Identification of Amino Acid Residues Involved in the Inactivation of Cytochrome P450 2B1 by Two Acetylenic Compounds: The Role of Three Residues in Nonsubstrate Recognition Sites

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ABSTRACT

The homologous rat cytochrome P450s 2B1 and 2B2 differ by 13 amino acids. A chimeric construct of P450 2B1/2B2 was used in conjunction with several site-directed mutants to identify key residues involved in the inactivation of P450 2B1 by two acetylenic compounds, 17α-ethynylestradiol (17EE) and tert-butyl 1-methyl-2-propynyl ether (tBMP). 17EE is a mechanism-based inactivator of P450 2B1 but not of P450 2B2. We show here that tBMP is also a mechanism-based inactivator of P450 2B1 but not of P450 2B2. Minimal loss in 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) activity was observed when P450 2B1 G478A was incubated with either inactivator, suggesting that this residue plays a role in the inactivation. However, P450 2B2 A478G behaved like wild-type P450 2B2, indicating that this residue alone is not sufficient for inactivation. A chimeric construct of P450 2B1/2B2 that is essentially P450 2B1 with five residues of P450 2B2 (including residue 478), was not inactivated by either tBMP or 17EE, suggesting that these five residues are important for inactivation. Sequential mutagenesis of the chimeric construct to quadruple (S407T-N417D-A419T-G478A) and triple (S407T-N417D-A419T) mutants of P450 2B1 did not result in inactivation by either inactivator. However, the triple mutant with mutations only in non-substrate recognition site (SRS) regions still exhibits wild-type P450 2B1 7-EFC O-deethylation activity with a $K_m$ value of 25 μM and $V_{max}$ of 8 nmol/min/nmol P450. These results demonstrate that substitution of three non-SRS residues in P450 2B1 leads to protection against inactivation of 2B enzymes by these two acetylenic compounds.

The phenobarbital inducible rat cytochrome P450 (P450) enzymes 2B1 and 2B2 are 98% identical, differing by only 13 amino acids. P450s 2B1 and 2B2 have similar substrate specificities; however, in most cases P450 2B1 has significantly higher catalytic activity than P450 2B2. Lidocaine and N-nitroso-N-methylaniline are examples of two compounds that are metabolized differentially by P450 2B1 and 2B2. (Stiborova et al., 1996; Hanna et al., 1998a). P450 2B1 catalyzes exclusively N-deethylation of lidocaine, whereas P450 2B2 catalyzes both N-deethylation and hydroxylation (Hanna et al., 1998a). P450 2B1 catalyzes both α-hydroxylation and denitrosation of N-nitroso-N-methylaniline, whereas P450 2B2 catalyzes only denitrosation. Due to their high sequence homology and different substrate specificities, P450s 2B1 and 2B2 have been used as a model system to study structure-function relationships.

Based on sequence alignments, six potential substrate recognition sites (SRSs) have been proposed for the CYP2 family (Gotoh, 1992). Site-directed mutagenesis and modeling of P450 2B enzymes have suggested important roles for several amino acid residues located in the SRSs in the metabolism of a number of substrates (Szklarz et al., 1995; Strobel et al., 1999; Lin et al., 2003). Six of the 13 amino acids that differ between P450s 2B1 and 2B2 are in the SRSs. These amino acids are at residues 303, 360, 363, 367, 473, and 478. The roles of these six residues in substrate specificity, reversible inhibition, and mechanism-based inactivation have been investigated extensively (He et al., 1992, 1994; Kent et al., 1997; Strobel and Halpert, 1997; Hanna et al., 1998a,b; Lin et al., 2003). Mutating residue 363 in P450 2B2 to the corresponding residue in P450 2B1 resulted in a switch in the metabolism of lidocaine from both N-deethylation and hydroxylation to only N-deethylation (Hanna et al., 1998a).

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ABBREVIATIONS: P450, cytochrome P450; SRS, substrate recognition site; 17EE, 17α-ethynylestradiol; tBMP, tert-butyl 1-methyl-2-propynyl ether; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; HPLC, high-performance liquid chromatography; ESI, electrospray ionization; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry.

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Residues 363 and 367 also play significant roles in the mechanism-based inactivation of P450 2B1 by secobarbital (He et al., 1986). Residue 478 is important in the inactivation of P450 2B1 by N-benzyl-1-aminobenzotriazole, steroid metabolism, and the metabolism of several other substrates and inactivators (He et al., 1992; Kent et al., 1997; Strobel and Halpert, 1997; Hanna et al., 1998a).

Mechanism-based inactivation is another useful tool for studying the structure-function relationships of individual P450 enzymes. The mechanism-based inactivation of P450 enzymes requires metabolic activation followed by covalent binding of the reactive intermediate to either the heme or the apoprotein rendering the enzyme inactive. So far, mechanism-based inactivators have been helpful in determining the orientation of the heme in the P450 active site and in the identification of peptides of the P450 enzyme involved in substrate binding (Kunze et al., 1983; Ortiz De Montellano et al., 1983; Yun et al., 1992; Roberts et al., 1993).

Many acetylenic compounds have been shown to act as mechanism-based inactivators of P450 enzymes (Ortiz De Montellano and Kunze, 1980; Foroozesh et al., 1997; Regal et al., 2000; Blobaum et al., 2002; Kent et al., 2002). The inactivation of P450 enzymes by acetylenic compounds occurs through metabolic oxidation of the acetylenic moiety, leading to the formation of a reactive intermediate that can covalently bind to either the apoprotein or the heme moiety (Ortiz De Montellano, 1985, 1986; Ortiz De Montellano and Reich, 1986; Osawa and Pohl, 1989). Oxygen insertion at the terminal acetylenic carbon results in covalent modification of the protein, whereas insertion of an oxygen atom at the internal acetylenic carbon leads to heme modifications. 17α-Ethynylestradiol (17EE) and tert-butyl 1-methyl-2-propynyl ether (tBMP) are two acetylenic compounds that differ significantly in size and structure (Fig. 1). 17EE, the major estrogenic component of oral contraceptives, is a mechanism-based inactivator of P450 2B1 that forms protein adducts (Kent et al., 2002). tBMP inactivates P450 2E1 through the formation of heme adducts (Blobaum et al., 2002). However, the ability of tBMP to act as a mechanism-based inactivator of P450s 2B1 and 2B2 has not yet been investigated.

In this study, we have investigated the ability of tBMP to act as a mechanism-based inactivator of P450s 2B1 and 2B2. A P450 2B1/2B2 chimera that is essentially P450 2B1 with five amino acids corresponding to P450 2B2, and sequential mutations to this chimera leading to a quadruple mutant and a triple mutant of P450 2B1 as well as single and double mutants of P450s 2B1 and 2B2, were investigated for their overall catalytic activity and for their ability to be inactivated by either tBMP or 17EE.

![Fig. 1. Chemical structures tBMP and 17EE.](image)

**Materials and Methods**

**Materials.** 17α-Difluorylphosphatidylcholine, δ-aminolevulinic acid, NADPH, bovine serum albumin, tert-butyl 1-methyl-2-propynyl ether, 17α-ethynylestradiol, sodium dithionite, glutathione, and catalase were purchased from Sigma-Aldrich (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was purchased from Molecular Probes (Eugene, OR). Trifluoroacetic acid (TFA) was obtained from Pierce Chemical (Rockford, IL). 7-Hydroxy-4-(trifluoromethyl)coumarin (HFC) was purchased from Oakwood Products Inc. (West Columbia, SC). Primers for the QuikChange site-directed mutagenesis and DNA sequencing were obtained from Invitrogen (Carlsbad, CA). Vent polymerase was purchased from New England Biolabs (Beverly, MA). DpnI was obtained from Roche Diagnostics (Indianapolis, IN).

**Site-Directed Mutagenesis and Purification of Enzymes.** Rat P450s 2B1 and 2B2 (cDNA provided by F. J. Gonzalez, National Institutes of Health, Bethesda, MD) and the chimeric construct of P450 2B1 and various mutants were expressed in *Escherichia coli* JM109 cells and purified using essentially the protocol for the wild-type P450 2B1. pCW plasmids containing the various mutants were transformed into *E. coli* JM109 cells. Cells were grown in 50 ml of LB media containing 100 μg/ml ampicillin at 37°C for 3 h before expanding into 1 liter of TB peptone containing ampicillin (100 μg/ml). The cells were grown at 37°C for 1 h before lowering the temperature to 27°C and adding δ-aminolevulinic acid (0.5 mM). The cells were induced with 1.5 mM isopropyl β-d-thiogalactoside when the optical density at 600 was ~0.6 and then allowed to grow for 48 h at 27°C. The cells were harvested and purified according to published protocols, except only a DEAE-Sepharose column and a SP-sephacel column were used for the purification (Hanna et al., 1998a). NADPH-P450-reductase (reductase) was expressed in *E. coli* TOP3 cells and purified to homogeneity by previously published methods (Hanna et al., 1998a). P450 concentrations were determined using the reduced CO spectra (Omura and Sato, 1964).

Construction of the mutants P450 2B1 M473K, P450 2B2 A478G-M473K, and P450 2B1 S407T-N417D-A419T (2B1 triple mutant) was done using Stratagene’s QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the following primers: 5′GACCTCACGCCCAAGGAGAGTGGCATT3′ (upstream) and 5′AATGCCTCCTCCTGGCCTGAGTC3′ (downstream).

P450 2B2 M473K was generated using wild-type P450 2B2 as a template, the P450 2B2 double mutant (A478G-M473K) was generated using the single mutant P450 2B2 A478G, and the 2B1 triple mutant was generated from the quadruple mutant described below.

The polymerase chain reaction products were sequenced at the University of Michigan DNA Sequencing Facility to ensure the absence of any errors in the sequence that may have occurred during amplification. The constructions of the P450 2B1/2B2 chimera, P450 2B1 S407T-N417D-A419T-G478A (2B1 quadruple mutant), and P450 2B2 A478G were previously reported by Hanna et al. (1998a), and P450 2B1 G478A has been reported by Kent et al. (2002). A schematic representation of the chimeric constructs and the mutants is shown in Fig. 2.

**Effect of tBMP on P450s 2B1 and 2B2.** The P450 enzymes (0.5 nmol) were reconstituted with reductase (1 nmol) and lipid (100 μg) for 45 min at 4°C. The primary reaction mixture contained 1 μM P450, 2 μM reductase, 0.1 mg/ml lipid, 26 U/μl catalase, 0 to 10 mM tBMP, and 1 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4). The primary reaction mixtures were incubated for 10 min at 30°C before the initiation of the reactions by the addition of NADPH (1 mM). At the indicated time points, aliquots (10 μl, containing 10 pmol of P450) were transferred to a secondary reaction mixture containing 40 μg/ml bovine serum albumin, 100 μM 7-EFC, and 1 mM NADPH in 50 mM potassium phosphate (pH 7.4) in a total volume of 990 μl. The secondary reactions were allowed to go for 10 min and were quenched with 334 μl acetonitrile. The conversion of 7-EFC to 7-hydroxy-4-(trifluoromethyl)coumarin (7-HFC) was mea-
sured on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD) at room temperature with an excitation wavelength of 410 nm and an emission wavelength of 510 nm.

The ability of alternate substrates to protect P450 2B1 from tBMP-dependent inactivation was investigated by incubating the reconstituted P450 2B1 together with tBMP (8 μM) and the alternate substrate in the absence or presence of NADPH. The alternate substrate used was 7-EFC (0:1, 1:0, and 1:10, tBMP/7-EFC). The primary reaction mixture containing tBMP and/or alternate substrate was incubated for 4 min after the addition of NADPH. 7-EFC O-deethylation activity was measured as described above. The effect of exogenous nucleophiles on tBMP-mediated inactivation was assessed by adding glutathione (10 mM) to the primary reaction mixture and measuring the residual activity as described above. The partition ratio of the inactivator was determined by incubating the primary reaction mixture containing tBMP (various concentrations ranging from 0 to 100 μM) for 30 min to ensure complete inactivation. Aliquots of the primary reaction (10 pmol of P450) were removed and assayed for residual activity as described above. The partition ratio was estimated by obtaining the percentage of activity remaining versus [tBMP]/[2B1]. The partition ratio was estimated from the intercept of the regression line obtained at low tBMP concentrations and the straight line obtained at saturating concentrations of tBMP.

Irreversibility and Determination of Heme Loss. P450 2B1 (1.4 nmol) and reductase (2.8 nmol) was reconstituted with phospholipid as described above. Primary reaction mixtures containing (±)-tBMP were incubated and assayed for 7-EFC O-deethylation activity. A portion of the sample (100 pmol of P450) was analyzed by reverse-phase HPLC with photodiode array detection to determine the amount of native heme in the samples (HPLC conditions listed below; system I). Another portion of the primary reaction mixture (100 pmol of P450) was added to 900 μl of an ice-cold quench buffer containing 40% glycerol and 0.6% Tergitol Nonidet P-40 in 50 mM potassium phosphate (pH 7.4) at 0 and 10 min. The reduced CO spectra were measured on a DW2 UV/VIS spectrophotometer (SLM Aminco, Urbana, IL) with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA) according to the method of Omura and Sato (1964). The remaining samples were dialyzed separately overnight using a Slide-A-Lyzer dialysis cassette at 4 °C against 1000 ml of 50 mM potassium phosphate (pH 7.4) with 20% glycerol. The next day, fractions of the dialyzed samples were assayed for activity in the presence or absence of fresh reductase. The dialyzed samples were also assayed for the reduced CO spectrum of the P450 and the amount of heme remaining by HPLC.

ESI-LC/MS and LC/MS/MS Analysis of Heme Adducts. ESI-LC/MS and ESI-LC/MS/MS were carried out with a ThermoQuest (ThermoQuest, Schaumburg, IL) ion trap mass spectrometer interfaced with a Hewlett Packard 1100 series HPLC system (Hewlett Packard, Palo Alto, CA). The reconstitution and incubations in the presence or absence of tBMP were carried out as indicated above except that the lipid content was reduced to 30 μg and the P450 2B1 to reductase ratio used was 1:1. Control or tBMP-inactivated samples (50 pmol of P450) were separated by reverse-phase HPLC (system II). The eluting peaks were analyzed in positive ion mode, and MS scans were acquired with the sheet gas set to 100 (arbitrary units) and the auxiliary gas set to 30 (arbitrary units). The spray voltage was 4.2 kV, the capillary voltage 19 V, and the capillary temperature was set at 220°C. MS/MS analysis of the control and tBMP-inactivated samples at m/z 705 was performed using 27% collision energy.

HPLC Conditions. For system I, a Phenomenex Jupiter C4, 10-μm, 300-Å column (250 × 4.6 mm; Phenomenex, Torrance, CA) was used to separate the heme components. The HPLC system consisted of a Waters (Milford, MA) 996 photodiode array detector, a Waters 600E controller, and a Waters 717 autosampler controlled by the Millenium software. The mobile phase consisted of 0.1% TFA in water (A) and 0.05% TFA in acetonitrile (B) at a flow rate of 1 ml/min. The elution was accomplished by holding the mobile phase at 70% A:30% B for 5 min followed by a linear gradient to 80% B in 25 min and then to 95% B in 5 min. Heme-containing peaks were monitored at 405 nm using the diode array detector and the areas under the heme containing peaks were integrated. For system II, a Phenomenex Jupiter C4, 5-μm, 300-Å column (150 × 2.0 mm; Phenomenex) that was equilibrated with 65% water containing 0.1% TFA (A) and 35% acetonitrile containing 0.1% TFA (B) was used for the LC/MS and LC/MS/MS analysis. The heme components were eluted by keeping the initial concentration at 65% A:35% B for 15
min followed by a linear gradient to 100% B in 10 min. The flow rate was 0.3 ml/min.

7-EFC O-Deethyllylation Activity of the Mutant Proteins. The wild-type and the mutant proteins (0.1 nmol of P450) were reconstituted with reductase (0.1 nmol) and lipid (20 μg) as described above. Catalase and potassium phosphate buffer were added to the reconstituted enzymes: 1 μM P450, 1 μM reductase, 0.2 mg/ml lipid, 14 U/μl catalase in 50 mM potassium phosphate (pH 7.4). The 7-EFC O-deethyllylation activity was measured as described for the secondary reaction under “Effect of tBMP on P450s 2B1 and 2B2.” The number of picomoles of HFC formed was calculated from an HFC standard curve. The kinetics of HFC formation were determined for the triple mutant by systematically varying the concentration of 7-EFC in the secondary reaction from 1 to 100 μM.

Effect of 17EE and tBMP on the P450 Mutants. The P450 2B1/2B2 chimeric construct and the mutants of P450s 2B1 and 2B2 were reconstituted with reductase and lipid as described above and incubated in the presence of 47 μM 17EE or 10 or 100 μM tBMP. Inactivation was initiated by adding 1 mM NADPH, and the reactions were allowed to proceed for 10 min at 30°C before transferring aliquots (10 pmol of P450) of the primary reaction into the secondary reaction to assay for the residual 7-EFC O-deethyllylation activity as previously described.

Results

Effect of tBMP on Wild-Type P450s 2B1 and 2B2. In a reconstituted system containing NADPH, tBMP caused a time- and concentration-dependent loss in P450 2B1 mediated 7-EFC O-deethyllylation activity (Fig. 3). The loss in 7-EFC O-deethyllylation activity was dependent on NADPH, indicating that catalytic activity is required for the inactivation of P450 2B1 by tBMP. The kinetic rate constants were determined from the slopes of the lines when the logarithm of the percentage of activity remaining (7-EFC O-deethyllylation) was plotted versus time. The reciprocals of the slopes were plotted against the reciprocals of the tBMP concentration (Fig. 3, inset) to determine the $k_{\text{inact}}$ and $t_{1/2}$. As can be observed in Fig. 3, the inactivation followed pseudo first order kinetics. The concentration of tBMP required to achieve one-half the maximum rate of inactivation ($K_i$) was 12 μM. The rate of inactivation, $k_{\text{inact}}$, was 0.28 min⁻¹ and the $t_{1/2}$ was 2.5 min. P450 2B2 was not inactivated by tBMP at concentrations as high as 1000 μM tBMP (data not shown).

The effect of the addition of an alternate substrate on tBMP-mediated inactivation of P450 2B1 was investigated. The addition of 7-EFC (1:10, tBMP/7-EFC) to the primary reaction resulted in a decrease in the level of inactivation of P450 2B1 (data not shown). With a 1:10 ratio of tBMP/7-EFC, a decrease in inactivation from 35 ± 5 to 58 ± 4% activity remaining was observed ($p < 0.005$). These results indicate that 7-EFC competes with tBMP for metabolism by P450 2B1 and thus protects the enzyme from inactivation.

Purified P450 2B1 was preincubated with tBMP and NADPH in the presence or absence of GSH and the 7-EFC O-deethyllylation activities remaining were measured. The addition of GSH (10 mM) to the primary incubations did not have an effect on the ability of tBMP to inactivate P450 2B1 adding to the evidence that tBMP acts as a mechanism based inactivator of P450 2B1 (data not shown). The partition ratio is the number of molecules of the inactivator metabolized per molecule of enzyme inactivated and is a measure of the efficiency of inactivation. The partition ratio for the inactivation of P450 2B1 by tBMP was approximately 12 (data not shown).

Irreversibility and Determination of Heme Loss. For a compound to be classified as a mechanism-based inactivator, the inactivation has to be irreversible. The losses in 7-EFC O-deethyllylation activity, reduced CO spectra and the amount of native heme were measured for both control and tBMP-inactivated samples before and after dialysis. In the presence of tBMP and NADPH, P450 2B1 lost 80% of its ability to mediate 7-EFC O-deethyllylation. In contrast, P450 2B1 lost only 25% of its ability to form a reduced CO complex (Table 1). The amount of native heme retained in the inactivated samples compared with control was also measured using HPLC with diode array detection. Native heme eluted at approximately 24 min and was present in both control and inactivated samples (Fig. 4). Inactivation by tBMP resulted in a 35% loss in native heme (Table 1). Two additional heme containing peaks were observed in the inactivated samples (Fig. 4). The diode array spectra of peaks A and B were similar to that of native heme, except a slight shift in the Soret peaks from 398 nm to approximately 407 nm was observed (data not shown).

**TABLE 1**  
Effect of tBMP inactivation on the P450 2B1-mediated 7-EFC O-deethyllylation activity, reduced CO spectrum, and HPLC-detected heme

Assay conditions were as described under Materials and Methods. Reconstituted P450 was inactivated with 10 μM tBMP for 10 min at 30°C prior to assaying for activity, reduced CO spectra, and heme. The values shown represent the mean and standard deviation from three separate experiments. The activity, reduced CO spectra, and heme were measured on the same day using the same inactivated sample. All values were calculated compared with control samples (~NADPH, 0 min).

<table>
<thead>
<tr>
<th>Percentage of Control</th>
<th>Activity Remaining</th>
<th>Reduced CO Spectrum</th>
<th>HPLC Heme Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before dialysis</td>
<td>20 ± 1*</td>
<td>75 ± 2</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>After dialysis</td>
<td>34 ± 6*</td>
<td>79 ± 6</td>
<td>69 ± 3</td>
</tr>
</tbody>
</table>

*p ≤ 0.005.
A very slight gain in activity was observed with tBMP-inactivated samples after dialysis (Table 1). However, there was no significant recovery of either the reduced CO spectrum or the native heme. A small change in the ratio of peak A to peak B was observed upon dialysis (data not shown). There seemed to be some loss of peak A, whereas peak B stayed constant before and after dialysis. No loss in activity, reduced CO spectrum, or heme was observed for P450 2B2 (data not shown).

LC/MS analysis of the two modified heme peaks (A and B) was performed. Both peaks have an m/z of 705, which is consistent with an iron-depleted heme with a tBMP molecule containing one oxygen atom attached to the heme (data not shown). The LC/MS/MS total ion chromatogram at m/z 705 for P450 2B1 inactivated by tBMP is shown in Fig. 5. The LC/MS/MS spectra for adducts A (peak A, retention time, 17.63 min) and B (peak B, retention time, 18.49 min) are shown in Fig. 5, B and C. The LC/MS/MS of m/z 705 showed a primary fragment of m/z 562, which corresponds to iron-depleted heme. Fragments with m/z 604, 632, and 648 were also observed and this suggests that the adducted tBMP moieties have oxygen attached to the internal acetylenic carbon. The proposed structure of the tBMP adducts is shown in the inset in Fig. 5A. No major difference was seen between the LC/MS/MS spectra for the two adducts. The loss in 7-EFC O-deethylation activity is much greater than the loss in both CO and the native heme for P450 2B1 inactivated by tBMP. This suggests that a protein adduct may be formed in addition to the two heme adducts that were observed. No adducted protein was detected in tBMP-inactivated P450 2B1 samples using ESI-LC/MS analysis. However, there was a loss in overall signal for P450 2B1 in the inactivated sample compared with the control sample.

7-EFC O-Deethylation Activity of Mutant Enzymes. The enzymes used for these studies are denoted in Fig. 2. All the mutants are either P450 2B1 with substitutions corresponding to residues in P450 2B2 or P450 2B2 with substitutions corresponding to residues in P450 2B1. Expression levels for the mutants ranged from 35 to 60 nmol/l. The ability of the mutants to catalyze the O-deethylation of 7-EFC was determined and compared with that of the wild-type enzymes (Fig. 6). As reported previously, P450 2B2 has a significantly lower rate of 7-EFC O-deethylation than P450 2B1. The single mutant P450 2B1 G478A has a 4-fold lower activity than wild-type P450 2B1. In comparison, the reverse mutant P450 2B2 A478G has a 2.5-fold increase in activity compared with wild-type P450 2B2. The single mutant P450 2B2 M473K has similar 7-EFC O-deethylation activity to wild-type P450 2B2, and the double mutant P450 2B2 A478G, M473K did not result in any additional increase in activity compared with the single mutant P450 2B2 A478G. The P450 2B1/2B2 chimeric protein, which is primarily P450 2B1 with five amino acids corresponding to P450 2B2, has a 23-fold lower 7-EFC O-deethylation activity than wild-type P450 2B1. The amino acids mutated in the chimera are 407, 417, 419, 473, and 478 (Fig. 2). By making a single mutation in the chimeric construct (quadruple mutant), changing amino acid 478 back to the P450 2B1 residue of glycine, a significant increase in activity is observed (p < 0.005) (Fig. 5). The triple mutant was obtained by mutating amino acid 473 in the quadruple mutant, generating the P450 2B1 S407T, N417D, A419T triple mutant. The activity of the triple mutant was similar to that of wild-type P450 2B1. The amino acids mutated in the triple mutant gave a V_{max} value for the triple mutant is comparable with the previously published value of 15 μM for wild-type P450 2B1 (Lin et al., 2003), and the V_{max} is similar to the previously published value for 7-EFC O-deethylation at 100 μM 7-EFC (Hanna et al., 1998b).

Comparative Effect of 17EE and tBMP on Chimeric P450 2B1/2B2 and Mutants. As shown in Table 2, 10 μM tBMP or 47 μM 17EE was sufficient to inactivate P450 2B1 more than 70% after 10 min of incubation. However, P450 2B2 is not inactivated by either tBMP or 17EE under these conditions. The 20% loss in activity observed with P450 2B2 in the presence of tBMP is dependent on NADPH but not on tBMP concentration. Increasing the concentration of tBMP 10-fold to 100 μM did not result in a large increase in inactivation of P450 2B1. A single mutation at residue 478, i.e., P450 2B1 G478A was sufficient to significantly reduce the inactivation by both 10 μM tBMP (from 73 to 28% loss) and 47 μM 17EE (from 69 to 24% loss). However, when P450 2B1 G478A was incubated with 100 μM tBMP, the activity loss was similar to wild type. Increasing the concentration of 17EE to 100 μM did not result in increased inactivation (data...
not shown). Conversely, when the reverse mutant, P450 2B2 A478G, was incubated with either tBMP or 17EE, minimal to no loss in activity was seen (Table 2). Similarly, the P450 2B2 M473K mutant (another residue in the SRS6 region) did not have any effect on the enzyme activity. The P450 2B1/2B2 chimeric construct, which is essentially P450 2B1 with five corresponding amino acids of P450 2B2 exhibits almost a complete loss of the 7-EFC O-deethylation activity. We actually observe a statistically significant increase in the 7-EFC O-deethylation activity upon exposure of the P450 2B1/2B2 chimera to either inactivator ($p < 0.005$); however, this phenomenon does not seem to be dependent on the concentration of inactivator (Table 2). Making subsequent mutations to this chimeric construct, thus changing it to a quadruple mutant (2B1 S407T-N417D-A419T-G478A) did not result in a protein that could undergo inactivation. Further mutation to a triple mutant (2B1 S407T-N417D-A419T-G478A) also did not result in a protein that could be inactivated by either inactivator, even though it had regained almost all of its catalytic activity for the deethylation of 7-EFC.

**Discussion**

Mechanism-based inactivators that undergo catalytic conversion to reactive intermediates have been used to study the catalytic specificity and active site structure of different P450 enzymes. Our current study shows the inactivation of P450 2B1 by tBMP in a reconstituted system containing P450 2B1, lipid, and reductase. The inactivation was characterized as mechanism-based by examining the following criteria: time,
NADPH, and concentration dependence (Fig. 3); effect of alternate substrate; irreversibility (Table 1); and effect of nucleophilic agents. The loss in enzyme activity of P450 2B1 inactivated by tBMP also resulted in losses in the reduced CO spectrum and native heme. The loss in native heme was accompanied by the appearance of two heme adducts (Fig. 4). LC/MS analysis of the heme adducts resulted in a parent ion with a m/z of 705 for both adducts, corresponding to iron depleted heme with one tBMP molecule and one oxygen atom covalently bound. LC/MS/MS analysis gave rise to fragmentation patterns that were essentially identical for the two adducts, indicating that both adducts probably stem from the same reactive intermediate that is attached to different atoms on the porphyrin ring (Fig. 5). The MS/MS data indicate that the heme is covalently attached to the oxidized tBMP molecule through the terminal acetylenic carbon with the oxygen atom located on the internal acetylenic carbon. The sites of modification on the heme molecule were not determined; however, we have previously shown that the two modified heme moieties generated from the inactivation of rabbit P450 2B4 by tBMP have the oxidized tBMP molecule attached to the pyrrole nitrogens (Von Weymarn et al., 2004). The loss in activity (80%) is greater than the loss of reduced CO spectra (25%) and the loss in native heme (35%), suggesting that a protein adduct may also have been formed. LC/MS analysis did not result in the identification of a protein adduct; however, a loss in the overall signal of P450 2B1 was observed upon inactivation. The loss in signal could be due to the formation of a protein adduct that causes the protein to fall out of solution after it is inactivated. A 14% recovery in the 7-EFC O-deethylation activity was observed after 24 h dialysis (Table 1). In addition, adduct A decreased relative to adduct B after dialysis indicating that adduct A may either be unstable or reversible. Reversible inactivation has been observed previously with a structurally similar inactivator, tert-butyl acetylene, and a mutant P450 2E1 (Blobaum et al., 2002).

Site-directed mutagenesis, homology modeling, and chimeric proteins have been used to identify and study the residues that play a role in metabolism and that are responsible for differences between closely related enzymes. In this study, we have taken advantage of the high degree of amino acid homology between P450s 2B1 and 2B2 (98%) and the differences in their metabolic profiles to study the effect of two structurally different acetylenic compounds, tBMP and 17EE, on a P450 2B1/2B2 chimera and various mutants. We have previously shown that 17EE is a mechanism-based inactivator of P450 2B1 with a Ki value of 11 μM and that 17EE inactivates P450 2B1 through the formation of protein adducts (Kent et al., 2002). Our results show that tBMP is also a mechanism-based inactivator of P450 2B1. P450 2B2, on the other hand is not inactivated by either tBMP (shown here) or 17EE (Kent et al., 2002).

A lot of work has been done to map substrate specificity within the 2B subfamily and other P450 subfamilies (He et al., 1992, 1996; Szklarz et al., 1996; Hanna et al., 1998a; Kent et al., 2002). Studies have shown that residues 114, 206, 209, 290, 294, 302, 363, 367, 477, 478, and 490, which are located in five different SRS regions affect specificities toward a number of substrates (He et al., 1992, 1996; Szklarz et al., 1996; Kobayashi et al., 1998). Although these studies support

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**TABLE 2**

Inactivation of P450 2B1, 2B2, and mutants by tBMP and 17EE

<table>
<thead>
<tr>
<th></th>
<th>10 μM tBMP</th>
<th>100 μM tBMP</th>
<th>47 μM 17EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of Activity Remaining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B1</td>
<td>27 ± 4</td>
<td>18 ± 4</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>2B1 G478A</td>
<td>72 ± 8</td>
<td>25 ± 1</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>2B2</td>
<td>81 ± 6</td>
<td>80 ± 8</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>2B2 A478G</td>
<td>89 ± 1</td>
<td>87 ± 4</td>
<td>101 ± 1</td>
</tr>
<tr>
<td>2B2 M473K</td>
<td>79 ± 4</td>
<td>78 ± 5</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>2B2 A478G, M473K</td>
<td>81 ± 6</td>
<td>74 ± 6</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>2B1/2B2</td>
<td>127 ± 4</td>
<td>125 ± 4</td>
<td>108 ± 2</td>
</tr>
<tr>
<td>Quadruple</td>
<td>88 ± 6</td>
<td>87 ± 8</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Triple</td>
<td>93 ± 7</td>
<td>89 ± 3</td>
<td>94 ± 2</td>
</tr>
</tbody>
</table>

Assay conditions were as described under Materials and Methods. Reconstituted wild-type or mutant P450 was inactivated with 10 μM tBMP, 100 μM tBMP, or 47 μM 17EE for 10 min at 30°C prior to assaying for 7-EFC O-deethylation activity. The values shown represent the mean and standard deviation from three separate experiments. All values were calculated compared with control samples (−NADPH, 0 min).
the significance of several residues in SRS regions, they do not provide much information regarding the importance of residues outside the SRSs domains.

Of the 13 amino acids that differentiate P450s 2B1 and 2B2, six are located within the SRSs. They include acid residues 303, 360, 363, 367, 473, and 478. Mutating amino acid 478 of P450 2B1 to the corresponding residue in P450 2B2 (2B1 G478A) abolishes inactivation by both acetylenic compounds (Table 2). This is not surprising because this residue has previously been reported to have a significant effect on P450 2B1-mediated metabolism (He et al., 1992; Kent et al., 2002). However, increasing the concentration of tBMP to 100 μM resulted in some inactivation, suggesting that the inactivation of P450 2B1 G478A by tBMP is not abolished. In contrast, increasing the concentration of 17EE to 100 μM did not result in inactivation (data not shown). The P450 2B1 G478A mutation seemed to have a more severe effect on the inactivation by the planar acetylenic compound 17EE than on the smaller tBMP molecule. The reverse mutant (2B2 G478A) was not inactivated, indicating that other residues also contribute to the ability of the enzyme to undergo inactivation. These results suggest that amino acid 478 is important for inactivation by tBMP and 17EE but not sufficient. P450 2B2 M473K and the double mutant P450 2B2 A478G-M473K behaved similarly to wild-type P450 2B2 when incubated with either inactivator, suggesting that residue 478, but not 473, is important for inactivation.

The P450 2B1/2B2 chimera construct was based on P450 2B1 with five amino acids derived from P450 2B2 (Hanna et al., 1998a). This chimera included two amino acids that are part of the SRSs (473 and 478) and three non-SRS residues (407, 417, and 419) (Fig. 2). When this chimera was incubated with either inactivator it behaved like P450 2B2, suggesting that these five residues are important for inactivation (Table 2). That this chimera did not undergo inactivation was not surprising because we have already demonstrated that a glycine residue at position 478 is necessary for inactivation by both tBMP and 17EE. This chimera construct was sequentially altered to yield a quadruple and a triple mutant.

In the quadruple mutant, residue 478 was mutated back to the glycine, which is present in wild-type P450 2B1. Surprisingly, the quadruple mutant was not inactivated by either tBMP or 17EE. The 7-EFC O-deethylation activity of the quadruple mutant was ~14-fold higher than that of the P450 2B1/2B2 chimera but ~1.7-fold lower than wild-type P450 2B1. The triple mutant, which does not contain any mutations in the SRS regions, exhibited a 7-EFC O-deethylation activity comparable with that of wild-type P450 2B1 with a $K_m$ value of 25 μM and a $V_{max}$ value of 8 nmol/min/nmol P450. The $K_m$ and the $V_{max}$ values obtained for the triple mutant are comparable with the previously published values for wild-type P450 2B1 ($K_m = 15$ μM, Lin et al., 2003; $V_{max} = 8$ nmol/min/nmol P450, Hanna et al., 1998b). However, the triple mutant was not inactivated by either tBMP or 17EE, suggesting that the remaining three residues, i.e., 407, 417, and 419, do play a critical role in the inactivation of the enzyme by 17EE/tBMP. That non-SRS residues can play an important role in metabolism has been reported by He et al. (1998), who has shown that two single non-SRS mutants of P450 2B5 can alter the progesterone hydroxylation. The work presented here provides another example of important roles of non-SRS residues.

From the recently published structures of P450s 2B4 and 2C5, it seems that residues 407, 417, and 419 lie on the proximal surface of the P450 molecule with the side chains exposed to solvent (Williams et al., 2000, 2003; Scott et al., 2003, 2004). Residues 407, 417, and 419 are located in the loop region between helices K' and K" (407) and K' and K" (417 and 419) (Scott et al., 2004). This region does not change when the enzyme goes from the open to the closed conformation but has been involved in the association of reductase with the P450 (Bridges et al., 1998; Scott et al., 2004). The mutations in these loop regions may cause a conformational change in P450 2B1 that does not change the overall fold of the enzyme but results in a perturbation of the active site changing the orientation of tBMP and 17EE, but not 7-EFC and therefore abolishing inactivation. Another possibility is that the triple mutant may have affected electron transfer rates from the reductase to the P450.

In conclusion, the results of the present study show that substitution of three residues in the non-SRS region of P450 2B1 with the corresponding residues in P450 2B2 completely abolish the inactivation by tBMP and 17EE. The results implicate these residues as being important determinants for the function of these 2B enzymes in regard to inactivation by these acetylenic compounds.

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References


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