Novel Reversible Inactivation of Cytochrome P450 2E1 T303A by tert-Butyl Acetylene: The Role of Threonine 303 in Proton Delivery to the Active Site of Cytochrome P450 2E1

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ABSTRACT

This report investigates and characterizes the mechanism for the novel reversible inactivation of a T303A mutant of rabbit cytochrome P450 (P450) 2E1 by tert-butyl acetylene (tBA). P450 2E1 T303A was inactivated in a time-, concentration-, and NADPH-dependent manner through the formation of two tBA adducts to the P450 heme. Interestingly, losses in enzymatic activity and in the reduced CO spectrum of the tBA-inactivated T303A mutant could be restored to the samples after an overnight incubation at 4°C. Removal of free tBA and NADPH from the tBA-inactivated T303A samples by spin column gel filtration demonstrated that the observed reversibility was time-dependent and was not significantly affected by the presence or absence of NADPH or tBA. Furthermore, the recovery of native heme was dependent on the native P450 enzyme structure. Electrospray ionization liquid chromatography-tandem mass spectrometry analysis under non-denaturing conditions of a pre-acidified tBA-inactivated T303A sample yielded two tBA adducts (m/z of 661 Da) with ion fragmentation patterns characteristic of a tBA adduct to the P450 heme. These adducts were absent in non-acidified samples subjected to the same conditions. In contrast, tandem mass spectrometry analysis of both non- and pre-acidified tBA-inactivated wild-type 2E1 samples yielded two tBA adducts (m/z of 661 Da) with ion fragmentation patterns similar to the preacidified T303A mutant adducts. These results lend insight into the reversible inactivation mechanism of the tBA-inactivated T303A mutant and suggest a role for the highly conserved threonine 303 residue in proton donation to the P450 2E1 active site and the stabilization of a reactive intermediate during substrate metabolism by P450.

The cytochrome P450 enzymes are critically important for the metabolism of numerous endogenous compounds and for the detoxification of a wide variety of xenobiotics (Nelson et al., 1996; Gonzalez, 1998). Cytochrome P450 2E1 metabolizes ethanol and other small molecules, including acetaminophen and nitrosamines (Perrot et al., 1989; Yang et al., 1990), and studies have suggested that P450 2E1 is involved in the metabolic activation of short-chain N-nitrosamines to carcinogens (Yang et al., 1990; Guengerich et al., 1991). Mutation of a highly conserved threonine residue (T303 in P450 2E1, T302 in P450cam) in the I helix of P450 2E1 has allowed for comparison studies between wild-type P450 2E1 and the mutant enzyme. Previous research has documented the differential effects of isothiocyanates on the activities of P450s 2E1 and 2E1 T303A, suggesting an important role for threonine 303 in the metabolism of these compounds (Kent et al., 1998, 2001; Moreno et al., 2001).

Active site models of human P450 2E1 predict a large majority of hydrophobic residues contained within a small active site, with the substrate binding pocket positioned directly over the A and D pyrrole rings of the heme moiety (Tan et al., 1997). Based on their model, Tan et al. (1997) have suggested that the conserved T303 residue may be involved in the binding of substrates in the 2E1 active site. Increasing experimental evidence has pointed to a role for this conserved threonine residue (T303 in P450 2E1, T302 in P450 2B4, and T252 in P450cam) as a participant in a proton delivery network in the enzyme active site that is involved in oxygen activation (Imai et al., 1989; Martinis et al., 1989; Vaz et al., 1996, 1998). Reduction of molecular oxygen in the P450 catalytic cycle involves progression from a peroxy-iron species to a hydroperoxo-iron species and ultimately to an oxenoid-iron complex. Cytochrome P450 2E1 has been shown to primarily use the putative oxenoid-iron species for substrate oxygenation.

ABBREVIATIONS: P450, cytochrome P450; tBA, tert-butyl acetylene; DLPC, dilauroyl-L-α phosphatidylcholine; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; ESI-LC-MS, electrospray ionization liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; LC-MS, liquid chromatography-mass spectrometry.
ation, whereas the hydroperoxy-iron species served as the primary oxidant for the 2E1 T303A mutant (Vaz et al., 1998). It was thought that the threonine to alanine mutation disrupted the proton delivery to the active site, thereby changing the rates of hydroxylation, deformylation, or epoxidation of various substrates.

Small acetylenic compounds have been shown to inactivate cytochrome P450 2E1 and the T303A mutant of 2E1 in a mechanism-based manner through the formation of adducts to the P450 heme and apoprotein (Blobaum et al., 2002). The structure of one of these inactivators, tert-butyl acetylene (tBA), is shown in Fig. 1. Inactivation of P450 2E1 with tBA resulted in the covalent modification of both the P450 apoprotein and the heme by a tBA reactive intermediate. When the 2E1 T303A mutant was incubated with tBA and NADPH, a loss in activity with a concurrent loss in native heme was observed, suggesting that the inactivation was primarily through tBA modification of the P450 heme. However, in contrast to the tBA-inactivated wild-type enzyme, the loss in activity was reversible with extensive dialysis (Blobaum et al., 2002). Such a unique reversible inactivation mechanism has not been reported previously with P450 enzymes.

Therefore, we have investigated the inactivation of P450 2E1 T303A by tBA in further detail to elucidate and characterize the mechanism behind this novel reversibility. Rabbit cytochrome P450 2E1 T303A was inactivated by tBA in a time-, concentration-, and NADPH-dependent manner with a Ki value of 2.0 mM. Losses in enzyme activity occurred with concurrent losses in native heme and were accompanied by the appearance of two different tBA-modified heme products (m/z of 661 Da) in each inactivated sample. Upon further exploration of the reversible inactivation mechanism, we have found that the tBA adducts to the P450 2E1 T303A heme are capable of reversal upon standing and that this reversibility is dependent on time and is not affected by the presence or absence of tBA and NADPH. Isolated tBA heme adducts are stable and do not revert to native heme in the absence of the native protein structure. Interestingly, the tBA-inactivated T303A mutant could not form stable heme adducts under nonacidic solvent conditions unless the samples were first acidified. In comparison, the tBA-inactivated wild-type P450 2E1 was able to form these two tBA adducts under the same conditions regardless of prior acidification. These data suggest an important role for the highly conserved threonine 303 as a participant in a proton relay network to the active site of P450 2E1. Our data imply that the conserved threonine residue may be involved in stabilization of the tBA reactive intermediate during P450-catalyzed metabolism.

Materials and Methods

Materials. tBA was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dilauroyl-L-/α-phosphatidylcholine (DLPC), NADPH, catalase, G-50 Sephadex matrix, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Molecular Probes (Eugene, OR). HPLC-grade acetonitrile was from Fisher Scientific Co. (Pittsburgh, PA) and trifluoroacetic acid (TFA) was from Pierce Chemical (Rockford, IL).

Enzymes. The cDNA for rabbit P450s 2E1 and 2E1 T303A (provided by M. J. Coon, University of Michigan) was expressed in Escherichia coli cells. Expression and purification of the proteins were carried out according to published methods (Larson et al., 1991) with some modifications (Kent et al., 1998). NADPH-P450 reductase was purified after expression in E. coli TOP3 cells as described previously (Hanna et al., 1998).

Enzyme Activity Assays. Purified rabbit cytochrome P450s 2E1 and 2E1 T303A (OM-7-EFC in dimethyl sulfoxide, 0.2 mM NADPH, and 40 μg bovine serum albumin/ml in 50 mM potassium phosphate buffer (pH 7.4) for a total reaction volume of 1 ml. Methanol (100%) was added to the control samples instead of tBA. At the indicated times, 25 μl of the P450 2E1 primary reaction mixture was transferred into 975 μl of a secondary reaction mixture containing 100 μM 7-EFC in dimethyl sulfoxide, 0.2 mM NADPH, and 40 μg bovine serum albumin/ml in 50 mM potassium phosphate buffer (pH 7.4). Samples were incubated for 10 min at 30°C in a shaking water bath, and enzyme activity was terminated by the addition of 334 μl of acetonitrile. Activity was assessed spectrofluorometrically by measuring the extent of O-deethylation of 7-EFC to 7-hydroxy-4-(trifluoromethyl)coumarin on a model SPF-500C spectrophotometer (SLM Amino, Urbana, IL) with excitation at 410 nm and emission at 510 nm (Buters et al., 1993).

Spectrophotometric Quantitation. P450 2E1 T303A was reconstituted and inactivated as described above for enzymatic activity. Then, 100-μl aliquots of control and tBA-inactivated P450 2E1 T303A primary reaction mixture were removed and diluted with 900 μl of ice-cold 50 mM potassium phosphate buffer (pH 7.7), containing 40% glycerol and 0.6% Tergitol Nonidet P-40. The samples were gently bubbled with carbon monoxide for approximately 90 s, and the spectrum was recorded from 400 to 500 nm on a DW2 UV/Vis spectrophotometer (SLM Amino) equipped with an OLI spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA). Dithionite was added and the reduced CO spectrum was recorded (Omura and Sato, 1964). The maximal absorbance at 450 nm was used to quantitate P450 heme.

HPLC Analysis. P450 2E1 T303A was reconstituted and inactivated as described above for enzymatic activity. A final concentration of 2 mM tBA was used. P450 2E1 T303A and reductase for control and tBA-inactivated samples were separated by HPLC on a 250 × 4.60-mm reverse phase C4 column (Phenomenex, Torrance, CA) (solvent A, 0.1% TFA and H2O; solvent B, 95% acetonitrile and 0.1% TFA). The flow rate was 1 ml/min and a linear gradient from 60% A and 40% B to 100% B over 45 min was used. The elution of proteins and heme was monitored using diode array detection.
ESI-LC-MS Analysis. P450 2E1 T303A was reconstituted and inactivated as described above for enzymatic activity. Primary incubation mixtures contained 0.4 nmol of P450 2E1 T303A, 0.8 nmol of reductase, and 30 μg of DLPC. A final concentration of 2 mM tBA was used. Control samples incubated with tBA in the absence of NADPH and tBA-inactivated P450 2E1 T303A samples were resolved on a 150 × 2.00-mm reverse phase C4 column (Phenomenex) (solvent A, 0.1% TFA and H2O; solvent B, 100% acetonitrile and 0.1% TFA) equilibrated with 40% B at a flow rate of 0.3 ml/min. Heme components were eluted using a linear gradient from 60% A and 40% B to 100% B over 25 min. The eluting peaks were subjected to mass analysis on an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Scans were acquired with the sheath gas set at 100 (arbitrary units) and the auxiliary gas set at 30 (arbitrary units). For acquisition, the spray voltage was 4.2 kV, the capillary voltage 19 V, and the capillary temperature 220°C.

Time-Dependent Reversibility of Inactivation. P450 2E1 T303A was reconstituted and inactivated as described above for enzymatic activity. A final concentration of 2 mM tBA was used. Small molecules such as NADPH and tBA were removed from the samples using a G-50 Sephadex spin column as described previously (Fry et al., 1978). Control samples and samples containing tBA-inactivated P450 2E1 T303A were loaded onto the column and spun sequentially for 10 min at 600 rpm and 3 min at 2800 rpm to determine whether the inactivation and losses in native heme were reversible. The samples were assayed concurrently for 7-EFC O-deethylation activity and percentage of heme remaining both before and after their placement on the spin column. The percentage of native heme remaining and the formation of tBA-adducts to the P450 2E1 T303A heme was measured by HPLC as described above.

ESI-LC-MS/MS. P450s 2E1 and 2E1 T303A were reconstituted and inactivated as described above for enzymatic activity. Primary incubation mixtures contained 0.4 nmol of P450 2E1 or P450 2E1 T303A, 0.8 nmol of reductase, and 30 μg of DLPC. A final concentration of 2 mM tBA was used. Both non- and preacidified (0.1% TFA) control samples incubated with tBA in the absence of NADPH and tBA-inactivated P450 2E1 and 2E1 T303A samples were resolved on a 150 × 2.00 Phenomenex reverse phase C4 column under nonacidic solvent conditions (solvent A, H2O; solvent B, 100% acetonitrile) equilibrated with 40% B at a flow rate of 0.3 ml/min. Heme components were eluted using a linear gradient from 60% A and 40% B to 100% B over 25 min. MS/MS analysis of the tBA-modified heme products (m/z of 661 Da) was performed on an LCQ ion trap mass spectrometer (Thermo Finnigan) with 0.4 m/z isolation width and 25% collision energy.

Results

Inactivation of P450 2E1 T303A by tBA. The kinetics for the inactivation of P450 2E1 T303A by tBA was studied by measuring the loss in 7-EFC O-deethylation activity. P450 2E1 T303A in the reconstituted system was inactivated by tBA in a time- and concentration-dependent manner (Fig. 2).
In the absence of NADPH, there was no significant loss in the activity of the enzyme with tBA (data not shown). As can be observed in Fig. 2, the inactivation followed pseudo-first order kinetics. The apparent kinetic constants were determined from a double reciprocal plot of the inverse of the initial rates of inactivation as a function of the reciprocal of the tBA concentration (Fig. 2, inset). The maximal rate of inactivation \( (k_{\text{inact}}) \) of P450 2E1 T303A by tBA was 0.38 min\(^{-1}\), the concentration of tBA required for half-maximal inactivation \( (K_I) \) was 2.0 mM, and the half-time for inactivation \( (t_{1/2}) \) was 1.8 min.

**HPLC and ESI-LC-MS Analysis of tBA-Inactivated P450 2E1 T303A.** Previous observations in our laboratory have indicated that a loss in 2E1 T303A activity after inactivation by tBA correlates with a similar loss in the P450 reduced CO spectrum (Blobaum et al., 2002). In this report, P450 2E1 T303A was inactivated by tBA and analyzed by HPLC to determine the amount of native heme remaining after inactivation and to determine whether heme-acetylene adducts could be detected. Figure 3I shows the HPLC profile at 405 nm of P450 2E1 T303A incubated with tBA and NADPH. In addition to the native heme eluting at approximately 21 min (A), two additional peaks (B and C) were observed eluting at approximately 25 and 27 min, respectively. The tBA-inactivated T303A mutant enzyme generated about 6 times more of peak B than peak C. Only peak A, corresponding to the unmodified heme, was observed in all of the control incubations where the P450 was incubated with tBA in the absence of NADPH (data not shown). The diode array spectra of peaks B and C were similar to the spectrum of the native heme, except that a shift of the soret peaks of B and C from 395 to 406 to 410 nm was observed (Fig. 3II). This suggested that both peaks were tBA-modified heme products. ESI-LC-MS analysis of the tBA-inactivated P450 2E1 T303A indicated that peak A had a mass consistent with unmodified heme (616.3 Da) and that peaks B and C had masses of 661.3 Da, presumably corresponding to the mass of an iron-depleted heme (562 Da) plus the additional masses of a tBA-reactive intermediate (83 Da) and one oxygen atom (16 Da). Because the two tBA adducts have similar masses (661 Da),
the different retention times of the two adducts are thought to reflect adduction to different pyrrole nitrogen rings of the P450 heme.

**Reversible Adduction of the P450 2E1 T303A Heme.**

We have previously reported that overnight dialysis completely restored both the enzymatic activity and the P450 content of tBA-inactivated P450 2E1 T303A and that losses in the native heme were restored after an overnight dialysis (Blobaum et al., 2002). Further studies were performed in an attempt to understand and characterize this novel reversible inactivation mechanism.

Control and tBA-inactivated 2E1 T303A samples were assayed for activity and reduced CO spectra both before and after incubation overnight at 4°C. As shown in Table 1, approximately 34% of T303A activity and 37% of the P450-reduced CO spectrum were restored upon overnight standing of the sample. This recovery of activity and CO spectral losses seems to be slower than what was previously observed with an overnight dialysis (Blobaum et al., 2002). Interestingly, enzymatic activity was restored to the levels observed with dialysis if the samples were allowed to stand for an additional night at 4°C (88 ± 5% activity remaining). Small molecules were removed from the control and tBA-inactivated T303A samples by spin column gel filtration to determine whether the reversible inactivation mechanism was time-dependent and whether the reversibility required the continuous presence of small molecules such as NADPH and tBA. Table 2 shows the effect of mini-gel filtration on the reversibility of tBA-inactivated P450 2E1 T303A. The data show a restoration of both enzymatic activity and native heme to the tBA-inactivated samples after gel filtration; however, this restoration is not nearly to the extent that was observed with overnight dialysis (14% compared with 92% for enzymatic activity). Therefore, the tBA-inactivated samples were allowed to sit overnight at 4°C after their collection off the spin column. If left for longer periods at 4°C, these samples continued to restore losses in activity to levels comparable with the overnight dialysis (83 ± 6% activity remaining). Similarly, samples that were allowed to sit overnight at 4°C before their placement on the spin column also restored these losses to nearly the same extent. Thus, the reversible inactivation mechanism seems to be primarily time-dependent and not influenced by the presence or absence of NADPH and tBA.

To determine whether the native enzyme structure was required for the reversibility of the inactivation, control and tBA-inactivated P450 2E1 T303A samples were resolved by reverse phase chromatography under denaturing conditions (Fig. 4I), and the native hemes and adducts B and C were collected. Overnight stability of the native heme and tBA adducts to the P450 2E1 T303A heme was investigated by allowing each collected peak to sit overnight at 4°C, followed by reinjection onto the HPLC. The native heme (peak A) was completely stable upon reinjection and did not show any restoration of previous losses (data not shown). Figure 4, II and III, show that the isolated tBA-modified heme products B and C are relatively stable, and only 10% losses in the adducts were observed after an overnight sit at 4°C. Previously, the reversible inactivation of chloroperoxidase by allylbenezene was characterized (Dexter and Hager, 1995). Chloroperoxidase inactivation was accompanied by a loss in native heme and the formation of N-alkyl heme adducts (Debrunner et al., 1996). Activity and native heme spectra were restored to the samples upon standing, and the authors demonstrated that the native chloroperoxidase structure was required for recovery of activity and heme. Thus, as reported by Dexter and Hager (1995) for allylbenezene-inactivated chloroperoxidase, the reversal of the inactivation of P450 2E1 T303A by tBA is dependent on time and on the presence of the native P450 enzyme structure.

**ESI-LC-MS/MS Analysis of tBA-Inactivated P450s 2E1 and 2E1 T303A under Nonacidic Solvent Conditions.** Threonine 303, corresponding to T252 in P450cam and T302 in P450 2B enzymes, has been proposed to be involved in a proton delivery network to the active site of P450 enzymes with an important role in oxygen activation. To determine whether our HPLC and ESI-LC-MS denaturing solvent conditions were contributing to the stability of the heme adducts in the tBA-inactivated 2E1 T303A samples, control and inactivated samples were analyzed by ESI-LC-MS/MS under nonacidic solvent conditions (water, acetonitrile). As discussed previously, the two tBA-modified T303A heme products (B and C) were found by ESI-LC-MS to have masses of 661 Da. ESI-LC-MS/MS analysis of the tBA-inactivated

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**TABLE 1**

Restoration of activity and reduced CO spectra of tBA-inactivated P450 2E1 T303A after incubation overnight at 4°C

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>% Control Activity Remaining*</th>
<th>% Control Reduced CO Spectrum Remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 2E1 T303A + tBA (before O/N)</td>
<td>17.4 ± 0.62</td>
<td>28.9 ± 1.2</td>
</tr>
<tr>
<td>P450 2E1 T303A + tBA (after O/N)</td>
<td>50.9 ± 2.0</td>
<td>66.2 ± 2.2</td>
</tr>
</tbody>
</table>

O/N, overnight incubation at 4°C.

* The 100% control values for the enzymatic activity and the P450 reduced CO spectrum were 4.8 ± 0.7 pmol of 7-HFC/min/pmol of P450 2E1 T303A and 100 pmol of P450 2E1 T303A.

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**TABLE 2**

Effect of mini-gel filtration on the reversibility of tBA-inactivated P450 2E1 T303A

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>% Activity Remaining*</th>
<th>% Heme Remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 2E1 T303A (B.S.)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P450 2E1 T303A + tBA (B.S.)</td>
<td>12.5 ± 2.1</td>
<td>49.9 ± 2.7</td>
</tr>
<tr>
<td>P450 2E1 T303A (A.S.)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P450 2E1 T303A + tBA (A.S.)</td>
<td>26.7 ± 2.6</td>
<td>64.3 ± 2.6</td>
</tr>
</tbody>
</table>

B.S. and A.S. refer to before spin and after spin, respectively.

* The 100% control values for the enzymatic activity and P450 heme by HPLC were 4.8 ± 0.7 pmol of 7-HFC/min/pmol of P450 2E1 T303A and 100 pmol of P450 2E1 T303A.
Fig. 4. Stability of the tBA adducts to the P450 2E1 T303A heme with overnight incubation. I, HPLC chromatogram of P450 2E1 T303A incubated with tBA and NADPH. The unmodified heme peak (A) precedes the two tBA-modified heme products (B and C), which elute at approximately 23 and 25 min, respectively. Each peak was collected off the column and allowed to sit overnight at 4°C. The peaks were then individually reinjected under the same solvent and gradient conditions as the complete tBA-inactivated sample to monitor their stability. II and III, tBA-modified heme products B and C decrease by approximately 10% after an overnight incubation at 4°C.
T303A mutant under nonacidic solvent conditions resulted in an absence of the 661-Da masses. Upon the addition of 0.1% TFA to the inactivated sample, the two tBA adducts with \( m/z \) values of 661 Da were observed. These data are presented in Table 3 for the first tBA adduct (B), although similar results were observed for the second adduct (C). When the 661-Da masses were subjected to MS/MS analysis, the adducts fragmented into ions corresponding to a tBA molecule with an inserted oxygen atom at the internal carbon of the acetylenic group. The MS/MS spectral data and ion fragmentation pattern for adduct B is shown in Fig. 5 and inset. The terminal carbon of the acetylenic group is shown attached to the heme moiety at one of the pyrrole nitrogens, analogous to the allylbenzene-adducted chloroperoxidase heme (Debrunner et al., 1996), although the exact location of the tBA adduction to the P450 heme is unknown at this time.

For comparison, tBA-inactivated P450 2E1 was also analyzed by ESI-LC-MS/MS under nonacidic conditions. Unlike the mutant enzyme, two distinct adducts with masses of 661 Da were observed for P450 2E1 inactivated by tBA under nonacidic conditions (Table 3). The MS/MS spectral data and ion fragmentation pattern of the two adducts were identical to those observed for the tBA-inactivated T303A enzyme when it was incubated with 0.1% TFA before analysis. The requirement for protons to stabilize the tBA adducts to the 2E1 T303A heme and the absence of this necessity in the wild-type enzyme suggest a probable role for the T303 residue in proton delivery to the active site of P450 2E1.

**Discussion**

The crystal structures of several bacterial P450s show a highly conserved threonine residue positioned over the proposed oxygen-binding pocket and within hydrogen bonding distance to the peroxy-iron activated oxygen species (Poulos et al., 1987; Ravichandran et al., 1993; Hasemann et al., 1994). Consistent with the proposed role of this residue in a proton delivery network, camphor hydroxylation by P450cam (Imai et al., 1989; Martinis et al., 1989) and fatty acid hydroxylation by BM-3 (Yeom et al., 1995) were significantly diminished when this residue was mutated to alanine. Makris and Sligar proposed that in the absence of the threonine hydroxyl group at position 252 in P450cam, the second proton could not be transferred to the reduced dioxygen complex and the oxyferryl intermediate could not be generated (Makris et al., 2002). More recent studies with this same

![Fig. 5. ESI-MSMS analysis of tBA-inactivated P450 2E1 T303A under nonacidic solvent conditions. P450 2E1 T303A was incubated with tBA and NADPH and acidified with 0.1% TFA before injection onto the mass spectrometer. The main panel shows the resulting MS/MS data from the first tBA-modified heme product (adduct B), which has an overall mass-to-charge ratio of 661 Da. The inset illustrates the proposed sites of fragmentation of this adduct based on the ion fragmentation pattern observed under the MS/MS conditions.](image-url)
T252A mutant indicate that a hydroperoxo-iron intermediate can be formed and is capable of catalyzing the epoxidation of olefins (Jin et al., 2003). Likewise, the peroxy- and hydroperoxo-heme complexes, respectively, have been used as the primary oxidants by the threonine to alanine mutants of P450s 2B4 (Vaz et al., 1996) and 2E1 (Vaz et al., 1998). In contrast, the wild-type 2B4 and 2E1 P450s used the classic oxenoid-iron species. Together, these data suggest a role for the highly conserved threonine residue in proton delivery to the enzyme active site and in the activation of molecular oxygen.

We have previously reported that small acetylenic compounds inactivated P450 2E1 and the T303A mutant by three distinct mechanisms: 1) covalent alkylation of the P450 heme, 2) a combination of heme alkylation and protein addition, and 3) a novel reversible alkylation of the P450 heme (Blobaum et al., 2002). For the tBA-inactivated P450 2E1 T303A, losses in enzymatic activity correlated with losses in the P450 native heme, suggesting that the inactivation occurred primarily through heme modification. An unexpected observation demonstrated that overnight dialysis reversed the losses in enzymatic activity, the P450 CO spectrum, and the native heme of the tBA-inactivated 2E1 T303A samples (Blobaum et al., 2002). Because this had not previously been observed for cytochrome P450 enzymes, the studies in this report were designed to investigate the nature of this reversible inactivation of the P450 2E1 mutant by tBA.

Cytochrome P450 2E1 T303A was inactivated by tBA in a mechanism-based manner (Fig. 2) through the formation of two tBA adducts (m/z of 661 Da) to the P450 heme (Fig. 3). Because the two adducts had identical masses, the difference in retention times observed with HPLC analysis is thought to reflect the addition of tBA to two different pyrrole nitrogen rings. Comparable with our results with the 2E1 T303A mutant and tBA, allylbenzene was found to reversibly inactivate chloroperoxidase through N-alkyl heme addition (Dexter and Hager, 1995; Debrunner et al., 1996). Interestingly, activity and native heme spectra were restored to the

Scheme 1. Reversible inactivation of P450 2E1 T303A by tBA. A tBA-reactive intermediate responsible for the reversible loss in the enzymatic activity of the 2E1 T303A mutant (in brackets) has two possible fates: 1) its formation is reversible and it will slowly decompose, leaving a reactivated enzyme with an unmodified heme and a tBA carboxylic acid reversal product; or 2) the intermediate is stabilized in the presence of protons (similar to the native proton transfer pathway involving T303 in the parent 2E1 enzyme) and will modify the P450 heme by N-alkylation.
samples upon standing, and the native enzyme structure was required for recovery of the native heme. Our current investigations into the reversible inactivation mechanism of the tBA-inactivated P450 T303A mutant have led to similar results. Losses in enzymatic activity, the reduced CO spectrum, and native heme of the tBA-inactivated T303A mutant could be spontaneously restored to the samples upon standing. Likewise, the reversibility was time-dependent and required an intact P450 enzyme structure. Although these experiments aided in the characterization of the novel reversible inactivation mechanism, they did not provide an answer as to the actual mechanism by which this reversibility was occurring. Because we have observed that stable heme adducts are obtained when the pH of the LC-MS solvents is between pH 2 to 3, we opted to inactivate the 2E1 T303A mutant with tBA and resolve the sample components under nonacidic conditions to test the requirement of protons in the formation of stable heme adducts. ESI-LC-MS/MS analysis under nonacidic conditions of a preacidified tBA-inactivated T303A sample yielded two tBA adducts (m/z of 661 Da) with ion fragmentation patterns characteristic of a TBA adduct to the P450 heme. These adducts were absent in nonacidified samples subjected to the same conditions. In contrast, both non- and preacidified tBA-inactivated wild-type 2E1 samples were able to form the two tBA adducts (m/z of 661 Da). The MS/MS ion fragmentation patterns of the two adducts are consistent with the insertion of one oxygen atom at the internal carbon of the acetylenic functional group with the terminal carbon adducted to the heme. Although we suspect that the tBA inactivator is adducted to one of the pyrrole nitrogens, as was observed with allylbenzene-adducted chloroperoxidase (Debrunner et al., 1996), we do not yet have definitive proof of this location. NMR studies are currently underway to determine the exact location of the TBA adduction to the heme.

The evidence presented herein indicates that a TBA-reactive intermediate is responsible for the reversible loss in the enzymatic activity of the 2E1 T303A mutant. This intermediate has two possible fates: 1) the formation of the reactive intermediate is irreversible, and it will slowly decompose leaving a reactivated enzyme with an unmodified heme; or 2) in the presence of exogenously supplied protons (similar to the native proton transfer pathway involving T303 in the parent 2E1 enzyme), the intermediate will modify the P450 heme by N-alkylation. It has been proposed that the T303A mutant of 2E1 uses a hydroperoxo-iron species as the primary oxidant for the oxygenation of substrate (Vaz et al., 1998). This hydroperoxo-iron complex is known to have both nucelophilic and electrophilic properties. An electrophilic hydroperoxoiron has been postulated to insert an OH into a C-H bond to generate a protonated alcohol product (Newcomb et al., 2000). Scheme 1 shows how a TBA-reactive intermediate capable of enzyme inactivation may be formed by the concerted attack of OH into the electrophilic hydroperoxo-iron species and one of the nitrogens of the heme. In the presence of an exogenous source of protons (or T303 in the native P450 2E1), cleavage of the inactivating intermediate leads to the formation of a stable, inactive, TBA-labeled heme product. The scheme also demonstrates how a slow, uncatalyzed cleavage of the bond to the heme nitrogen in the absence of protons in 2E1 T303A could lead to the formation of a TBA-reactive intermediate capable of rearrangement to a ketene. Addition of water to the ketene forms a TBA carboxylic acid reversal product. Because this candidate reversal product should be detectable in TBA-inactivated 2E1 T303A samples that have spontaneously reversed, we are currently investigating the presence of potential TBA reversal products by LC-MS analysis.

Together, these data provide insight into the reversible inactivation of the T303A mutant of P450 2E1. The MS/MS results suggest that protons are necessary to stabilize the TBA adduct to the T303A heme, whereas the wild-type 2E1 enzyme possesses an internal source of protons to stabilize the adduct. The preacidification of our samples through the addition of TFA may allow for the directed delivery of protons to occur via an alternate pathway within the active site of the T303A mutant; a greater access of water molecules to the T303A active site may restore proton transfer. We hypothesize that the conserved T303 in P450 2E1 is a participant in a proton delivery network to the enzyme active site and/or that the threonine hydroxyl may be responsible for stabilizing the TBA reactive intermediate during P450 metabolism. Our results now suggest a role for this highly conserved residue in proton donation to the active site of P450 2E1 and stabilization of a reactive intermediate during P450 metabolism. Homology modeling of the P450 2E1 active site with acetylenic inactivators and characterization of the NMR structures of the TBA adducts will aid in our understanding of the active site structure of P450 2E1. Comparative studies using alternate oxidants and site-specific mutants of 2E1 active site residues are in progress to address why a larger acetylenic inactivator, tert-butyl 1-methyl-2-propynyl ether, forms adducts to the T303A heme that are stable to dialysis, whereas addition by the smaller tBA is reversible. In this regard, we hope to define more precisely both the role of T303 and nearby 2E1 active site residues in proton delivery and substrate stabilization as well as the role that distinct differences in inactivator structure may play in influencing P450 metabolism and inactivation.

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