Formation of a Novel Reversible Cytochrome P450 Spectral Intermediate: Role of Threonine 303 in P450 2E1 Inactivation†

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ABSTRACT: *tert*-Butyl acetylene (tBA) is a mechanism-based inactivator of cytochromes P450 2E1 and 2E1 T303A; however, the inactivation of the T303A mutant could be reversed by overnight dialysis. The inactivation of P450 2E1 T303A, but not the wild-type 2E1 enzyme, by tBA resulted in the formation of a novel reversible acetylene−iron spectral intermediate with an absorption maximum at 485 nm. The formation of this intermediate required oxygen and could be monitored spectrally with time. Although the alternate oxidants *tert*-butyl hydroperoxide (tBHP) and cumene hydroperoxide (CHP) supported the inactivation of wild-type P450 2E1 by tBA in a reductase- and NADPH-free system, only tBHP supported the inactivation of the 2E1 T303A mutant. The losses in enzymatic activity occurred concomitantly with losses in the native P450 heme, which were accompanied by the formation of tBA-adducted heme products. The inactivations supported by tBHP and CHP were completely irreversible with overnight dialysis. Spectral binding constants (K_s) for the binding of tBA to the 2E1 P450s together with models of the enzymes with the acetylenic inactivator bound in the active site suggest that the T303A mutation results in increased hydrophobic interactions between tBA and nearby P450 residues, leading to a higher binding affinity for the acetylene compound in the mutant enzyme. Together, these data support a role for the highly conserved T303 residue in proton delivery to the active site of P450 2E1 and in the inactivation of the 2E1 P450s by small acetylenic compounds.

The cytochrome P450[1] enzymes belong to a superfamily of heme-containing mono-oxygenases that are involved in the metabolism of numerous endogenous and exogenous substrates (*1*, *2*). The ethanol-inducible cytochrome P450 2E1 catalyzes the oxidation of a large number of drugs and hepatotoxic xenobiotics, including halogenated alkanes, acetaminophen, nitrosamines, benzene, and styrene (*3*, *4*). Evidence from homology models of P450 2E1 and studies in which a conserved threonine residue in the I helix of several different P450s was mutated to an alanine points to a role for this residue in the orientation of substrate in the P450 active site and as a possible proton donor in acid−base reactions (*5*−*7*). Site-specific mutation of this threonine residue (T303A) in P450 2E1 has allowed for comparisons between wild-type P450 2E1 and the mutant enzyme. Previous studies have documented the differential effects of isothiocyanates on the activities of P450 2E1 and the T303A mutant, suggesting an important role for threonine 303 in the metabolism of these compounds. *tert*-Butyl isothiocyanate (tBITC) was shown to be a mechanism-based inactivator of the 2E1 P450s in the reconstituted system (*8*). Covalent binding of tBITC to critical amino acid residues in the active site of P450 2E1 irreversibly inactivated the enzyme and resulted in the formation of an adduct to the apoprotein (*9*). Benzyl and phenethyl isothiocyanate (BITC and PEITC, respectively) also inactivated wild-type P450 2E1 in a mechanism-based manner (*10*, *11*). However, similar studies with the T303A mutant produced only competitive inhibition by BITC, while PEITC inactivated the enzyme in a manner comparable to that of the wild type. These data suggest a role for the T303 residue in the metabolism of isothiocyanates.

The activation of molecular oxygen in the P450 catalytic cycle is thought to involve a stepwise progression from the peroxo−iron to the oxenoid−iron species. Although the oxenoid−iron species is generally considered to be the predominant oxidant in P450-catalyzed reactions, other activated oxygen species may also oxygenate substrates (*12*, *13*). In addition to the generally accepted oxenoid−iron species, the peroxo− and hydroperoxo−iron species may also be considered competent oxidants, and any one of the three

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[1] Abbreviations: P450, cytochrome P450; tBA, *tert*-butyl acetylene; tBMP, *tert*-butyl 1-methyl-2-propynyl ether; reductase, NADPH-cytochrome P450 reductase; DLPC, dilauroyl-L-α-phosphatidylcholine; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; 7-HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; *p*-NP, *p*-nitrophenol; BSA, bovine serum albumin; tBHP, *tert*-butyl hydroperoxide; CHP, cumene hydroperoxide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; ESI-LC−MS, electrospray ionization liquid chromatography−mass spectrometry.

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species derived from the reduction of molecular oxygen may serve as the primary oxidant, depending on the particular substrate or P450 isozyme that is involved (*14*, *15*). However, a multiple-oxidants model may not be the only explanation for the existence of multiple reaction pathways. Shaik and co-workers have reported computational results that have implicated two accessible spin states for the oxenoid−iron species that have the ability to react differently, providing a "two-state rebound mechanism" alternative for multiple reaction pathways (*16*, *17*).

There is experimental evidence to support a role both for multiple oxidants in P450-catalyzed reactions and for a highly conserved threonine residue in delivery of protons to the P450 active site. For instance, Vaz and colleagues (*15*) have reported that P450 2E1 primarily oxygenates substrates through the use of the oxenoid−iron species, while the hydroperoxo−iron species is believed to be the primary oxidant for the T303A mutant of P450 2E1. From these data, the authors concluded that the mutation of threonine to alanine in P450 2E1 disrupted the delivery of protons to the enzyme active site and thus altered the rates for the hydroxylation, deformylation, or epoxidation of various substrates. Support for a role of the conserved threonine residue in proton delivery also comes from studies with bacterial P450s. Camphor hydroxylation by $P450_{cam}$ (*6*, *18*) and fatty acid hydroxylation by BM-3 (*19*) were significantly inhibited when the corresponding threonine residue in these enzymes was mutated to alanine. In agreement with the report of Vaz and co-workers for P450 2E1 (*15*), Makris and Sligar (*20*) have recently proposed that a loss of the threonine hydroxyl group at position 252 in $P450_{cam}$ prevents the transfer of the second proton to the reduced dioxygen complex and blocks the formation of the oxyferryl intermediate. Additional studies with this same T252A mutant have indicated that a hydroperoxo−iron intermediate can be formed and that it is capable of catalyzing the epoxidation of olefins (*21*).

The mechanism-based inactivation of P450s 2E1 and 2E1 T303A by *tert*-butyl acetylenic compounds has been reported previously (*22*). The characterization of a novel mechanism for the reversible inactivation of the T303A mutant by *tert*-butyl acetylene (tBA) has supported a role for the conserved T303 residue in delivery of protons to the active site of cytochrome P450 2E1 (*23*). We report here the formation of a novel tBA−Fe spectral intermediate during the inactivation of the T303A mutant of P450 2E1 that demonstrates reversibility with time and an absolute requirement for NADPH and oxygen. Additionally, studies with alternate oxidants capable of supporting enzyme inactivation in the absence of NADPH and reductase suggest the formation and utilization of a hydroperoxo−iron species by the T303A mutant for substrate oxygenation, confirming the disruption of proton delivery to the active site of this enzyme. The spectral binding constants (K_s) for the binding of tBA and *tert*-butyl 1-methyl-2-propynyl ether (tBMP) by the wild-type and mutant 2E1 P450s were determined and show a higher binding affinity for the acetylenic compounds in the active site of the T303A mutant than in the wild-type enzyme. Active site models of P450 2E1 and 2E1 T303A with tBA bound in the active site confirm the tighter binding of the acetylenic inactivators in the active site of the T303A mutant. A comparison of these models of the two 2E1 P450s with the docked tBA inactivator also supports the mechanism for the inactivation of these enzymes by tBA and lends new insights into the role of T303 in the inactivation event and in delivery of protons to the 2E1 active site.

EXPERIMENTAL PROCEDURES

Materials. tert-Butyl acetylene (tBA), *tert*-butyl 1-methyl-2-propynyl ether (tBMP), *tert*-butyl hydroperoxide (tBHP), and cumene hydroperoxide (CHP) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dilauroyl-L-α-phosphatidylcholine (DLPC), *p*-nitrophenol (*p*-NP), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)-coumarin (7-EFC) was obtained from Molecular Probes, Inc. (Eugene, OR). HPLC-grade acetonitrile was from Fisher (Pittsburgh, PA), and trifluoroacetic acid (TFA) was from Pierce (Rockford, IL).

Enzymes. The cDNAs for rabbit P405s 2E1 and 2E1 T303A (provided by M. J. Coon, The University of Michigan) were expressed in *Escherichia coli* cells. Expression and purification of the proteins were carried out according to published methods (*24*) with some modifications (*8*). NADPH-P450 reductase was purified after expression in *E. coli* Topp3 cells as previously described (*25*).

Enzyme Activity Assays. Purified rabbit cytochrome P450s 2E1 and 2E1 T303A were reconstituted with reductase and lipid for 45 min at 4 °C. Primary incubation mixtures contained 1 nmol of P450, 2 nmol of reductase, 166 μg of DLPC, 2000 units of catalase, tBA (in 1 μL/mL CH_3OH), and 1.2 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4) for a total reaction volume of 1 mL. Methanol was added to the control samples instead of tBA. At the indicated times, 25 μL of the P450 primary reaction mixture was transferred into 975 μL of a secondary reaction mixture containing 100 μM 7-EFC, 0.2 mM NADPH, and 40 μg/mL BSA in 50 mM potassium phosphate buffer (pH 7.4). Samples were incubated for 10 min at 30 °C in a shaking water bath, and the enzyme activity was terminated by the addition of 334 μL of acetonitrile. Enzymatic activity was assessed spectrofluorometrically by measuring the extent of *O*-deethylation of 7-EFC to 7-HFC on a Shimadzu model RF-5301PC spectrofluorophotometer (Shimadzu Scientific Instruments, Columbia, MD) with excitation at 410 nm and emission at 510 nm (*26*).

Spectral Analysis. P450s 2E1 and 2E1 T303A were reconstituted as described above for enzymatic activity, and methanol was added to the control samples instead of tBA. NADPH was added to both the control and tBA-containing samples to start the reaction. Equal volumes from the control and tBA-inactivated samples were placed into a reference and sample cuvette, respectively. A difference spectrum between the two cuvettes was recorded from 350 to 700 nm on a DW2 UV−Vis spectrophotometer (SLM Aminco, Urbana, IL) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA). The reversibility of the resulting tBA−Fe spectral intermediate in the T303A mutant enzyme was investigated by adding the competitive substrate *p*-nitrophenol (*p*-NP), at 5 times the tBA concentration, to both the control and tBA-inactivated samples. The difference spectrum was recorded both prior to and following incubation with *p*-NP for 10 min

at 30 °C. To prevent spectral interference by *p*-NP and its hydroxylated product, these molecules were removed from the samples using a G-50 Sephadex spin column as previously described (*27*). Additionally, the reversibility of the spectral intermediate was examined by incubating the control and tBA-inactivated samples overnight at 4 °C and recording the difference spectrum both prior to and following the incubation. For some experiments, NADPH was added to the control and tBA-inactivated samples as described above and the difference spectrum at 485 nm between the cuvettes was recorded at 0.5 s intervals for times ranging from 10 to 20 min on a Shimadzu model UV-2501PC UV−Vis recording spectrophotometer (Shimadzu Scientific Instruments) with an attached CPS temperature control set to 30 °C. Additionally, the difference spectrum between the two cuvettes was recorded from 450 to 530 nm at 90 s intervals following the addition of NADPH to both cuvettes on a DW2 UV−Vis spectrophotometer (SLM Aminco) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc.).

Spectral Analysis under Anaerobic Conditions. P450 2E1 T303A was reconstituted as described above for enzymatic activity. Control and tBA-containing samples were placed into separate anaerobic cuvettes. NADPH was added to the sidearms of the anaerobic cuvettes and kept isolated from the samples. Each sample underwent 15 cycles of alternating gas (argon) and vacuum to make the contents of the cuvette anaerobic. A baseline scan was taken between the control and tBA-inactivated sample on a Shimadzu model UV-2501PC UV−Vis recording spectrophotometer (Shimadzu Scientific Instruments) with an attached CPS temperature control set to 30 °C. The NADPH in the sidearms of the cuvettes was then mixed with the sample contents, and the difference spectra between the two cuvettes were recorded at 0.5 s intervals for 20 min. Following these scans, oxygen was allowed back into the system and the samples were dialyzed overnight at 4 °C against 2 × 500 mL of 50 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol, to determine if the tBA spectral intermediate could be re-formed. Following dialysis, the samples were reconstituted with lipid for 45 min at 4 °C, and fresh tBA and NADPH were added. Samples were assayed for 7-EFC *O*-deethylation activity, and scans were recorded at 0.5 s intervals for 20 min.

Support of Catalytic Activity and Inactivation Using Alternate Oxidants. Assay conditions for the support of 7-EFC enzymatic activity by the alternate oxidants were optimized prior to proceeding with the inactivation studies. P450s 2E1 and 2E1 T303A were incubated with lipid for 45 min at 4 °C. Primary incubation mixtures contained 1 nmol of P450, 166 μg of DLPC, tBA (in 1 μL/mL CH_3OH), and 10 μM *tert*-butyl hydroperoxide (tBHP) or 10 μM cumene hydroperoxide (CHP) in 50 mM potassium phosphate buffer (pH 7.4) for a total reaction volume of 1 mL. Methanol was added to the control samples instead of tBA. The reactions were initiated by the addition of the alternate oxidant. At the indicated times, 25 μL of the P450 primary reaction mixture was transferred into 975 μL of a secondary reaction mixture containing 100 μM 7-EFC, 40 μg/mL BSA, and 10 μM tBHP or 10 μM CHP 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. Enzymatic activity was assessed spectrofluorometrically by measuring the extent of *O*-deethylation of 7-EFC to 7-HFC on a Shimadzu model RF-5301PC spectrofluorophotometer (Shimadzu Scientific Instruments) with excitation at 410 nm and emission at 510 nm (*26*).

HPLC Analysis. HPLC with diode array analysis of the eluate was used to detect the formation of tBA adducts to the hemes of P450 2E1 and the T303A mutant of 2E1 in the alternate oxidant-supported system. P450s 2E1 and 2E1 T303A were inactivated as described in Support of Catalytic Activity and Inactivation Using Alternate Oxidants, and the control or tBA-inactivated samples were separated by HPLC on a 250 mm × 4.60 mm Phenomenex reverse phase C4 column (solvent A, H_2O and 0.1% TFA; solvent B, 100% 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.

Irreversibility of Inactivation. P450 2E1 and the T303A mutant of 2E1 were inactivated as described in Support of Catalytic Activity and Inactivation Using Alternate Oxidants. Control samples and samples containing tBA-inactivated P450 2E1 or 2E1 T303A (0.5 mL) were dialyzed overnight at 4 °C against 2 × 500 mL of 50 mM potassium phosphate buffer (pH 7.4), containing 20% glycerol, to determine whether the inactivation and losses in native heme were reversible. Following overnight dialysis, samples were reconstituted with lipid for 45 min at 4 °C. Samples were assayed concurrently for 7-EFC *O*-deethylation activity and heme content as described above both prior to and following overnight dialysis.

Binding Spectra and Binding Constants. P450s 2E1 and 2E1 T303A were reconstituted with 100 μg of lipid for 45 min at 4 °C. The reconstituted proteins were then diluted to 0.5 μM with 50 mM potassium phosphate (pH 7.4). The samples were divided, and equal volumes were added to a reference cuvette and a sample cuvette. Samples were scanned from 350 to 500 nm on a DW2 UV−Vis spectrophotometer (SLM Aminco) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc.). Following the addition of 1 μL aliquots of tBA or tBMP dissolved in methanol to the sample cuvette and 1 μL aliquots of methanol to the reference cuvette, scans were recorded for tBA or tBMP concentrations between 10 and 200 μM. The binding constant (K_s) was calculated from a plot of the inverse of the tBA or tBMP concentration versus the inverse of the change in absorbance (380−420 nm). The K_s was obtained from the *x*-intercept of the linear regression line (*28*).

Homology Modeling of Cytochromes P450 2E1 and P450 2E1 T303A. The three-dimensional structures of rabbit P450 2E1 and the T303A mutant of 2E1 were built on the basis of the crystal structure of rabbit P450 2C5 using Quanta/Protein Modeler (Accelrys Inc.). The Protein Data Base (PDB) entry for P450 2C5 is 1DT6 (*29*). The high degree of sequence identity between rabbit P450s 2C5 and 2E1 (54%) facilitated the generation of the models and limited the number of necessary amino acid insertions or deletions. The crystallographic coordinates of rabbit P450 2C5 were obtained from the Brookhaven Protein Data Bank (*30*). The sequence of rabbit P450 2E1 was obtained from the Protein

Information Resource (http://pir.georgetown.edu/). The sequence alignment of P450 2E1 and P450 2C5 was performed using the alignment tool in Quanta/Protein Modeler. Homology models of wild-type 2E1 and the 2E1 T303A mutant were constructed from rabbit P450 2C5 crystallographic coordinates based on amino acid residue replacement according to the sequence alignment. The missing portion of the F−G loop (a small stretch of 11 residues) in the rabbit P450 2C5 crystal structure was inserted into the original crystal structure of rabbit P450 2C5 through protein data bank loop search procedures implemented in Sybyl 6.9.1 Biopolymer (Tripos Associates, St. Louis, MO). According to the alignment, minimal insertions or deletions of amino acid residues were performed. The modified enzyme structure was energy minimized (1000 iterative cycles of molecular mechanics) using the Tripos force field and then was used as a template in Quanta/Protein Modeler to build the homology models. The initial models were energy minimized using CHARMm in Quanta to yield low-energy and stable structures for the wild-type and mutant 2E1 P450s.

Docking of tBA into the Active Site. The inactivator, tBA, was docked into the active sites of the P450 2E1 and T303A mutant models using AutoDock 3.0, and the Lamarckian Genetic Algorithm (LGA) method was used for conformational searching in this study (*31*). The macromolecules and ligand were prepared following the original publication protocols (*31*). The proteins were assigned AMBER united-atom charges using SYBYL 6.9.1. The structure of tBA was constructed using SYBYL 6.9.1 and was energy minimized using Tripos force fields. The ligand was assigned MMFF94 charges using SYBYL 6.9.1. Each LGA run was performed with a crossover ratio of 0.80, a mutation ratio of 0.20, and an elitism ratio of 0.10. During docking, all the rotatable single bonds in the ligand (i.e., sp3−sp3 and sp3−sp2) were allowed to rotate except those whose rotations did not result in different conformations, such as the ones connecting a terminal CH_3 group. Searching steps for translation, rotation, and torsions were set to 0.5 Å, 15°, and 15°, respectively. The size of the docking box was 15 Å × 15 Å × 15 Å, and grid spacing inside the docking box was 0.25 Å. Other miscellaneous parameters were assigned the default values given by the AutoDock program. The empirical scoring function, X-Score, was used to estimate the binding affinity of the P450 enzymes for the tBA and tBMP inactivators (*32*, *33*). On the basis of this scoring function, the smaller or more negative the X-Score value, the higher the binding affinity is predicted to be.

RESULTS

Spectral Analysis. Clorgyline was previously shown to inactivate P450 2B1 through the formation of a metabolic intermediate (MI) complex that could be reversed to regenerate the active enzyme (*34*). Incubation with clorgyline and NADPH resulted in an absorbance peak with a maximum at 455 nm in the difference spectrum that is characteristic of a MI complex. To test whether the reversible formation of a tBA adduct to the heme of the 2E1 T303A mutant (*23*) was occurring through the formation of a MI complex or some other type of spectral intermediate, the difference spectra between control and tBA-inactivated P450 2E1 and 2E1 T303A samples were recorded from 350 to 700 nm. As

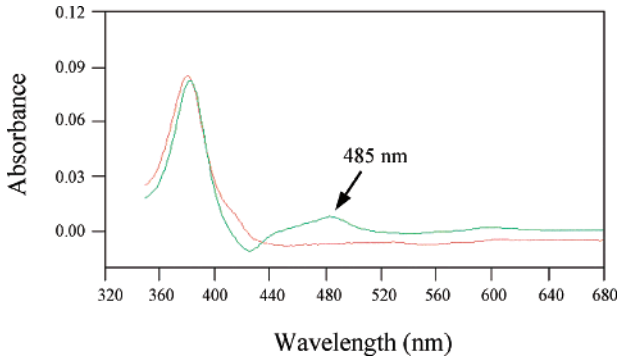


FIGURE 1: Comparison of the difference spectra between wild-type P450 2E1 and the 2E1 T303A mutant. For both enzymes, the control and tBA-inactivated samples were scanned simultaneously as described in Experimental Procedures to record a difference spectrum between the two samples. The difference spectra for P450 2E1 and the T303A mutant are shown in red and green, respectively. The tBA−Fe spectral intermediate with an absorption maximum at 485 nm is denoted with an arrow.

shown in Figure 1, the difference spectrum for the inactivated wild-type 2E1 sample exhibited an absorbance maximum at 385 nm. Although a similar absorbance maximum at 385 nm was observed for the inactivated T303A mutant, the difference spectrum also showed a peak with an absorbance at 485 nm that was not present in the spectrum of the wild-type enzyme (Figure 1). Since this absorbance peak had a maximum at 485 nm rather than at 455 nm, which is diagnostic for a MI complex, this new peak appears to be due to a novel tBA−Fe spectral intermediate. To test whether the formation of this novel tBA−Fe spectral intermediate was reversible, control and tBA-inactivated 2E1 T303A samples were incubated overnight at 4 °C and the difference spectra were recorded both prior to and following the incubation. As has been previously shown for tBA-inactivated 2E1 T303A samples (*23*), a spontaneous recovery of enzymatic activity was observed that occurred concomitantly with a decrease in the level of spectrally detectable tBA−Fe intermediate (data not shown). In a similar fashion, the addition of *p*-NP, a substrate for 2E1, to both the control and inactivated samples resulted in a loss of the spectrally detectable intermediate (data not shown). To monitor the time course for the formation of the spectral intermediate at 485 nm, scans were taken every 0.5 s at 30 °C for a period of 20 min following the addition of NADPH to both control and tBA-containing samples. The difference spectrum between the control and tBA-inactivated P450 2E1 T303A samples showed an increase in absorbance at 485 nm over time with the most rapid rate of increase occurring in the first 10 min of recording (Figure 2, red). Wild-type P450 2E1 samples did not show an increase in absorbance at 485 nm over that same time (Figure 2, blue). Scans from 450 to 530 nm were recorded every 90 s between control and tBA-inactivated P450 2E1 T303A samples to monitor the formation of the tBA−Fe spectral intermediate with time (Figure 2, inset). Together, these data suggest the existence of a newly discovered reversible spectral intermediate of tBA with the heme of the 2E1 T303A mutant enzyme.

Anaerobic Spectral Analysis. Control and tBA-inactivated P450 2E1 T303A samples were made anaerobic through repetitive cycles of sparging with argon gas and applying a vacuum to determine if the formation of the tBA spectral

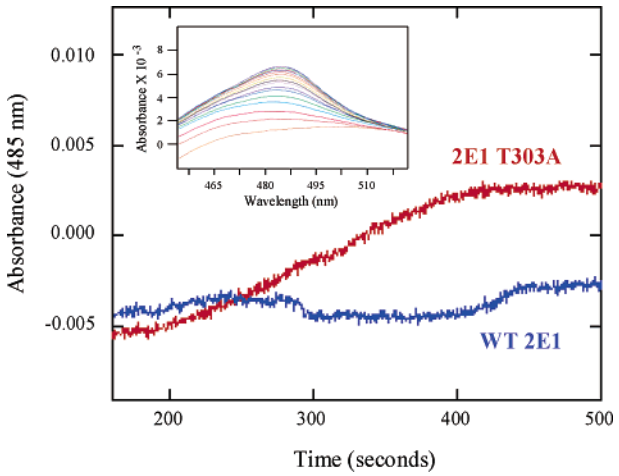


FIGURE 2: Time-dependent formation of the tBA−Fe spectral intermediate at 485 nm. For both the wild-type 2E1 and the 2E1 T303A mutant enzymes, the control and tBA-inactivated samples were scanned simultaneously at 0.5 s intervals for approximately 10 min. The formation of the tBA−Fe spectral intermediate was monitored at 485 nm. The results for the wild-type and T303A mutant enzymes are shown in blue and red, respectively. The inset shows scans of the difference spectrum between the control and tBA-inactivated T303A samples taken at 90 s intervals and illustrates the time-dependent formation of the 485 nm peak.

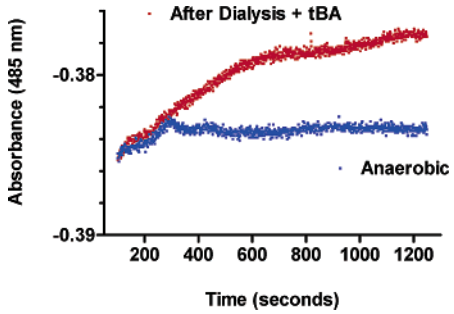


FIGURE 3: Oxygen requirement for the formation of the tBA−Fe spectral intermediate. Control and tBA-inactivated 2E1 T303A samples were made anaerobic through repeated cycles of argon gas and vacuum and then scanned simultaneously at 0.5 s intervals for approximately 20 min (blue). The formation of the tBA−Fe spectral intermediate was monitored at 485 nm. The samples were dialyzed overnight at 4 °C and reconstituted with lipid. Fresh tBA and NADPH were added as described in Experimental Procedures, and the samples were again scanned at 0.5 s intervals for 20 min. The resulting formation of the tBA−Fe spectral intermediate is shown in red.

intermediate with the T303A mutant enzyme was dependent on the presence of oxygen. Figure 3 shows scans taken at 0.5 s intervals between a control and tBA-inactivated T303A sample that was made anaerobic and then mixed with NADPH. Under anaerobic conditions, it appears that the spectral intermediate may form in extremely small quantities within the first 300 s, but then no further increase in the absorbance at 485 nm is observed. We could not detect the formation of the spectral intermediate at 485 nm by simply exposing these samples to oxygen in the air after the anaerobic scans were complete (data not shown). To perfuse these samples with oxygen, the contents of the cuvettes were then dialyzed overnight at 4 °C and fresh tBA and NADPH were added to determine if the 2E1 T303A enzyme could be re-inactivated and to assess whether the tBA spectral intermediate could be re-formed. Interestingly, fresh tBA and NADPH led to the re-inactivation of the T303A mutant

Table 1: Effect of the Alternate Oxidants *tert*-Butyl Hydroperoxide and Cumene Hydroperoxide on the Inactivation of P450s 2E1 by tBA[a]

	tBHP-supported inactivation		CHP-supported inactivation	
	% activity remaining	type of adduct	% activity remaining	type of adduct
P450 2E1	50	heme	71	heme
P450 2E1 T303A	53	heme	98	none

[a] Assay conditions were as described in Experimental Procedures. P450s 2E1 and 2E1 T303A were incubated with DLPC in the absence of reductase. The alternate oxidants, tBHP and CHP, were used to support the enzymatic activity of the P450s and their inactivation by tBA in a NADPH-free system. The given values are the average from two separate experiments in which the results differed by less than 5%. The value obtained for the non-inactivated sample under each condition was assigned to 100%, and the given values were calculated as percents of control activity remaining. The formation of tBA heme adducts was detected by HPLC and diode array analysis as described in Experimental Procedures.

(averages of 81% activity remaining after dialysis and 16% activity remaining after the addition of fresh tBA and NADPH in the presence of oxygen), and the cumulative formation of the tBA spectral intermediate was once again detectable (Figure 3, red). These data suggest a requirement for oxygen in the formation of the tBA spectral intermediate and in the ability to generate the intermediate following dialysis and re-inactivation.

Alternate Oxidant-Supported Activity and Inactivation. A variety of alternate oxidants, including tBHP, hydrogen peroxide, CHP, and iodosobenzene, can replace the NADPH and molecular oxygen required in P450 catalysis and have been utilized to support the formation of different oxidant species in the P450 catalytic cycle. In fact, tBHP (*35*−*37*) and CHP (*38*−*40*) have been used repeatedly in studies to support the catalytic activity and/or inactivation of several different P450s. Research investigating hydroperoxide-supported oxidations has provided valuable information regarding the mechanisms by which P450s activate oxygen and form dioxygen P450 species (*41*). It is thought that these hydroperoxide oxidants can form one or more of the activated oxygen species in the P450 catalytic cycle. To investigate what effect alternate oxidants might have in our system, we initially examined the ability of tBHP and CHP to support the 7-EFC *O*-deethylation activity of P450s 2E1 and 2E1 T303A and then investigated the inactivation of these enzymes by tBA in a reductase- and NADPH-free system. The optimal concentrations of tBHP and CHP required to support the P450 activity were found to be approximately 10 μM. Therefore, these concentrations were used in subsequent experiments to determine if the two alternate oxidants could support the inactivation of the 2E1 P450s by tBA. Table 1 shows that tBHP was able to support the inactivation of both the wild-type and 2E1 T303A mutant enzymes by tBA (50 and 53% enzymatic activity remaining, respectively). The approximate 50% loss in the activities of the enzymes upon inactivation by tBA in the tBHP-supported system was a result of a 10 min incubation with tBA and tBHP and represented the time at which the maximal amount of enzyme inactivation was achieved. Interestingly, CHP was able to support the inactivation of the wild-type enzyme by tBA, but did not support the inactivation of the T303A mutant. These data suggest that tBHP and CHP may be

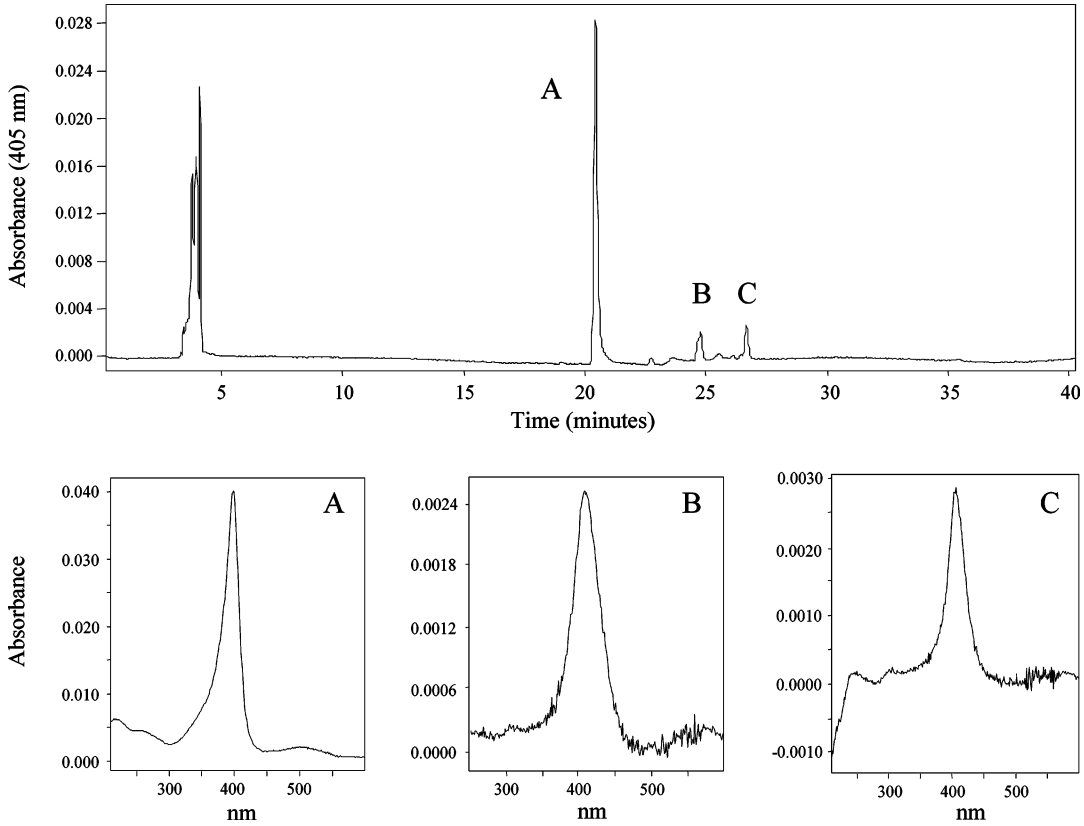


FIGURE 4: HPLC analysis of the hemes from tBA-inactivated P450 2E1 T303A in the system supported by tBHP. The top panel shows the HPLC chromatogram of P450 2E1 T303A incubated with tBA and tBHP. The unmodified heme peak (A) elutes at 21 min, and the two tBA-modified heme products (B and C) elute at approximately 25 and 27 min, respectively. The bottom panel shows the individual diode array spectra of the unmodified heme (A) and the two heme adducts (B and C).

supporting the formation of distinct oxidant species in the P450 catalytic cycle. Although the observed differences between tBHP and CHP and the 2E1 P450s may be dependent upon the enzymes and/or substrates or inactivators in question, we propose that while CHP favors the formation of the oxenoid−iron species, tBHP may potentially favor the formation of the hydroperoxo− or peroxo−iron species. Vaz and co-workers proposed that mutation of threonine 303 to alanine in P450 2E1 resulted in a disruption in proton transfer leading to the formation of the oxenoid−iron species (*15*). They reported decreases in the extents of hydroxylation reactions and increases in the extents of epoxidation reactions with this mutant enzyme. From these data, the authors concluded that the T303A mutant of P450 2E1 utilized the hydroperoxo−iron species for the oxidation of various substrates, whereas the wild-type 2E1 primarily used the oxenoid−iron intermediate for substrate oxygenation (*15*). HPLC with diode array analysis demonstrated that the mechanism for the inactivations of the 2E1 P450s by tBA in the alternate oxidant-supported system was through the formation of tBA adducts to the P450 hemes (Table 1). Figure 4 shows a HPLC chromatogram monitored at 405 nm for P450 2E1 T303A incubated in the presence of tBA and tBHP. The native P450 heme (A) elutes first and is followed by two additional peaks of nearly equal area (B and C). The diode array spectra of the native heme and the tBA-modified hemes (peaks B and C) were similar with the exception of a small shift toward longer wavelengths for the modified hemes, consistent with the formation of *N*-alkylated heme products. tBA has been previously shown to inactivate both the wild-type and T303A mutant P450s through the formation of adducts to the P450 heme (*22*).

Irreversibility of Inactivation. The inactivation of P450 2E1 T303A by tBA in a reconstituted system with reductase and NADPH has been shown previously to be reversible with dialysis (*22*), while the inactivation of wild-type 2E1 by tBA is completely irreversible. The losses in enzymatic activity, the P450 reduced CO spectrum, and the native P450 heme could be restored to the tBA-inactivated T303A samples with extensive dialysis. To test whether the inactivations by tBA and the losses in the native hemes of P450 2E1 and the T303A mutant of 2E1 were reversible in the tBHP-supported system, control and tBA-inactivated samples were dialyzed extensively. Interestingly, the losses in enzymatic activity and native heme of both the tBA-inactivated wild-type and T303A mutant enzymes were shown to be completely irreversible with dialysis when the reactions were supported by tBHP. This is in stark contrast to what has been previously observed for the tBA-inactivated T303A mutant in the reductase and NADPH system (*22*). Table 2 shows that prior to dialysis, inactivation of the T303A mutant by tBA in the tBHP-supported system left approximately 46% of the enzymatic activity remaining and 27% of the native P450 heme remaining. Following overnight dialysis of the tBA-inactivated T303A sample, approximately 52% of the enzymatic activity and 29% of the native heme remained, illustrating that the tBHP-supported inactivation was completely irreversible. Similar results were obtained for the wild-type 2E1 enzyme in the artificial oxidant-supported system (data not shown). The abrupt change in the status of

Table 2: Irreversibility of the Inactivation of Cytochrome P450 2E1 T303A by tBA When the Reaction Is Supported by tBHP[a]

	% control activity remaining	% control P450 heme remaining
before dialysis	45.9 ± 0.2	27.3 ± 2.4
after dialysis	51.6 ± 1.8	29.3 ± 1.6

[a] Assay conditions were as described in Experimental Procedures. P450 2E1 T303A was incubated with DLPC in the absence of reductase. The alternate oxidant tBHP was used to support the enzymatic activity and the inactivation by tBA of P450 2E1 T303A in a NADPH-free system. The given values represent the mean and standard deviation from three separate experiments. The values obtained for the non-inactivated sample in each condition were assigned to 100%, and the given values were calculated as percents of control activity or native heme remaining. Native P450 heme was detected by HPLC with diode array analysis as described in Experimental Procedures.

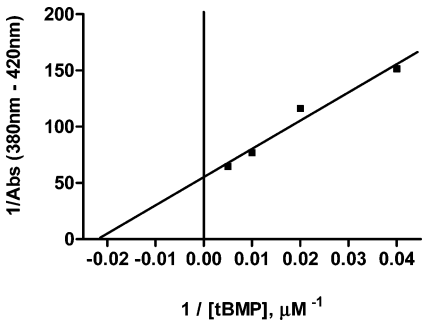


FIGURE 5: Determination of the binding constant for binding of tBMP to cytochrome P450 2E1. The conditions for the spectral titration were as described in Experimental Procedures. The inverse of the change in the P450 2E1 difference spectrum (380−420 nm) was plotted vs the inverse of the tBMP concentration. The binding constant (K_s) was calculated from the *x*-intercept.

reversibility between the reductase/NADPH- and tBHP-supported systems may be the result of a change in chemistry in the enzyme active site. Previously, we have shown that an external source of protons was necessary for formation of the tBA heme adducts in the T303A mutant, but not the wild-type enzyme (*23*). It is possible that the chemistry of tBHP may be contributing a source of protons to the tBA reactive intermediate that would potentially lead to the formation of a stable, irreversible adduct.

Binding Spectra and Binding Constants. The binding of substrates to P450 enzymes may be accompanied by changes in the optical absorbance spectra of these proteins (*28*). The binding of tBA and tBMP to P450s 2E1 and 2E1 T303A produced spectral changes characteristic of type I compounds. Spectral titrations of the two P450s were performed with tBA and tBMP, and the change in absorbance (380−420 nm) in the difference spectrum was determined at each concentration of substrate. The resulting changes in absorbance were plotted as the inverse of the change in absorbance versus the inverse of the tBA or tBMP concentration. From these plots, the spectral binding constants (K_s) were determined. The K_s is defined as the concentration of the compound that results in 50% of the theoretical maximal spectral change (*28*). Figure 5 shows the determination of the binding constant for the binding of tBMP to P450 2E1. The K_s values for the binding of tBA and tBMP to the P450s 2E1 are shown in Table 3. Interestingly, the 2E1 T303A mutant seems to bind tBA and tBMP with higher affinity (27 and 23 μM, respectively) than the wild-type enzyme (55 and 46 μM, respectively). From these data, it appears that the threonine to alanine mutation has resulted in a change in the architecture of the P450 active site that has brought about interactions between the acetylenic inactivators and nearby amino acid residues which results in tighter binding of tBA and tBMP to the T303A mutant.

Table 3: Substrate Binding Constants for tBA and tBMP with P450s 2E1[a]

	type of binding spectrum	K_s (μM)
P450 2E1 with tBA	type I	55
P450 2E1 T303A with tBA	type I	27
P450 2E1 with tBMP	type I	46
P450 2E1 T303A with tBMP	type I	23

[a] Binding spectra were determined as described in Experimental Procedures. The binding constants (K_s) were determined from plots of the inverse of the change in the P450 absorption in the difference spectrum (380−420 nm) vs the inverse of the tBA or tBMP concentration.

Homology Modeling of P450s 2E1 and Docking of tBA into the Enzyme Active Sites. Homology models of wild-type P450 2E1 and the T303A mutant of P450 2E1 were constructed from the rabbit P450 2C5 crystallographic coordinates based on amino acid residue replacement according to the sequence alignment of the two enzymes. The tBA inactivator was docked into the active sites of the 2E1 and 2E1 T303A enzymes using AutoDock 3.0 (*31*). Figure 6 shows the docking of tBA (yellow, space-filling molecule) into the active site of the wild-type enzyme (A) and the 2E1 T303A mutant (B). Amino acid residues within 4 Å of the inactivator are colored light blue, and the P450 heme moiety with its central iron atom is in red. From these models, we can see that the tBA inactivator is oriented quite differently in the active site of the wild-type enzyme when compared to that of the T303A mutant. Using an empirical scoring function called X-Score (*32*, *33*), we were able to estimate the relative binding affinity for tBA and tBMP in the enzyme active sites. X-Score calculated binding scores of −6.16 for tBA binding in the T303A mutant and −5.86 for tBA binding in the wild-type enzyme. The more negative score (i.e., −6.16) indicates a higher binding affinity. These results are in agreement with our determination of the spectral binding constants for binding of tBA and tBMP to the P450s 2E1 (Table 3).

Interestingly, the calculated distances between the internal and terminal acetylenic carbons of tBA and the heme iron vary dramatically between the 2E1 and T303A mutant enzymes (Table 4). The internal and terminal acetylenic carbons of tBA are within 3.6 and 3.0 Å, respectively, of the central iron atom in the active site of the wild-type 2E1 enzyme and are within 4.8 and 5.4 Å, respectively, in the T303A mutant. Ortiz de Montellano and co-workers have previously reported that acetylenic compounds modifying the heme will have an oxygen inserted into the internal acetylenic carbon whereas compounds modifying the apoprotein will have the oxygen atom inserted into the terminal carbon (*42*−*44*). The inactivation of wild-type P450 2E1 by tBA was previously shown to occur through a combination of heme and protein adduction (*22*). In the 2E1 models, the closer distances of the acetylenic carbon groups to the iron atom in the wild-type enzyme when compared to the mutant (a difference of 2.4 Å for the terminal acetylenic carbon) indicate that distance may be a factor in determining the

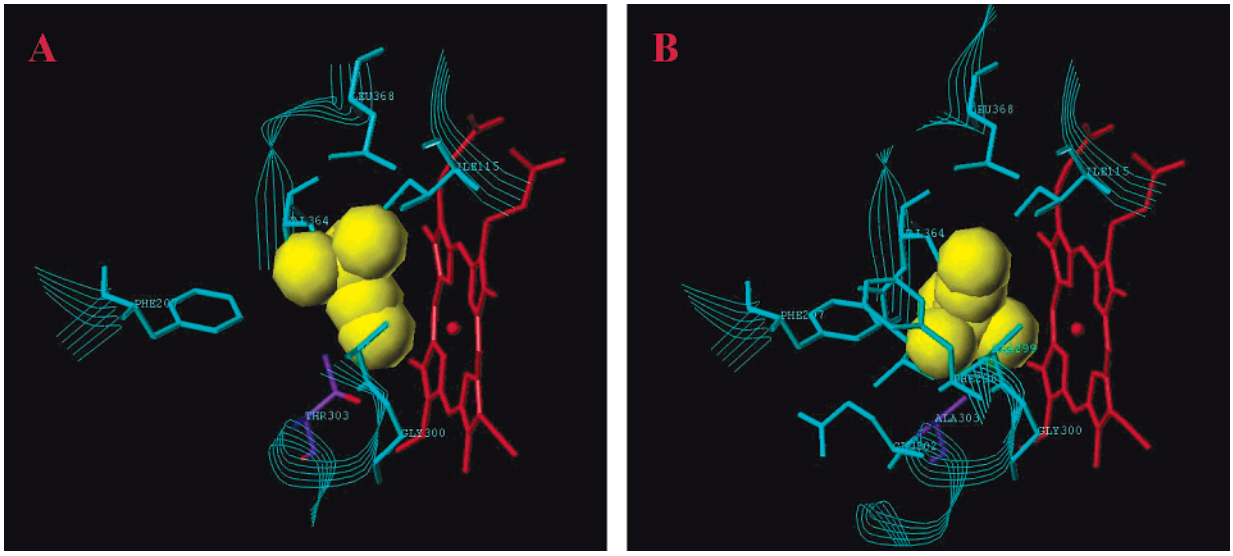


FIGURE 6: Active site docking models of P450 2E1 (A) and P450 2E1 T303A (B) with tBA. The P450 heme moiety with the central iron atom is given in red. Amino acid residues within 4 Å of the inactivator are shown in light blue. The conserved threonine 303 residue in P450 2E1 (A) and the mutated alanine residue in the 2E1 T303A mutant (B) are shown in dark purple. The inactivator, tBA, is represented by a space-filling molecule in yellow.

Table 4: Distances between the Internal and Terminal Acetylenic Carbons of tBA and the Heme Iron[a]

	internal acetylenic carbon	terminal acetylenic carbon
P450 2E1	3.6 Å	3.0 Å
P450 2E1 T303A	4.8 Å	5.4 Å

[a] The distances between the acetylenic carbons of tBA and the central iron atom of the heme group were calculated as described in Experimental Procedures.

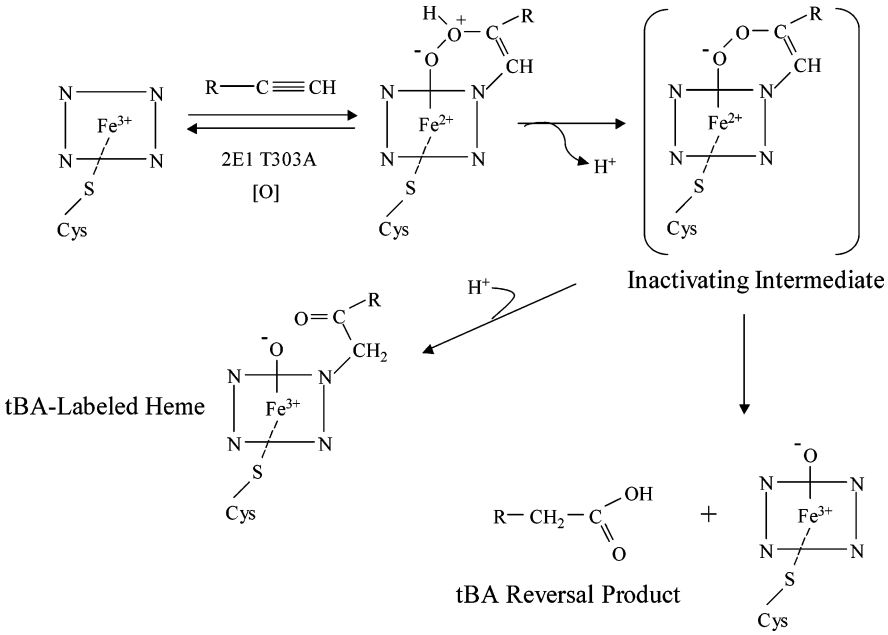
mechanism for inactivation. The position of the conserved T303 residue in P450 2E1 (Figure 6A) also supports this mechanism of inactivation and suggests that the T303 residue may be the site of apoprotein modification for the tBA reactive intermediate. In comparison, the acetylenic carbon groups of tBA are much farther from the heme iron in the T303A mutant model (Table 4). Although we are able to support the formation of tBA adducts to the P450 heme in the 2E1 T303A mutant by supplying an external source of protons to the enzyme (*23*), the tBA intermediates formed in this enzyme can be completely reversed by dialysis and overnight incubation at 4 °C (*22*, *23*). The absence of the conserved T303 residue in this mutant and the altered orientation of tBA in the active site (Figure 6B) support our previous inactivation and reversibility data as well as the data presented here concerning the formation of the reversible tBA−Fe spectral intermediate.

DISCUSSION

The mechanism-based inactivation of cytochromes P450 2E1 and 2E1 T303A by *tert*-butyl acetylene (tBA) and *tert*-butyl 1-methyl-2-propynyl ether (tBMP) has been previously investigated (*22*). Interestingly, the T303A mutant of 2E1 was inactivated by tBA in a reversible manner (*22*, *23*). Losses in enzymatic activity, the reduced CO spectrum, and native heme of the tBA-inactivated T303A mutant could be restored to the samples with dialysis and overnight incubations. The reversibility was further demonstrated to be time-dependent and to require an intact P450 enzyme structure. ESI-LC−MS/MS analysis under nondenaturing conditions of a pre-acidified tBA-inactivated T303A sample yielded two tBA adducts (*m*/*z* 661 Da) with ion fragmentation patterns characteristic of a tBA adduct to the P450 heme. These adducts were absent in samples subjected to the same conditions that had not been pre-acidified. In contrast, both non- and pre-acidified tBA-inactivated wild-type 2E1 samples formed the two tBA adducts (*m*/*z* 661 Da), suggesting that the T303A mutant of P450 2E1 was deficient in the delivery of protons to the enzyme active site. Thus, one of the critical goals of this study was to determine what role threonine 303 plays in the inactivation and the reversibility of inactivation of cytochrome P450 2E1 and the T303A mutant of 2E1 by *tert*-butyl acetylenic compounds.

Although metabolic intermediate (MI) complexes have been well documented in the literature and are known to have an absorbance maximum at 455 nm, there has also been evidence for the formation of other types of spectral intermediate complexes that are characterized by absorbance maxima at higher wavelengths (*45*, *46*). To investigate the possible formation of a metabolic intermediate complex or some other type of spectral intermediate by the tBA-inactivated T303A mutant, extensive spectral analyses were performed. Interestingly, a peak having an absorption maximum at 485 nm was observed with the 2E1 T303A samples; however, a similar peak was not detected in the spectrum of the wild-type enzyme. The formation of the 485 nm peak in the T303A enzyme was monitored with time and showed a rapid accumulation during the first 10 min with a plateau after recording had been carried out for 20 min. Importantly, the formation of the peak at 485 nm required oxygen, and following reversion to the active enzyme after overnight dialysis, the peak could be regenerated by the addition of fresh tBA and NADPH. Given that typical MI complexes absorb at 455 nm, this spectral intermediate having a maximum absorption at 485 nm is believed to be a newly discovered tBA−Fe intermediate. Of further interest was the fact that the tBA−Fe spectral

Scheme 1: Formation of a Reversible, Spectrally Detectable tBA-Inactivating Intermediate of P450 2E1 T303A[a]

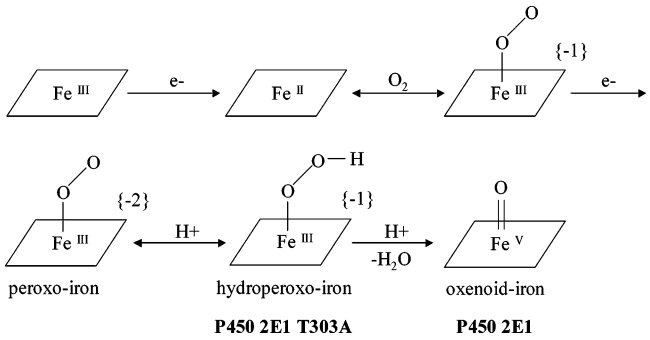


[a] A chelated intermediate that includes the heme iron, an oxygen-inserted acetylene intermediate, and a pyrrole nitrogen may be responsible for the reversible loss in the enzymatic activity of the 2E1 T303A mutant. The intermediate having an absorbance at 485 nm (in brackets) can either lead to inactivation or slowly decompose to regenerate the native catalytically active enzyme and a tBA carboxylic acid product. An exogenous source of protons for the T303A enzyme (*23*) results in *N*-alkylation of the P450 heme. This second pathway is identical to the sequence of steps involved in the irreversible inactivation of the wild-type 2E1 enzyme.

intermediate was also reversible with time or by the addition of a 2E1 substrate, supporting our previous T303A reversibility data (*22*, *23*). Since protons are required to support the formation of the *N*-alkylated tBA heme adducts (*23*), this acetylene−iron spectral intermediate may be a chelated structure in which the oxygenated acetylene forms a bridge between the heme iron atom and one of the pyrrole nitrogens of the heme moiety. Scheme 1 proposes the formation of such a bridging intermediate between the heme iron, an oxygenated tBA, and a pyrrole nitrogen in the tBA-inactivated T303A mutant enzyme. In the absence of an exogenous source of protons, the inactivating spectral intermediate can be reversed to regenerate native heme and a tBA reversal product, presumably the tBA acid (Scheme 1). In contrast, the wild-type 2E1 enzyme rapidly forms *N*-alkylated heme adducts that cannot be reversed. Further characterizations of possible tBA reversal products are ongoing using mass spectrometry.

The reduction of molecular oxygen in the P450 catalytic cycle is believed to involve a progression from the peroxo−iron species to a hydroperoxo−iron intermediate and finally to an oxenoid−iron complex (Scheme 2). Cytochrome P450 2E1 has been suggested to primarily utilize the putative oxenoid−iron species for substrate oxygenation, whereas the hydroperoxo−iron species is believed to serve as the primary oxidant for the 2E1 T303A mutant (*15*). In the P450 catalytic cycle, the addition of one electron, a molecule of oxygen, and a second electron is equivalent to the addition of oxygen as a peroxide or hydroperoxide. In support of this, it has been demonstrated that some P450s are able to use alternate oxidants such as hydroperoxides, peracids, and iodosobenzene as oxidants in place of molecular oxygen and NADPH (*47*, *48*). To further investigate the possible role of T303 in proton delivery and inactivation, we used the alternate

Scheme 2: P450 Catalytic Cycle and Resulting Activated Oxygen Species[a]



[a] The P450 catalytic cycle involves (1) a one-electron reduction of the P450 from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state, (2) binding of molecular oxygen to generate the oxyferrous P450, (3) transfer of a second electron to form the peroxo−iron species, (4) addition of one proton to form the hydroperoxo−iron species, and (5) addition of the second proton and loss of water to form the putative oxenoid−iron P450. Alternate oxidants have been used to replace the requirement for NADPH in P450 reactions and may form any one of these three activated oxygen species. Presumably, our data suggest that P450 2E1 T303A is deficient in delivering protons to the active site and cannot form the oxenoid−iron complex; thus, this enzyme may primarily utilize the hydroperoxo−iron species for inactivation by tBA.

oxidants *tert*-butyl hydroperoxide (tBHP) and cumene hydroperoxide (CHP) as replacements for NADPH and molecular oxygen to support the formation of distinct oxidant species in the P450 catalytic cycle. Although both alternate oxidants supported the inactivation of P450 2E1 by tBA, only tBHP supported the inactivation of the T303A mutant by tBA. Although the observed differences between tBHP and CHP and the 2E1 P450s may be dependent upon the enzymes and/or substrates or inactivators in question, we believe that CHP and tBHP may be forming different acti-

vated oxygen species in the P450 catalytic cycle. From this, we have proposed that the oxidant species utilized for substrate oxygenation in the 2E1 T303A mutant is the hydroperoxo−iron species and that proton delivery from the threonine residue is necessary for progression to the active oxenoid−iron species in the wild-type enzyme (Scheme 2). These data support our previous ESI-LC−MS/MS observations showing that an external source of protons was necessary to support the formation of stable tBA heme adducts in the T303A mutant enzyme while the wild-type P450 2E1 was able to generate stable adducts in the absence of protons (*23*). Taken together, 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.

Since we have previously observed reversibility with acetylenic inactivators when NADPH and oxygen were used to support the inactivation (*22*, *23*), we investigated whether the inactivations of the 2E1 P450s in the alternate oxidant system were reversible with dialysis. Interestingly, the inactivations of both P450 2E1 and 2E1 T303A by tBA in the tBHP- and CHP-supported systems were not reversible. The losses in enzymatic activity and native P450 heme could not be restored to the samples following extensive dialysis. The irreversibility of the tBA-inactivated 2E1 T303A mutant in the alternate oxidant-supported system may be due to the contribution of protons in the active site of the enzyme by the *tert*-butyl hydroperoxide. Although the overall pH of the reaction mixtures was 7.4 during the inactivation and dialysis, the environment of the active site may have been altered through the use of tBHP to generate the hydroperoxo−iron activated oxygen species. Additionally, the extra hydrogen atom present on the hydroperoxo−iron species may be contributing to the irreversibility in the mutant enzyme. Interestingly, when the inactivation of the 2E1 T303A mutant by tBA was supported by tBHP and monitored spectrally, the characteristic tBA spectral intermediate at 485 nm in the reductase- and NADPH-supported system was not observed (data not shown). Along with the noted irreversibility of inactivation of the 2E1 T303A mutant in the tBHP-supported system, these observations suggest that tBHP itself or the resultant hydroperoxo−iron intermediate may be contributing to the formation of a stable tBA heme adduct in the active site of the T303A enzyme.

The crystal structures of several bacterial P450s show a highly conserved threonine residue which is located over the proposed oxygen-binding pocket and within hydrogen bonding distance of the activated oxygen species (*49*−*51*). Our active site models of rabbit cytochromes P450 2E1 and 2E1 T303A confirm these observations. Although the tBA molecule is oriented differently in the active site models of the wild-type and mutant 2E1 enzymes, it is located above the heme plane and is closely surrounded by Leu368, Phe207, Val364, Ile115, Gly300, and Thr303 or Ala303 (Figure 6A,B). Interestingly, the threonine to alanine mutation in the 2E1 T303A enzyme has shifted the location of Glu302 and Phe298 to bring them within 4 Å of the tBA inactivator, increasing the number of residues in close contact with tBA (Figure 6B). The orientation of the tBA inactivator within the enzyme active site also changes as a result of the T303A mutation. Substrate binding data (Table 3) and calculations from an empirical scoring function, X-Score (*32*, *33*), both indicate a higher binding affinity for tBA in the T303A active site than in the active site of the wild type. This is most likely due to an increased level of hydrogen bonding interactions with nearby residues in the mutant active site, including Glu302 and Phe298. A comparison of the orientation of tBA in the active site models of the T303A mutant and wild-type enzymes also demonstrates what may be a significant shift in the distances between the heme iron and the internal and terminal acetylenic carbons of the tBA inactivator (Table 4). The absence of T303 for the delivery of protons to the active site may be partially responsible for the reversible heme adduction of tBA to the T303A mutant enzyme that we observe.

Together, the data presented here provide insights into the reversible inactivation of the T303A mutant of P450 2E1. Our studies with artificial oxidants confirm that protons are necessary for the formation of a stable tBA adduct to the T303A heme and suggest that a disruption in proton delivery to active site of the 2E1 T303A mutant has occurred. Comparison models of P450 2E1 and 2E1 T303A with tBA positioned in the active site confirm a modification in the active site architecture as a result of the T303A mutation, leading to an altered orientation of tBA and a higher binding affinity for the acetylene in the active site. The spectral characterization of the reversible tBA−Fe spectral intermediate aids in our understanding of the novel mechanism for the reversible inactivation of the 2E1 T303A mutant by tBA. Collectively, these results strongly support a role for the highly conserved T303 residue in the donation of protons to the active site of P450 2E1 as well as a role in the irreversibility or reversibility of inactivation of the 2E1 P450s by small acetylenic compounds such as tBA.

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