has demonstrated that 17EE can inactivate rat P450 2B1, but not rabbit P450 2B4 (Kent et al., 2002). The metabolism of 17EE by rat P450 2B1 and rabbit P450 2B4 was evaluated to test this hypothesis. The profile of the three major metabolites generated by rat P450 2B1 was essentially similar to the WT with metabolite C being one of the major metabolites, whereas metabolite C was almost undetectable following metabolism by rabbit P450 2B4. The metabolite profiles for these six P450 isoforms shown in Fig. 6 confirm that inactivation by 17EE is not observed with those P450s where the formation of metabolite C is negligible. This finding suggests that the reactive intermediate leading
to the formation of metabolite C may be responsible for the 17EE-dependent inactivation of P450 2B1 as well as the Ser- and Ala-mutants.

**GC-MS Analysis of Metabolite C.** In order to determine the identity of metabolite C and to see if metabolite C produced by the WT, Ser- and Ala-mutants was identical, the corresponding HPLC fractions were collected, derivatized with trimethylsilane (TMS) and subjected to GC-MS analysis. The GC retention times, total ion chromatograms (TIC), the \( m/z \) of the molecular ion, as well as the \( m/z \) of ion fragments of metabolite C formed by all three P450s were identical. As was seen previously with metabolites from rat P450 2B1, two prominent signals at scan number 1312 - 1313 and at scan numbers 1321 - 1323 were generated in each case (Kent et al., 2002). Both signals for all three samples gave rise to the same \( m/z \) ions of 456, 441, 428, 371, 338, 323, 298, 143, and 73. The mass of 73 corresponds to TMS. The HPLC and GC retention times as well as the \( m/z \) ions observed for metabolite C did not match any of the known standards tested such as hydroxyestradiol or hydroxyestrone. The TMS-derivatized parent with a mass of 456, corresponding to compound with mass of 312 plus 2 molecules of TMS, suggested that the mass of the unknown was 312, which is a possible product from the insertion of one oxygen into the ethynyl group of 17EE with the mass of 296. This parent mass in conjunction with the \( m/z \) ions 441 and 428 suggests a loss of a methyl group and carbon monoxide and would be most consistent with the formyl-D-homoestrone observed by Schmid and co-workers (Schmid et al., 1983). The two GC signals that were seen for metabolite C from all three P450s were identical in mass and \( m/z \) ion patterns and may correspond to the same molecule existing in equilibrium between the homoestrone
alcohol and the aldehyde. Fig. 7 illustrates a scheme for the postulated metabolic pathways giving rise to the reactive intermediates of 2EN and 17EE that result in the inactivation of P450 2B1 (Schmid et al., 1983; Roberts et al., 1993; Kent et al., 2002). The scheme suggests that the ethynyl group in both inactivators is oxidized by P450 2B1 to a 2-oxo or 17α-oxirene reactive intermediate in 2EN and 17EE, respectively. The reactive intermediate can then attack the target residue(s) on the apoprotein. Alternatively, the ketene intermediate formed during 2EN metabolism could react with water to form the carboxylic acid product 2NA. The 17α-oxirene intermediate obtained during the metabolism of 17EE could rearrange through D-homoannulation to form a formyl-D-homosterone which can exist as an equilibrium mixture of the alcohol and aldehyde products.
Discussion

The importance of Thr-205 in WT P450 2B1 has been demonstrated (Lin et al., 2003a). Three site-directed mutants were generated and analyzed with six substrates including two mechanism-based inactivators. When Thr-205 was converted to Val, the testosterone 16β-hydroxylase activity was abolished and the susceptibility to inactivation by 2EN and 17EE was largely suppressed. These two inactivators were chosen for further investigation of the functional role of Thr-205 because mechanism-based inactivation by these two ethynyl substrates has previously been well characterized (Roberts et al., 1993; Kent et al., 2002). Our results show that: (a) the inactivations of all P450s by 2EN and 17EE were concentration- and time-dependent; (b) substitution of Thr-205 with Ala or Ser did not significantly alter the susceptibility to inactivation by 2EN or 17EE; (c) the Val-mutant had a 6-fold decrease in efficiency of 2EN-inactivation and 30-fold decrease in efficiency of 17EE-inactivation when compared to WT; (d) high levels of covalent binding of radiolabeled 2EN and 17EE to the apoprotein were observed in the WT, but not in the Val-mutant; and (e) all of the P450s metabolized 2EN and 17EE to relatively similar extents, except that the Val-mutant exhibited slower initial rates with 2EN and generated very low levels of metabolite C from 17EE when compared to the WT.

The ability of 2EN and 17EE to inhibit the EFC deethylation in these four P450s with various amino acid substitutions at position 205 was Thr ≅ Ser ≅ Ala > Val. Although both Ala and Val are relatively hydrophobic, their effects on the inactivation by 2EN and 17EE were quite different. The Ala-mutant was similar in function to the WT. Perhaps Ala, a small residue, is less likely to induce helical distortion or interfere
with substrate orientation compared to the more bulky Val residue. After comparing the metabolite profiles for testosterone, 2EN and 17 EE formed by the WT, Ala-, Ser- and Val-mutants, the contribution of the hydroxyl group of Thr to the metabolism of 2EN and 17EE was not as obvious as with testosterone (Lin et al., 2003a). We performed docking studies of 2EN and 17EE with the WT protein to aid in interpreting the possible structure/function relationships of Thr-205 in the mechanism-based inactivation of P450 2B1.

Previous work in our laboratory has established that a ketene intermediate is produced during the metabolism of 2EN. This intermediate may then react covalently with a nucleophilic group of an I helix residue and inactivate the enzyme or it may react with water to form 2NA (Roberts et al., 1993). Although the Val-mutant was able to oxidize 2EN to 2NA, it was not inactivated and covalently modified as extensively as the WT. Thr-205 is not absolutely necessary for 2EN oxidation but is required in to achieve the maximal rate of inactivation and covalent binding. The decrease in efficiency of inactivation is 6-fold, but the decrease in the initial rate of metabolism is only ~40% when Thr-205 is replaced by Val. Factors other than just a decrease in the rate of metabolism may account for the decreased efficiency of inactivation. Modeling studies involving docking of 2EN into the P450 2B1 active site suggest that the distance between Thr-205 and 2EN is more than 5 Å and may not have a direct effect on catalysis. However, Phe-206 is only 3.15 Å or 3.57 Å away, depending on the two different orientations of 2EN. Replacing Thr-205 with a bulky hydrophobic residue such as Val may move Phe-206 away from the substrate binding pocket resulting in a significant geometrical alteration in the active site. Although such a change may not
significantly affect the metabolism of 2EN, it may lead to an increase in the mobility of the 2EN reactive intermediate in the active site thereby altering the interaction with the target residue in the I helix. The smaller Ser and Ala residues may not influence the conformation of the F helix enough to significantly interfere with the covalent binding of the reactive intermediate.

The metabolite profiles of 17EE incubated with the six different P450s revealed several interesting findings. All the P450s that were effectively inactivated by 17EE generated metabolite C in high levels. Thus, the reactive intermediate leading to the formation of metabolite C may be responsible for the 17EE-dependent inactivation. The formation of both 2-OH-17EE, a result of A ring oxidation (2α) and metabolite C, an oxidation product of the D ring (17α-ethynyl group), suggests that either 17EE can enter the substrate access channel oriented in two opposite directions or that the P450 2B1 substrate binding pocket is large enough to accommodate two superimposed 17EE molecules as has been described for the hydroxylation of testosterone at the 2α– and 16α– position by P450 2C11 (Schenkman, 1992). Another possibility is that the steroid molecule may exist in a dynamic equilibrium with multiple orientations in the P450 active site as suggested for 2A4/2A5 (Negishi et al., 1996). The metabolite profiles for 17EE reported here suggest that the orientation of 17EE in the P450 active site is such that: (1) the A or D ring can approach the heme iron of the WT enzyme with about the same frequency, (2) in the Ser- and Ala-mutants, the D ring preferentially orients towards the heme iron, and (3) the A ring is preferentially oriented towards the heme iron in the Val-mutant. The docking model shown in Fig. 8B provides additional valuable information. The model suggests that: (a) the orientation of 17EE is such that the D ring
is favored to face the heme iron; (b) there is not enough room for two 17EE molecules in
the substrate-heme pocket; and (c) the A ring is in close contact with Thr-302 (I helix)
whereas the B ring is in close contact with Phe-206 (F helix), and the D ring is in close
contact with Val-367 (K helix) and Phe-206 (F helix). A possible explanation for
hydroxylation of the 2α position (A ring) and the oxidation of the ethynyl group (D ring)
in P450 2B1 is that the substrate may enter the active site with two distinct binding
orientations with either the A or the D ring facing the heme iron. Thr-205 is
approximately 6 Å away from 17EE in the model whereas Phe-206 is only approximately
3.24 Å from C-7 (B ring) and 3.56 Å from C-15 (D ring). The results from the
metabolism of 17EE and the information obtained from the docking model lead us to
postulate the following. The mutation of Thr-205 to Val could alter the critical helical
conformation, thereby loosening the interaction of Phe-206 with the B and D rings. The
change in the substrate binding region in the F helix could steer the ethynyl moiety away
from the heme iron reducing the formation of metabolite C. Ala or Ser at position 205
may modify the F helix leading to a closer contact with 17EE and stabilize the
interaction between the ethynyl group and molecular oxygen at the active site.
Substituted residues in the mutant P450s may alter the motion of the F helix to such an
extent as to be incompatible with the substrate achieving the correct orientation for
entering the substrate access channel. The different profiles of the metabolites generated
by WT and mutant P450s clearly indicate that the microenvironment of residue 205 and
its immediate neighboring residue(s) have a marked influence on the conformation of the
F helix and can impact on its control of substrate binding orientation in the active site.
Our P450 2B1 homology model was based on the crystal structure of the substrate-bound
conformation of P450 2C5 rather than the crystal structure of the open conformation of P450 2B4 because the open conformation may represent an inactive form of the enzyme (Scott et al., 2003). A recently published report of the crystal structure of the closed conformation of P450 2B4 indicates that the substrate-bound structures of P450s 2C5 and 2B4 are similar (Scott et al., 2004).

A mutation of Thr-205 to Val has differential effects on the inactivation of P450 2B1 by 2EN and 17EE. The Thr to Val conversion decreased the $k_i$, but did not significantly alter the $K_I$ for EFC deethylation activity after incubation with 2EN. The mutation dramatically decreased both the $k_i$ and the $K_I$ for the inactivation of EFC deethylation activity after incubation with 17EE. The decrease in the efficiency of inactivation following the Val conversion is greater with 17EE than 2EN. The 2EN and 17EE docking models suggest that the substrate pocket can accommodate two 2EN molecules, but only one 17EE molecule. Therefore, the modification of the three dimensional structure of the F helix resulting from the Thr to Val conversion had a more profound effect on 17EE than on 2EN. The decrease in the efficiency of inactivation by 2EN was in part due to a local conformational change that resulted in a decrease in the interaction of the ketene intermediate with a target residue in the I helix. The resistance to inactivation of the Val-mutant by 17EE was primarily due to a modification of the F helix that resulted in severely impaired oxidation of the ethynyl group at the D ring. The stable product of the 2EN reactive intermediate is a carboxylic acid whereas the stable product of the 17EE reactive intermediate appears to be an alcohol or aldehyde, the isomer of the formyl-D-homoestrone. The loss in susceptibility to 17EE-inactivation by the Val-mutant lead us to suggest that metabolite C contributes to the inactivation.
The findings in this report demonstrate that although the F helix is believed to be spatially distant from the heme iron, interactions with residues(s) in the SRS-2 or a change in the F helix structure could readily affect the active site region and strongly control the binding orientation and site of oxidation of substrates. These observations underscore the importance of the F helix in catalysis by mammalian P450s.
Acknowledgments

Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility.


Hasemann CA, Kurumbail RG, Boddupalli SS, Peterson JA and Deisenhofer J (1995)


Roberts ES, Hopkins NE, Zaluzec EJ, Gage DA, Alworth WL and Hollenberg PF (1994) Identification of active-site peptides from 3H-labeled 2-ethynlnaphthalene-


Footnotes

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Figure Legends

Fig. 1. Loss of EFC deethylation activity of P450s due to mechanism-based inactivation by 2EN (A) and 17EE (B). The data shown are representative from the experiments in which samples were incubated for 8 min with four concentrations of 2EN or 17EE as described in Materials and Methods. The P450s were WT (●), Ser mutant (□), Ala-mutant (■), and Val-mutant (○).

Fig. 2. Time-dependent inactivation of the EFC deethylation activity of P450s by 2EN (A) and 17EE (B). The results presented are from experiments in which the P450s were inactivated by 5 µM 2EN or 50 µM 17EE as described under Materials and Methods. The P450s were WT (●), Ser mutant (□), Ala-mutant (■), and Val-mutant (○).

Fig. 3. HPLC elution profile of P450 WT and the Val-mutant apoprotein from the reconstituted systems incubated with radiolabeled 2EN (A) or 17EE (B). The control sample (-NADPH) or the inactivated sample (+NADPH) were separated by reverse phase HPLC, and the fractions were counted as described in Materials and Methods. The WT P450 samples were incubated with (●) or without (○) NADPH, and the Val-mutant samples were incubated with (●) or without (○) NADPH.

Fig. 4. Autoradiography of the SDS-PAGE separations of the proteins in the reconstituted system. An incubation mixture containing reconstituted P450, reductase, catalase and radiolabeled 17EE in the absence (-) or in the presence (+) of NADPH was separated on SDS-PAGE. P450 (25 pmol) was loaded in each lane of a 10% polyacrylamide gel. The gel was dried and analyzed by autoradiography as described in Materials and Methods.
Fig. 5. Formation of 2NA from the metabolism of 2EN by the WT and Val-mutant proteins. The reaction mixtures were initiated with NADPH, quenched at the time points indicated with 30% phosphoric acid, and then extracted with ethyl acetate. Product formation was analyzed by reverse phase HPLC using a C18 column as described in Materials and Methods.

Fig. 6. Representative HPLC chromatogram of 17EE metabolites generated by six P450 isoforms. Reconstituted P450s were incubated with 100 µM 17EE and 1 mM NADPH at 30 °C for 30 min and the products were extracted with methylene chloride as described in Materials and Methods. The metabolites were separated by HPLC and the relative absorbance was recorded at 280 nm. Metabolites B, C, and D and the substrate 17EE eluted at 13, 14, 17, and 20 min, respectively.

Fig. 7. Scheme proposed for the inactivation of P450 2B1 and the formation of the reactive intermediates and stable products generated during the metabolism of 2EN and 17EE by P450 2B1.

Fig. 8. Docking of 2EN (A) and 17EE (B) into the active site of the P450 2B1 model to illustrate the geometrical relationship of Thr-205, Phe-206, the F helix, I, and K helices, and the heme with the inactivators. The active site can accommodate two binding orientations of the 2EN molecule and one orientation of 17EE molecule with the ethynyl moiety (indicated by an arrow) facing toward the heme and the K helix.
Table 1. Inactivation of WT and mutant P450s by 2EN and 17EE<sup>a</sup>

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>P450</th>
<th>$K_i$ (µM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$k_i$ (min&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>$t_{1/2}$ (min)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>$k_i/K_i$ (min&lt;sup&gt;-1&lt;/sup&gt; µM&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2EN</td>
<td>WT</td>
<td>1.5</td>
<td>0.09</td>
<td>8</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Ser-mutant</td>
<td>2.2</td>
<td>0.08</td>
<td>9</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Ala-mutant</td>
<td>1.3</td>
<td>0.06</td>
<td>12</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Val-mutant</td>
<td>1.8</td>
<td>0.02</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>17EE</td>
<td>WT</td>
<td>9</td>
<td>0.06</td>
<td>12</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Ser-mutant</td>
<td>10</td>
<td>0.09</td>
<td>7</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>Ala-mutant</td>
<td>8</td>
<td>0.06</td>
<td>12</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Val-mutant</td>
<td>53</td>
<td>0.01</td>
<td>65</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>The incubation mixtures were described under Materials and Methods. Four concentrations of inactivators ranging from 0.5 to 5 µM for 2EN and from 5 to 200 µM for 17EE were used to determine the loss of EFC deethylation activity after 2, 4, 8, and 18 min of incubation with either inactivator.

<sup>b</sup>The concentration for inactivator required to give the half-maximal rate of inactivation.

<sup>c</sup>The maximal rate constant for inactivation at a saturating concentration of the inactivator.

<sup>d</sup>The time required for half of the P450 to be inactivated at a saturating concentration of the inactivator.

<sup>e</sup>The efficiency of inactivation (Roberts et al., 1998).
JPET #71670 Figure 2

A

B

Log % Activity Remaining vs. Time (min)

Time (min)
JPET #71670 Figure 4

WT  Val-mutant  Ala-mutant
-    +       -    +       -    +

Reductase

P450
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JPET #71670 Figure 6

Absorbance (280 nm)

Elution Time

B  C  D  17EE

WT

Val-mutant

Ser-mutant

Ala-mutant

Rat P450 2B1

Rabbit P450 2B4
Inactivator

Unstable Intermediate

Stable Product

2EN

H₂O

2-oxo Carboxylic Acid

Active P450

Inactive P450

17EE

D-Homoannulation

Rearrangement +
Ring expansion

17-α-oxirine

Alcohol

Keto-Enol
Tautomerization

Aldehyde

inactive P450

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Figure 8

(A) F helix, G helix, I helix

(B) F helix, G helix, I helix, K helix