ABSTRACT

Rabbit cytochromes P-450 (P-450) 2B4 and 2B5 differ by only 12 amino acid residues yet they exhibit unique steroid hydroxylation profiles. Previous studies have led to the identification of active site residues that are determinants of these specificities. In this study, mechanism-based inactivators were identified that discriminate between the closely related 2B4 and 2B5 enzymes. A previously characterized inhibitor, 2-ethynylnaphthalene (2EN), was found to be selective for 2B4 inactivation. As inhibitor metabolism and the partition ratio affect susceptibility, molecular dynamics simulations were performed to assess the stability of the productive binding orientation of 2EN within 2B4 and 2B5 three-dimensional models. Although 2EN was stable within the 2B4 model, it exhibited substantial movement away from the heme moiety in the 2B5 model. However, heterologously expressed 2B5 was found to catalyze the oxidation of 2EN to the stable product 2-naphthylacetic acid. Thus, the increased mobility of 2EN may result in reduced susceptibility of 2B5 by increasing the probability that the reactive ketene intermediate hydrolyzes with water instead of reacting with active site residues. Another compound, 1-adamantyl propargyl ether (1APE), selectively inactivated 2B5. The structural basis for 2EN and 1APE susceptibility was assessed using active site mutants. Interconversion of 2EN susceptibility was observed for 2B4 or 2B5 mutants containing a single alteration at residue 363. Single substitutions in 2B4 also conferred susceptibility to 1APE; however, multiple alterations were required to reduce the susceptibility of 2B5. These alterations may influence inhibitor susceptibility by affecting the stability of the productive binding orientation.

Recent advances in the discovery and design of isoform-selective cytochrome P-450 (P-450) inhibitors have contributed greatly to the identification of individual cytochromes responsible for particular detoxification and bioactivation reactions (Rendic and Di Carlo, 1997). Mechanism-based inactivators are especially useful for differentiation of P-450 enzymes, and progress has been limited in the identification of inhibitors able to distinguish between P-450 enzymes of high structural identity (Halpert, 1995). In recent years, homology modeling has become an important tool to study P-450 function. Molecular models have been used in combination with site-directed mutagenesis to identify and confirm active site residues and to analyze binding of inhibitors, as well as to explain alterations in enzyme inactivation due to residue replacement (Skłarz and Halpert, 1997). Modeling of secobarbital in the P-450 2B1 active site led to the identification of residues 302 and 363 as determinants of heme alkylation (He J. et al., 1996). In addition, docking of N-benzyl-1-aminobenzotriazole (BBT) in the 2B1 G478A active site suggested that the presence of Ala 478 and the altered heme pocket contributed to the decreased susceptibility of the mutant (Szklarz and Halpert, 1997). In this study, mechanism-based inactivators were identified that discriminate between the closely related 2B4 and 2B5 cytochromes P-450 (P-450). A previously characterized inhibitor, 2-ethynylnaphthalene (2EN), was found to be selective for 2B4 inactivation. As inhibitor metabolism and the partition ratio affect susceptibility, molecular dynamics simulations were performed to assess the stability of the productive binding orientation of 2EN within 2B4 and 2B5 three-dimensional models. Although 2EN was stable within the 2B4 model, it exhibited substantial movement away from the heme moiety in the 2B5 model. However, heterologously expressed 2B5 was found to catalyze the oxidation of 2EN to the stable product 2-naphthylacetic acid. Thus, the increased mobility of 2EN may result in reduced susceptibility of 2B5 by increasing the probability that the reactive ketene intermediate hydrolyzes with water instead of reacting with active site residues. Another compound, 1-adamantyl propargyl ether (1APE), selectively inactivated 2B5. The structural basis for 2EN and 1APE susceptibility was assessed using active site mutants. Interconversion of 2EN susceptibility was observed for 2B4 or 2B5 mutants containing a single alteration at residue 363. Single substitutions in 2B4 also conferred susceptibility to 1APE; however, multiple alterations were required to reduce the susceptibility of 2B5. These alterations may influence inhibitor susceptibility by affecting the stability of the productive binding orientation.

ABBREVIATIONS: P-450, cytochrome P-450; DMSO, dimethyl sulfoxide; EPh, ethynylphenanthrene; 1APE, 1-adamantyl propargyl ether; 5Ph1P, 5-phenyl-1-pentyne; 2EN, 2-ethynylnaphthalene; 2NA, 2-naphthylacetic acid; BBT, N-benzyl-1-aminobenzotriazole.
instead of Gly would hinder the oxidation at the 1-amino nitrogen due to van der Waals overlaps, explaining the resistance of this mutant to inactivation (Kent et al., 1997). Another approach for inactivation studies is to use data in a predictive manner. For example, residue 478 has been shown to be a determinant of susceptibility to inactivation by N-(2-p-nitrophenethyl)chlorofluoroacetamide, based on the resistance of the 2B1 G478A variant to this inhibitor (Kedzie et al., 1991; He et al., 1992). This led to the prediction that 2B2, which also contains an Ala at this position, would not be inactivated by this compound. This prediction was confirmed for the heterologously expressed 2B2 enzyme (Strobel and Halpert, 1997).

The above findings suggest that one can rationally design or predict selective inactivators based on the identity of known active site residues. One goal of this study was to determine whether computer homology models can be used to accurately identify determinants of susceptibility and explain how inhibitors distinguish between two closely related enzymes. To address this question, P-450 2B4 and 2B5 were selected as the targets of potential inactivators. These two enzymes differ by only 12 residues, yet exhibit distinct substrate specificities. We reasoned that based on subtle differences in the active sites of 2B4 and 2B5, selective inactivators could be identified. In prior studies BBT derivatives inactivated both enzymes, whereas phenacyclidine was selective for 2B4 (Grimm et al., 1994, 1995).

Several recent studies have used a series of aryl acetylenes to assess differences in P-450 susceptibility to mechanism-based inactivation (Robert et al., 1996a; Foroozesh et al., 1997). These compounds form a reactive ketene intermediate that attacks either the heme moiety or a side chain to inactivate the enzyme, or alternatively, reacts with water to form stable product (Ortiz de Montellano and Kunze, 1980). The partition ratio is a measure of the efficiency with which the reactive species inactivates the enzyme, as opposed to rearranging to a stable product (Ator and Ortiz de Montellano, 1990). The activity of aryl acetylenes to inactivate P-450s 2B1, 2B4, 2B6, 2B11, 1A1, 1A2, and 1B1 varied with the size and shape of the aromatic ring systems, and the placement of the carbon-carbon triple bond in the molecule (Hopkins et al., 1992; Robert et al., 1996a; Foroozesh et al., 1997; Shimada et al., 1998). Thus, these compounds showed promise for selective inactivators of 2B4 and 2B5.

This study identifies 2-ethylnaphthalene (2EN) and 1-adamantyl propargyl ether (1APE) as selective inactivators of 2B4 and 2B5, respectively. The molecular basis for the susceptibility to inactivation was assessed. Molecular dynamics simulations suggest that differences in 2EN mobility and its preferred orientation in the active site may determine susceptibility to inactivation. In addition, based on the ability of 2B5 to catalyze oxidation of 2EN to 2-naphthylacetic acid (2NA), this reduced susceptibility is not due to a loss of inhibitor metabolism, but to an increase in product formation relative to enzyme inactivation.

**Experimental Procedures**

**Materials.** Lyria-Bertani Broth and Terrific Broth media for bacterial growth was purchased from Gibco-BRL (Grand Island, NY). The *Escherichia coli* strain Top3 was purchased from Stratagene (La Jolla, CA). NADPH, dilauroyl-t-3-phosphatidylcholine, BioMax MR-1 film, benzoyloxyresorufin, pentoxyresorufin, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid, benzoic acid, and 2NA were purchased from Sigma Chemical Co. (St. Louis, MO). HEPES was purchased from Calbiochem Co. (La Jolla, CA). [4-14C]Androstenedione and [4-14C]progesterone were purchased from DuPont-NEB (Boston, MA), and [4-14C]testosterone was obtained from Amersham Life Sciences (Arlington Heights, IL). Thin-layer chromatography plates [silica gel, 250 μM, Si 250PA (19C)] were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Rat NADPH-P-450 reductase was expressed in *E. coli* as described previously (Harlow and Halpert, 1997). 1-Adamantanol (1-hydroxymantane), sodium hydride, and 80% propargyl bromide in toluene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Reagent grade tetrahydrofuran, methylene chloride, and petroleum ether were obtained from Fisher Scientific (Pittsburgh, PA). 9-Ethynylphenanthrene (9EPh), 3EPh, 3EPh, and 9-1-propynylphenanthrene were synthesized as described previously (Hall et al., 1990; Hopkins et al., 1992). 4-Phenyl-1-butyne and 5-phenyl-1-pentene (5Ph1P) were obtained from Farchan Laboratories (Gainesville, FL). All other chemicals and supplies used were of the highest grade commercially available.

**Synthesis of 1APE.** 1-Adamantanol (3.04 g, 0.02 mol) was dissolved in 30 ml of freshly distilled tetrahydrofuran under N₂ atmosphere. Fresh sodium hydride (3 eq) was added slowly, followed by 2 eq of propargyl bromide. The reaction mixture was left to stir for 1 week under N₂ at room temperature. The reaction was quenched with 50 ml of deionized water and was extracted twice, each time with 30 ml of methylene chloride. The organic layers were combined and washed with 10% HCl followed by water. The crude product was then dried over anhydrous MgSO₄. The solvent was evaporated and the crude product was purified by flash silica gel column chromatography, using petroleum ether as solvent. The pure fractions were combined. The yield after purification was 47%. Gas chromatography-mass spectroscopy showed >99% purity; m/z (%): 190 (39), 94 (100). 1H NMR (CDCl₃): δ 1.60 (q, 6H), δ 1.79 (s, 6H), δ 2.25 (s, 3H), δ 2.37 (m, 1H), δ 4.30 (m, 2H).

**Heterologous Expression.** *E. coli* Top3 cells were used for P-450 expression. Preparation of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid-solubilized membranes and enzyme reconstitution were performed as described (John et al., 1994; Hasler et al., 1994). The 2B4 and 2B5 mutants analyzed in this study were constructed previously (He Y.Q. et al., 1996; Szklarz et al., 1996). The total P-450 concentration was measured by reduced CO difference spectra (Omura and Sato, 1964). In some experiments, phenobarbital-induced rabbit microsomes were used as a source of P-450 enzymes (Grimm et al., 1995).

**Assays of Substrate Oxidation.** Androstenedione, testosterone, and progesterone hydroxylase activities were measured as described (John et al., 1994; He et al., 1995; Szklarz et al., 1996). The final 150 μl reaction mixture contained 7.5 pmol of P-450, 15 pmol of NADPH P-450 reductase (Harlow and Halpert, 1997), 15 pmol of rat liver cytochrome b₅, 30 μg dilauroyl-t-3-phosphatidylcholine/ml, 1 mM NADPH, in 50 mM HEPES (pH 7.6), 15 mM MgCl₂, and 0.1 mM EDTA. Steroid substrate concentrations were 25 μM for [14C]androstenedione and [14C]progesterone, and 200 μM for [14C]testosterone. After addition of 1 mM NADPH, reactions proceeded for 5 min, except for 2B4 V367A and 2B5 A367V mutants, which were incubated for 10 min. Hydroxylated metabolites of androstenedione, testosterone, and progesterone were separated on thin-layer chromatography plates as described previously (John et al., 1994; He et al., 1995; Szklarz et al., 1996). Benzoyloxyresorufin and pentoxyresorufin O-dealkylase activities were measured using fluorometric analysis (excitation at 550 nm and emission at 585 nm) as described previously (Hasler et al., 1994; He et al., 1995).

**Mechanism-Based Inactivation.** Inactivation studies were performed as described previously (Kedzie et al., 1991; He et al., 1992, 1995). Inhibitors were added from a 100X dimethyl sulfoxide (DMSO) stock solution. Reconstituted P-450 samples were preincu-
bated with or without inhibitor for 2 or 5 min at 37°C. After addition of NADPH to start reactions, aliquots were removed at 0.5, 1, 1.5, 2, and 2.5 min into the secondary incubation with substrate. For experiments using rabbit microsomes, the protein concentration was 2 μg/μl, with a total of 20 μg used per time point. Aliquots of either 10 μl for steroid substrates (150 μl final reaction volume) or 25 μl for resorufin derivatives (500 μl final reaction volume) were removed at each time point. Dilutions from the primary to secondary incubations helped to minimize enzyme inhibition. Due to differences in the activities of the 2B4 and 2B5 mutants, several substrates were used for the inactivation studies. Benzoxylresorufin O-dealkylase activity was measured for 2B4, 2B4 I363V, 2B4 V367A, and 2B5 F114I-T294S-V363I-A367V. Testosterone hydroxylase activity was determined for 2B4 S294T, 2B4 I363V-S294T-V367A, 2B5 T294S, 2B5 A367V, and 2B5 F114I-T294S-V363I-A367V. Progestosterone hydroxylase activity was measured for 2B5 V363I, and both pentoxysorurin O-dealkylase and androstenedione hydroxylation activities were determined for 2B5. When microsomes were used as a source of P-450, the marker activities of benzoxylresorufin O-dealkylase and androstenedione hydroxylation activities were performed for 2B5. When microsomes were used as a source of P-450, the marker activities of benzoxylresorufin O-dealkylase (for 2B4) and androstenedione 15α-hydroxylase (for 2B5) were measured (Grimm et al., 1994, 1995). Rate constants for inactivation were calculated by linear regression analysis of the natural logarithm of the residual activity as a function of time. The rate constants of inactivation for independent experiments were averaged. In the few cases where k i values between independent experiments differed by more than 0.04 min⁻¹, additional experiments were performed to ensure the accuracy of values reported. The extent of reversible (competitive) inhibition was estimated from the decrease in the extrapolated activity at zero preincubation time compared with the methanol or DMSO control. Enzyme inactivation is measured as the loss of activity over time, and is substrate-independent. However, due to differences in relative affinities of a particular inhibitor and substrate for various 2B4 and 2B5 enzymes, competitive inhibition may differ between substrates.

**Computer Modeling of 2EN in 2B4- and 2B5-Active Sites.** The models of P-450 2B4 (Szklarz et al., 1996) and 2B5 (He Y.Q. et al., 1996) were constructed previously based on the model of P-450 2B1. The structure of 2EN was constructed using the Builder module of InsightII modeling package (Biosym/MSI, San Diego, CA). The three-dimensional structures were displayed on a Silicon Graphics workstation. Energy minimization and molecular dynamics simulations were carried out with the Discover program (version 2.97; Biosym/MSI), using consistent valence force field. The parameters for heme and ferryl oxygen were as described previously (Paulsen and Ornsbite, 1991, 1992). 2EN was docked into the P-450 2B4 or 2B5 active site in a productive binding orientation leading to substrate oxidation at the terminal carbon of the acetylenic group (C₁₂). The distance between this carbon and ferryl oxygen was 3 Å, the angle between the iron, oxygen, and carbon was close to 120°, and that between oxygen, C₁₂, and C₁ of 2EN was 90°. This orientation permits electron abstraction from the substrate. 2EN, a rigid molecule, was docked manually in the active site and the nonbond interaction energy between the compound and the protein was evaluated with the Docking module of InsightII to find low-energy binding orientations. To optimize enzyme-substrate interactions, 2EN was then fixed and the side chains of residues in contact with the substrate (within 5 Å) were minimized using the steepest descent method and harmonic potential, with a nonbond cutoff of 10 Å until the gradient was less than 5 kcal mol⁻¹ Å⁻¹ (Szklarz et al., 1995).

To evaluate the stability of 2EN in the reactive binding orientation in P-450 2B4 and 2B5 models, molecular dynamics simulations were performed. In these simulations the P-450 protein molecule was kept fixed whereas 2EN was allowed to move freely in the active site. The starting structure for the simulations was the optimized enzyme-substrate assembly obtained as described above. The conditions for molecular dynamics simulations were similar to those described previously (He Y.Q. et al., 1996; Kent et al., 1997). The leap-frog algorithm was used, the system was equilibrated for 0.1 ps, and the simulations continued for 5 ps at 300 K using 1 fs time steps. Structures were saved every 0.1 ps to provide an ensemble of possible substrate orientations that would represent movement of 2EN in the active site.

**Metabolism of 2EN by 2B4 and 2B5.** Metabolism of 2EN by heterologously expressed 2B4 and 2B5 was assayed as described previously (Roberts et al., 1993, 1996b). Briefly, reconstituted enzymes were incubated with 10 μM 2EN, and 100-μl aliquots containing 50 pmol of enzyme were removed at 5, 10, or 15 min. Reactions were quenched by the addition of 30% phosphoric acid. After addition of the benzoic acid internal standard, samples were extracted four times in ethyl acetate, dried under a stream of nitrogen, and resuspended in 100 ml of 20% buffer B. Buffer A was 0.5% trifluoroacetic acid in water, buffer B was 95% acetonitrile, 5% water, and 0.5% trifluoroacetic acid. The entire sample was injected onto a Beckman ODS HPLC column (4.6 mm × 25 cm, 5 μ). Detection was at 225 nm on a Beckman HPLC with a Spectroflow 757 detector and a Spectra Physics SP 4270 integrator. Retention times were 15 min for the benzoic acid standard and 27 min for 2NA. The 2NA product peak was confirmed by coelution with standard.

**Results**

**Inhibitor Selection and Molecular Dynamics of 2EN in Active Site of 2B4 and 2B5.** In recent studies, a series of aryl acetylenes were found to inactivate P-450 enzymes, including members of the 2B subfamily (Roberts et al., 1996a; Foroozesh et al., 1997). Thus these compounds were promising candidates for selective inactivators of 2B4 and 2B5. In addition, the mechanism of inactivation by acetylenic compounds has been determined, making it possible to correctly orient the inhibitors in the active site of computer homology models. An initial screening of compounds was performed using microsomes as a source of P-450 enzymes, and the established marker activities for 2B4 and 2B5. These studies showed that the known 2B4 inactivator, 2EN, was likely to be a selective inactivator. This small inhibitor molecule with a 2-ring structure might be expected to behave differently in the slightly larger active site of 2B5 compared with 2B4. Therefore, we performed molecular dynamics simulations to determine whether differences in orientation or mobility of 2EN could be observed in active site models.

First, 2EN was docked into the active sites of the 2B4 and 2B5 enzyme models in a productive binding orientation (see Experimental Procedures). In molecular dynamics simulations the protein molecule remained fixed whereas 2EN was allowed to move freely within the active site. In the case of P-450 2B4, the substrate orientation remained quite stable over the 5-ps run and the compound remains close to heme and active oxygen, as shown in Fig. 1. The results of similar simulations with 2EN docked into the active site of P-450 2B5 were quite different (Fig. 2). The inhibitor molecule moves out of the reactive binding orientation within the first 60 fs, and assumes an orientation further from the ferryl oxygen. As shown in Fig. 2, once 2EN is in the new orientation, it remains stable within the 5-ps time frame. Moreover, the orientation of 2EN relative to residue 302, the proposed site of enzyme modification (Roberts et al., 1994, 1996b), differs between the two simulations. In the P-450 2B4 model, the compound remains close to residue 302 throughout the 5-ps simulation whereas in the 2B5 model, movement of 2EN away from this residue occurs after 60 fs.

**Differential Susceptibility of 2B4 and 2B5 to 2EN and Analysis of Active Site Mutants.** Two different lines
of investigation suggested that 2B5 would be less sensitive
than 2B4 to inactivation by 2EN-inactivation experiments
with rabbit microsomes, and the high mobility of 2EN in the
2B5 active site in the molecular dynamics simulations. To
confirm this, the ability of 2EN to inactivate heterologously
expressed 2B4 and 2B5 enzymes was determined. As previ-
ously determined, 2B4 inactivation was time- and concentra-
tion-dependent (Fig. 3 and data not shown). Based on pre-
liminary inactivation experiments using a range of
concentrations, 10 μM 2EN was chosen for further experi-
ments as there was selective inactivation without high com-
petitive inhibition at this concentration. As shown in Fig. 3
and Table 1, 2B4 was inactivated by 2EN, whereas 2B5 was
resistant to 2EN at this concentration.

To assess the structural basis for this selectivity, active
site mutants were tested for susceptibility to 2EN. These
mutant P-450s had been previously constructed and assayed
for substrate specificity (He Y.Q. et al., 1996; Szklarz et al.,
1996). Based on the finding that alteration of positions 114,
294, 363, and 367 was required to interconvert most steroid
substrate specificities of 2B4 and 2B5, mutants containing
alterations at these positions were tested. Thus, single mu-
tants with substitutions at positions 294, 363, and 367 and
the quadruple mutants with the additional substitution at
residue 114 were assayed. Inactivation of the 2B4 I114F and
2B5 F114I enzymes could not be determined due to their low
basal activities. Alteration of residues 294 or 367 in 2B4 to
those present in 2B5 did not substantially alter susceptibility
to inactivation by 2EN. However, the 2B4 I363V mutant was
resistant to inactivation (Fig. 3 and Table 1); it is likely that
this alteration is largely responsible for the resistance also
observed in the 2B4 I114F-S294T-I363V-V367A mutant.

Both the 2B5 V363I and 2B5 A367V mutants exhibited in-
creased susceptibility to inactivation by 2EN; however, the
rate constants of inactivation were only about half that of
wild-type 2B4 (Table 1). The 2B5 T294S mutant did not acquire susceptibility to inactivation by 2EN.

Metabolism of 2EN. As lack of susceptibility to mecha-
nism-based inactivation can be the result of poor inhibitor
metabolism, the ability of 2B5 to produce 2NA from 2EN was assessed. Samples were taken at various time points after incubation of either 2B4 or 2B5 with 2EN to allow for direct comparisons between the two enzymes (Fig. 4). Initially, 2B4 exhibited a slightly higher rate of 2NA production compared with 2B5. However, the rate of 2NA formation decreased as 2B4 was inactivated, and leveled off by 10 min. In contrast, 2B5 continued to form product at a constant rate, and by 15 min the amount of 2NA produced by 2B5 exceeded that of 2B4. Thus, the reduced susceptibility to 2EN is not due to the inability of 2B5 to metabolize this inhibitor.

Identification of A 2B5-Selective Inactivator. After establishing 2EN as a selective 2B4 inactivator, the goal was to find a selective inactivator of 2B5 as well. To achieve this, additional aryl acetylenes were screened in microsomes by assessing the marker activities of 2B4 and 2B5; benzoxycresolurin O-dealkylation and androstenedione 15α-hydroxylation, respectively (Grimm et al., 1995). First, the compounds 2EPh, 3EPh, and 9-(1-propynyl)phenanthrene were tested, as 2B4 had been previously shown to be resistant to inhibition of these inhibitors led to inactivation of 2B5, as rate constants of inactivation (k_i) were all less than 0.02 min⁻¹ at 10 μM inhibitor concentrations. The 2B1 inactivator, 4-phenyl-1-butyne (Foroozesh et al., 1997) also failed to inactivate either 2B4 or 2B5. Inactivation of 2B5 was observed for the two compounds 9EPh and 5Ph1P; however, 2B4 also exhibited susceptibility to these inhibitors. Rate constants of inactivation for 9EPh (10 μM) were 0.23 min⁻¹ for 2B4 and 0.07 min⁻¹ for 2B5. Inactivation of 5Ph1P (50 μM) gave k_i values of 0.17 min⁻¹ for 2B4 and 0.07 min⁻¹ for 2B5. Finally, a newly synthesized compound, 1APE, was found to be selective for 2B5 inactivation with a k_i value of 0.24 min⁻¹ at an inhibitor concentration of 100 μM. 2B4 exhibited substantial competitive inhibition at this concentration (5% of control activity remaining), but did not exhibit susceptibility to inactivation. At 5 μM, 1APE resulted in lower competitive inhibition of 2B4 (25% of control activity remaining), without observable levels of inactivation.

Differential Susceptibility of 2B4 and 2B5 to 1APE and Analysis of Active Site Mutants. 1APE has a tricyclo structure and thus is likely to have a different orientation and movement in the enzyme-active site compared with the planar 2EN molecule. Heterologously expressed 2B4 and 2B5 enzymes were assessed for susceptibility to 1APE inactivation, confirming inactivation of 2B5 but not 2B4 (Fig. 5 and Table 2). 2B4 and 2B5 mutants containing substitutions in active site residues were tested to determine the structural basis of 1APE selectivity. These results are shown in Fig. 5 and Table 2. Of the 2B5 mutants examined, only 2B5 T294S and 2B5 F114I-T294S-V363I-A367V exhibited a loss of susceptibility to inactivation. All of the 2B4 substitutions tested conferred an increased susceptibility to 1APE inactivation, with 2B4 I363V, 2B4 S294T, and the 2B4 I114F-S294T-I363V-V367A mutants exhibiting higher susceptibility than 2B4 V367A. The 2B4 S294T mutant was very sensitive to both inactivation and inhibition by 1APE.

As observed with microsomes, the extent of reversible inhibition of 2B4 activity by 1APE was very high (7% control activity remaining, Table 2). For enzymes that exhibited strong competitive inhibition (low percentage of control activity remaining), a lower concentration of 1APE was also tested. A 10-fold lower 1APE concentration for 2B4 wild-type and 2B5 F114I-T294S-V363I-A367V enzymes increased the percent control activity remaining to 30%, but did not affect the rate constants of inactivation for 2B4, and only slightly increased it for 2B5 F114I-T294S-V363I-A367V (0.04 min⁻¹). The three single mutants of 2B4 were tested at a 25-fold lower concentration of 1APE (2 μM). 2B4 I363V and 2B4 V367A were not inactivated at this concentration, and very little inhibition was observed (90% or greater activity remaining). The 2B5 S294T mutant was very susceptible to 1APE, and at the 50 μM concentration the high percentage of inhibition (21% control activity remaining) masked inactivation. In Table 2, the data for 2B4 S294T is shown for the 2B5 I363V, 2B4 S294T, and the 2B4 I114F-S294T-I363V-V367A mutant. 2B5 T294S and 2B5 F114I-T294S-V363I-A367V exhibited a loss of susceptibility to 1APE inactivation. All of the 2B4 substitutions tested conferred an increased susceptibility to 1APE inactivation, with 2B4 I363V, 2B4 S294T, and the 2B4 I114F-S294T-I363V-V367A mutants exhibiting higher susceptibility than 2B4 V367A. The 2B4 S294T mutant was very sensitive to both inactivation and inhibition by 1APE.

Discussion

This study used mechanism-based inactivators to differentiate between two highly related enzymes, P-450 2B4 and 2B5. 2EN, which was known to inactivate 2B4, was found to be selective for this enzyme whereas the recently synthesized
compound 1APE was selective for 2B5 inactivation. Both orientation and movement of inhibitor or their reactive intermediates in the active site will determine whether enzyme inactivation will occur. As a means to understand differential susceptibility to 2EN inactivation, we used molecular modeling to demonstrate differences in the stability of 2EN bound in a productive orientation within the active sites of 2B4 and 2B5.

During the time course of the molecular dynamics simulation, 2EN remained in the productive binding orientation in the 2B4 active site. However, in the 2B5 active site it exhibited substantial movement away from the productive binding orientation and from the proposed site of enzyme modification, Thr302. This occurred within early time frames of the simulation, suggesting that the productive binding orientation is very unstable and that 2EN would preferentially bind further from heme in the 2B5 active site. In this case, enzyme susceptibility to inactivation would be affected by either reducing inhibitor metabolism (reducing formation of the ketene intermediate), or by increasing the partition ratio (ketene intermediate reacting more readily with water than with the enzyme). The ability of 2B5 to catalyze the oxidation of 2EN to 2NA suggests that reduced susceptibility is due not to decreased metabolism, but to an increase in product formation relative to enzyme adduct formation. Preferential binding of 2EN further away from residue 302 in the 2B5 simulation suggests that the ketene intermediate might be less likely to interact with this side chain, thus resulting in reduced enzyme inactivation. In addition, based on the homology models, the 2B5 active site can accommodate more water molecules than 2B4, which could also lead to an increase in product formation over enzyme inactivation. In fact, 2B5 is similar to the 2B4 T302A mutant, which is resistant to inactivation by 2EN but is still capable of inhibitor metabolism (Roberts et al., 1996b).

Molecular dynamics simulations indicate that 2EN binds close to residues 363 and 367 in the 2B5 active site. As these are two of the residues that differ between 2B4 and 2B5, they are good candidates for determinants of inhibitor susceptibility. Indeed, alteration of residue 363 interconverted susceptibility to 2EN, and alteration of residue 367 also affected 2B5 susceptibility. Presence of the larger Ile or Val side chains at these positions in 2B4 may reduce the mobility of 2EN in the active site, conferring sensitivity to inactivation. Residue 363 is also a strong determinant of steroid hydroxylation profiles. Alteration of this residue in 2B4 leads to an increase in hydroxylation of androstenedione at the 16α and 15α positions, and the reciprocal mutant of 2B5 exhibits a loss of 16α hydroxylation (Szklarz et al., 1996). In contrast with residue 363, alteration of residue 294 in either 2B4 or 2B5 did not interconvert susceptibility to 2EN. Modeling predictions concerning the role of these key residues are thus consistent with experimental results. Moreover, these studies indicate that molecular dynamics simulations show promise for situations such as mechanism-based inactivation where excessive mobility of the inhibitor within the enzyme active site may influence inhibitor metabolism and partition ratios.

Previous studies of the acryl acetylenes 1-ethynylpyrene and phenylacetylene demonstrated large differences in their binding affinities with P-450 1A1 (Chan et al., 1993). The authors reasoned that inhibitor binding and orientation in the 1A1 binding site influenced whether the terminal or internal carbon of the triple bond would be oxidized. This in turn affects the mechanism of inactivation, as heme modification is favored after oxidation at the internal carbon of the inhibitor, and protein modification after oxidation at the terminal carbon. In the case of 1A1 inactivation, the lower affinity phenylacetylene leads to heme modification, and the higher affinity compound, 1-ethynylpyrene, results in protein modification. In the present study, differences in binding and mobility of 2EN affect the ratio of carboxylic acid product formation relative to enzyme inactivation. This suggests that less restrictive binding of potential inhibitors can influence the partition ratio in addition to affecting the mechanism of inactivation (heme or protein modification).

The striking feature upon analysis of 2B4 and 2B5 active site mutants was the number of single mutants that acquired either resistance or susceptibility to inactivation by 2EN and/or 1APE. Although previous studies have shown that single mutants exhibit altered hydroxylation profiles for steroid substrates, multiple alterations are often required to interconvert these substrate specificities. Analysis of these mutants also revealed that it was easier to confer 1APE sensitivity to 2B4 than to make 2B5 resistant to inactivation. This may be related to the high competitive inhibition observed for 2B4, which suggests tight binding of 1APE in the active site. Alterations of single active site residues appear sufficient to alter the orientation of the inhibitor, resulting in enzyme inactivation. In addition, the determinants of susceptibility to 1APE differed from those of 2EN, with alteration of residue 294 having the strongest effect on 1APE susceptibility, followed by residue 363. Alterations at position 367 had little effect on susceptibility. It is interesting to note the high susceptibility of 2B4 S294T to both 1APE and 2EN. This mutant also exhibited higher activity than wild-type 2B4 or 2B5 with ethoxycoumarin (Szklarz et al., 1996). It would be of interest to assess whether this mutant displays increased sensitivity to inactivation by other aryl acetylenes.

Based on studies with secobarbital, BBT, and chloramphenicol derivatives, the active site residues 114, 302, 363, 367, and 478 have been identified as determinants of inactivator susceptibility (He et al., 1992, 1994, 1995; He J. et al., 1996b; Halpert and He, 1993; Kent et al., 1997). The present work identified an additional determinant, residue 294, and again established that substitutions at positions 363 and 367 affect P-450 susceptibility to inactivation. The combination of homology models, mutagenesis, and inhibitor metabolism

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>2B4</th>
<th>2B5</th>
<th>2B4</th>
<th>2B5</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μM IAPE</td>
<td>50 μM IAPE</td>
<td>50 μM IAPE</td>
<td>50 μM IAPE</td>
<td></td>
</tr>
<tr>
<td>2B4 WT</td>
<td>0.12 (78)</td>
<td>0.15 (77)</td>
<td>2B5 WT</td>
<td>0.12 (78)</td>
</tr>
<tr>
<td>2B4 S294T</td>
<td>0.04 (23)</td>
<td>0.15 (77)</td>
<td>2B5 A367V</td>
<td>0.04 (23)</td>
</tr>
<tr>
<td>2B4 I363V</td>
<td>0.15 (77)</td>
<td>2B5 A367V</td>
<td>0.15 (77)</td>
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<tr>
<td>2B4 V367A</td>
<td>0.04 (23)</td>
<td>2B5 A367V</td>
<td>0.04 (23)</td>
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</tr>
<tr>
<td>2B4 QUAD</td>
<td>0.15 (77)</td>
<td>2B5 A367V</td>
<td>0.15 (77)</td>
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</tr>
</tbody>
</table>

* k values (min⁻¹) are the means of two independent experiments with the indicated enzyme, conducted as described in Experimental Procedures. Zero (0) is used to indicate k values ≤0.01 min⁻¹. Control values (no inhibitor) were all ≥0.02 min⁻¹.

** All enzymes were incubated with 50 μM IAPE with the exception of 2B4 S294T, which was incubated with 2 μM IAPE.

Numbers in parentheses represent the percent activity remaining at the zero time point, as determined by linear regression analysis.

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has determined that inhibitor mobility plays a role in enzyme inactivation most likely by influencing the partitioning between product formation and enzyme inactivation. This study demonstrates that molecular dynamics simulations can contribute to our understanding of the molecular basis of enzyme inactivation and hold promise for our ability to rationally design enzyme inhibitors and inactivators.

References


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