Spectral Studies of *tert*-Butyl Isothiocyanate-Inactivated P450 2E1†

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ABSTRACT: Inactivation of cytochrome P450 2E1 by *tert*-butyl isothiocyanate (tBITC) resulted in a loss in the spectrally detectable P450−reduced CO complex. The heme prosthetic group does not appear to become modified, since little loss of the heme was observed in the absolute spectra or the pyridine hemochrome spectra, or in the amount of heme recovered from HPLC analysis of the tBITC-inactivated samples. Prolonged incubations of the inactivated P450 2E1 with dithionite and CO resulted in a recovery of both the CO complex and the enzymatic activity. Inactivated samples that were first reduced with dithionite for 1 h prior to CO exposure recovered their CO spectrum to the same extent as samples not pretreated with dithionite, suggesting that the major defect was an inability of the inactivated sample to bind CO. Spectral binding studies with 4-methylpyrazole indicated that the inactivated P450 2E1 had an impaired ability to bind the substrate. Enzymatic activity could not be restored with iodosobenzene as the alternate oxidant. EPR analysis indicated that approximately 24% of the tBITC-inactivated P450 2E1 was EPR-silent. Of the remaining tBITC-inactivated P450 2E1, approximately 45% exhibited an unusual low-spin EPR signal that was attributed to the displacement of a water molecule at the sixth position of the heme by a tBITC modification to the apoprotein. ESI-LC−MS analysis of the inactivated P450 2E1 showed an increase in the mass of the apoprotein of 115 Da. In combination, the data suggest that tBITC inactivated P450 2E1 by binding to a critical active site amino acid residue(s). This modified amino acid(s) presumably acts as the sixth ligand to the heme, thereby interfering with oxygen binding and substrate binding.

Cytochrome P450 enzymes (P450s)[1] belong to a superfamily of heme-containing monooxygenases with a similar catalytic mechanism (*1, 2*). Bacterial P450s are soluble proteins, and high-resolution crystal structures for several isoforms have been determined (*3*−*6*). Crystal structure determinations for mammalian P450s have so far been elusive since these P450s are membrane-bound. Recently, the first low-resolution structure of a modified microsomal P450 has been elucidated (*7*). P450 enzymes are found in great abundance in hepatic microsomal membranes and are involved in the phase I metabolism of a wide variety of endobiotics and xenobiotics such as steroids, prostaglandins, fatty acids, drugs, pesticides, and carcinogens (*2*). The metabolism of these compounds by P450 enzymes generally involves the insertion of an oxygen atom which results in a more polar and therefore more readily excretable product (*8*). In most instances, the metabolism of xenobiotics by P450s is therefore considered to be a detoxification process. However, in some instances, these reactions have been shown to generate a more toxic or carcinogenic metabolite (*9*, *10*).

P450 2E1 is constitutively expressed in liver and in lower levels in kidney, lung, intestine, nasal mucosa, lymphocytes, and brain. P450 2E1 is the major ethanol and starvation inducible isoform in mammalian liver and metabolizes ethanol as well as many other small compounds such as halogenated alkanes, acetaminophen, nitrosamines, benzene, and styrene (*10*−*12*). In particular, P450 2E1 is thought to play a role in the activation of short-chain *N*-nitrosamines to carcinogens (*13*). Isothiocyanates such as phenethyl isothiocyanate (PEITC) have been shown to inhibit *N*-nitrosobenzylamine-induced esophageal cancer in rodents (*14*). The administration of benzyl isothiocyanate (BITC) and PEITC in combination has also been suggested as a preventative measure for the treatment of lung cancer in humans (*15*, *16*). In recent reports, we have shown that isothiocyanates such as BITC and *tert*-butyl isothiocyante (tBITC) are potent mechanism-based inactivators of P450 2E1 in the reconstituted system (*17*, *18*). A mechanism-based inactivator is a compound that in the course of metabolism

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[1] Abbreviations: P450, cytochrome P450 [nomenclature from Nelson et al. (*1*)]; reductase, NADPH−cytochrome P450 reductase; tBITC, *tert*-butyl isothiocyanate; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; BSA, bovine serum albumin; DLPC, L-α-dilauroylphosphatidylcholine; DTT, dithiothreitol; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; 4-MP, 4-methylpyrazole; EPR, electron paramagnetic resonance; ESI-LC−MS, electrospray ionization liquid chromatography−mass spectrometry.

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forms a more reactive intermediate that prior to its release binds covalently to the active site of the metabolizing enzyme, thereby inactivating the catalytic activity of the enzyme (*19*). The inactivation of P450 2E1 by isothiocyantes could occur by several different mechanisms. The isothiocyante reactive intermediate could alkylate and destroy the heme moiety; the modified heme could become cross-linked to the apoprotein, or the reactive intermediate could modify an amino acid residue critical for catalysis or substrate binding (*20*−*22*). With isothiocyanates, a fourth possibility also needs to be considered. Oxidative desulfuration could lead to the release of sulfur which in turn could bind to the P450, resulting in a loss of enzymatic activity (*23*). Previous observations with BITC and P450 2E1 indicated that the primary defect leading to inactivation was the binding of a BITC reactive intermediate to the P450 2E1 apoprotein (*18*). The inactivation of P450 2E1 by BITC did not result in a loss in the spectrum of the P450−reduced carbon monoxide complex, an observation that has been used to implicate inactivation via adduction of a BITC reactive intermediate to the apoprotein. However, P450 2E1 samples treated with tBITC in the presence of NADPH exhibited a considerable decrease in the CO spectrum that was similar in extent to the loss in enzymatic activity (*17*).

In this report, we have extended our analysis of the tBITC-inactivated P450 2E1. We present novel data supporting the notion that tBITC binds to a critical amino acid residue in the active site of P450 2E1. The major defect incurred by the binding of a reactive tBITC metabolite to the P450 2E1 apoprotein resulted in a reduced ability of CO or substrate to bind. EPR spectroscopic analysis indicated that the heme iron of 24% of the tBITC-inactivated P450 2E1 was in the Fe^{2+} EPR silent state, thereby prohibiting completion of the P450 catalytic cycle. The remaining tBITC-inactivated P450 fraction exhibited a unique low-spin EPR signal that may result from the displacement of a water molecule from the sixth coordinate to the heme iron by a tBITC-derived apoprotein adduct.

EXPERIMENTAL PROCEDURES

Materials

L-α-Dilauroylphosphatidylcholine (DLPC), NADPH, BSA, and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained form Molecular Probes Inc. (Eugene, OR), and 7-hydroxy-4-(trifluoromethyl)coumarin was from Enzyme Systems Products (Livermore, CA). HPLC-grade acetonitrile was from Fisher (Pittsburgh, PA) and trifluoroacetic acid from Pierce (Rockford, IL). tBITC was purchased from Trans World Chemicals (Rockville, MD).

Methods

Purification of P450 and Reductase. Rat NADPH−cytochrome P450 reductase (reductase) was purified from *Escherichia coli* as previously described (*24*). Shortened, bacterially expressed rabbit P450 2E1 was purified as described by Larson et al. with modifications (*17*, *25*).

Enzyme Activity Assays. The 7-EFC activity of purified reconstituted P450 2E1 was measured essentially as previously described for P450 2B1 (*26*). P450 2E1 and reductase were reconstituted with lipid for 1 h at 4 °C. Primary incubation mixtures contained 1 μM P450 2E1, 1 μM reductase, 200 μg of DLPC/mL, 250 units of catalase/mL, 1 μM tBITC (unless otherwise indicated in 1 μL of CH_3OH/mL), or 1 μL/mL CH_3OH, and 1.2 mM NADPH in 150 mM potassium phosphate (pH 7.4) containing 40 μg of BSA/mL. The 7-EFC activity was measured in a secondary reaction mixture as previously described, except that the samples were incubated for 15 min at 30 °C (*17*).

Iodosobenzene-Supported Activity. Iodosobenzene-supported activity was evaluated as previously described (*27*). P450 2E1 was reconstituted and inactivated with tBITC as described previously, except that the NADPH concentration in the primary incubation mixtures was 0.5 mM. Aliquots (115 pmol) from control samples incubated only with NADPH and from tBITC-inactivated samples were transferred to 950 μL of a secondary reaction mixture containing 0.2 mM 7-ethoxycoumarin in 50 mM potassium phosphate (pH 7.4), 40 μg of BSA/mL, and either 0.2 mM NADPH or 0.8 mM iodosobenzene. The increase in fluorescence of the coumarin product was monitored for 99 s with excitation at 390 nm and emission at 440 nm.

Spectrophotometric Quantitation of P450 2E1. P450 2E1 and reductase were reconstituted with lipid for 1 h at 4 °C. The primary reaction mixtures contained 1 μM 2E1, 1 μM reductase, 20 μg of DLPC, 125 units of catalase, 1 μM tBITC (in 5 μL of CH_3OH/500 μL), and 50 mM potassium phosphate (pH 7.4) in a total volume of 500 μL. Reactions were initiated with 1.2 mM NADPH. Control samples received an equal volume of H_2O instead of NADPH. After 10 min at 30 °C, 10 μL of the primary reaction mixtures was assayed for 7-EFC activity and 100 μL of the primary reaction mixture was diluted with 900 μL of 50 mM ice-cold potassium phosphate (pH 7.4) containing 40% glycerol and 0.6% Tergitol NP-40 (quench buffer). Dithionite and CO were added, and the reduced carbon monoxide spectra were recorded from 400 to 500 nm (*28*).

Reduced Pyridine Hemochrome Spectra. The reduced pyridine hemochrome was assessed essentially as described by Koop (*29*). Aliquots (400 μL) containing 0.4 nmol of P450 2E1 were added to 800 μL of 0.11 M NaOH in 4 M pyridine. The samples were reduced with dithionite, and the spectrum was recorded from 500 to 600 nm. The pyridine hemochrome was quantified using an extinction coefficient of 34.4 mM^{-1} cm^{-1} (*30*).

Visible Spectra of P450 2E1. Reconstituted P450 2E1 was inactivated with tBITC as described above. Exposed control samples, incubated with tBITC but without NADPH, and tBITC-inactivated samples were dialyzed for 18 h at 4 °C against two 500 mL changes of potassium phosphate (pH 7.4) containing 20% glycerol. The samples were diluted with fresh dialysis buffer to 1 μM P450 and scanned from 350 to 700 nm. Prior to dialysis, P450 2E1 was primarily in the low-spin state with a 394 nm/424 nm ratio of 0.7. This was presumably due to the inclusion of 50 μM 4-MP during the initial purification steps which was shown by Larson et al. to stabilize the enzyme, resulting in higher yields of active P450 2E1 (25). Addition of tBITC resulted in a shift to the high-spin state with a 394 nm/424 nm ratio of 1.6 and the appearance of a charge-transfer band at 650 nm. Incubations of the P450 2E1 in the presence of tBITC and NADPH followed by dialysis resulted in a change in the spin state

with a 394 nm/424 nm ratio of 0.9 and a concurrent disappearance of the charge-transfer band.

HPLC Analysis of P450 2E1. P450 2E1 (1 nmol) and reductase (1 nmol) were reconstituted together with 25 μg of DLPC for 45 min at 4 °C. The sample was diluted to a final volume of 200 μL with potassium phosphate (pH 7.4) containing 250 units of catalase and 2 μM tBITC in 1 μL of CH_3OH. One-half of the sample received 1.2 mM NADPH, and the control sample received an equal volume of H_2O. Both samples were incubated for 15 min at 30 °C. Each sample (0.2 nmol) was injected onto a Vydac C4 HPLC reverse phase column (Hesperia, CA) equilibrated with 40% CH_3CN. The samples were resolved with a linear gradient of 40 to 90% CH_3CN at a flow rate of 1 mL/min. The samples were monitored from 200 to 500 nm using a diode array detector.

4-Methylpyrazole Binding Spectra. P450 2E1 was inactivated and dialyzed as described above for visible spectra. Spectra were recorded from 350 to 500 nm after the addition of increasing concentrations of 4-methylpyrazole. The difference in the absorbance between 427 and 390 nm was measured to calculate the percent increase in the level of binding between the inactivated sample and the exposed control sample treated with 40 μM 4-methylpyrazole. All spectra were recorded on a DW2 UV−vis spectrophotometer (SLM Aminco, Urbana, IL) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Inc., Bogart, GA).

EPR Spectra. For EPR spectral analyses, P450 2E1 (10 or 20 nmol of 2E1/sample) was reconstituted for 45 min on ice with an equimolar amount of reductase and 100 or 200 μg of DLPC. The reconstituted samples received 625 or 1250 units of catalase and 0.5 mM tBITC and were diluted to 1.8 or 3.65 mL with 50 mM potassium phosphate (pH 7.4). The reaction mixtures were incubated for 30 min at 30 °C with or without 1.4 mM NADPH. Enzymatic activity was measured with 7-EFC as described above. Exposed and inactivated samples were concentrated on a Centricon-30 concentrator (Amicon, Beverly, MA) and washed three times with 500 μL of potassium phosphate buffer (pH 7.4) containing 20% glycerol. Samples were diluted to approximately 200−300 μL and transferred to EPR tubes. Some samples also received 1 mM tBITC at this point. The protein samples were frozen with liquid N_2 and stored in liquid N_2 until they were analyzed. EPR measurements were taken as previously described using a Varian Century Line X-band (9 GHz) EPR spectrometer equipped with a cryogenic Dewar system (*31*). The conditions for detection of ferric ions were as follows: microwave power, 20 mW for high-spin ferric and 0.2 mW for low-spin ferric; microwave frequency, 9.17 GHz; modulation frequency, 100 kHz; amplitude, 0.1 mT; and temperature, 25−15 K. The analogue output was recorded digitally on the computer for each spectrum via a data acquisition board obtained from Computer Board Inc. (Mansfield, MA). Theoretical predictions were calculated from the currently accepted model for heme EPR spectra (*32*).

Dithionite Treatment of tBITC-Inactivated P450 2E1. P450 2E1 was reconstituted and incubated with tBITC as described above. The final concentrations for P450 2E1 and tBITC ranged from 0.5 to 5 μM and from 1 to 500 μM, respectively. Inactivations were carried out for 10−15 min at 30 °C. The 7-EFC activity of control samples and tBITC-inactivated samples was measured using the 7-EFC assay as described. Aliquots were removed and diluted with quench buffer, and the reduced CO spectra were recorded. A few crystals of solid dithionite were added to the tBITC-inactivated samples, and the samples were incubated for 1 h at room temperature. Following this treatment, aliquots were again removed for 7-EFC activity and reduced CO spectral analysis.

Effect of Dithionite Pretreatment on the Rate of CO Binding. P450 2E1 (2.5 μM) was reconstituted with reductase (2.5 μM) and lipid (60 μg) and incubated with 100 μM tBITC in the absence or presence of 1.2 mM NADPH for 10 min at 30 °C. The 7-EFC activity of the samples was determined as described above. The control sample (100 μL containing 0.25 nmol of P450) was transferred to 900 μL of cold quench buffer, bubbled gently with CO for 90 s, and reduced with solid dithionite. The reduced carbon monoxide spectrum was recorded between 400 and 500 nm. The inactivated sample (200 μL containing 0.5 nmol of P450) was transferred into 1800 μL of quench buffer. The sample was divided into two portions. One was bubbled gently with CO for 90 s and reduced with solid dithionite, and the spectra were recorded for 60 min. The second inactivated sample was immediately reduced with solid dithionite and allowed to sit for 60 min at room temperature. After the 60 min dithionite preincubation, the sample was gently bubbled with CO for 90 s, and reduced carbon monoxide spectra were recorded for an additional 60 min. A final spectrum was taken for each sample after 2 h.

ESI-LC−MS Analysis of P450 2E1. P450 2E1 was incubated with tBITC or inactivated with tBITC and NADPH for 10 min at 30 °C. Samples contained 1 μM P450 2E1, 1 μM reductase, 80 μg/mL DLPC, 5 μg/mL catalase, and 50 μM tBITC in 50 mM potassium phosphate buffer (pH 7.4). The samples (50 pmol of P450 2E1) were injected onto a 2.1 mm × 150 mm C18 column (5 μm, Vydac Advances) equilibrated with 40% CH_3CN and 0.1% TFA at a flow rate of 0.3 mL/min. After a 10 min wash under the initial conditions, the column flow was diverted into the LCQ mass spectrometer (Thermoquest, Schaumburg, IL), and spectra were recorded. The proteins were resolved by raising the acetonitrile concentration to 90% over the course of 30 min. The column was washed for an additional 30−45 min to wash off all the lipid. The ESI source conditions were optimized using horse myoglobin. Scans were acquired with the sheath gas set at 90 (arbitrary units) and the auxiliary gas set at 30 (arbitrary units). The spray voltage was 4.2 kV, and the capillary temperature was 230 °C. The protein envelopes were deconvoluted using the Thermoquest Excalibur 1.0SR1 Qual Browser to obtain the masses associated with each protein envelope.

RESULTS

Effect of tBITC Inactivation on the P450 2E1 Heme. Previous studies showed that P450 2E1 activity was lost concurrently with a similar loss in the P450 CO spectrum when P450 2E1 was incubated together with tBITC and NADPH (*17*). However, HPLC analysis of tBITC-inactivated P450 2E1 suggested that the majority of the P450 heme was not destroyed or modified (*17*). Since it could not be ruled out that an acid labile heme adduct was removed with the

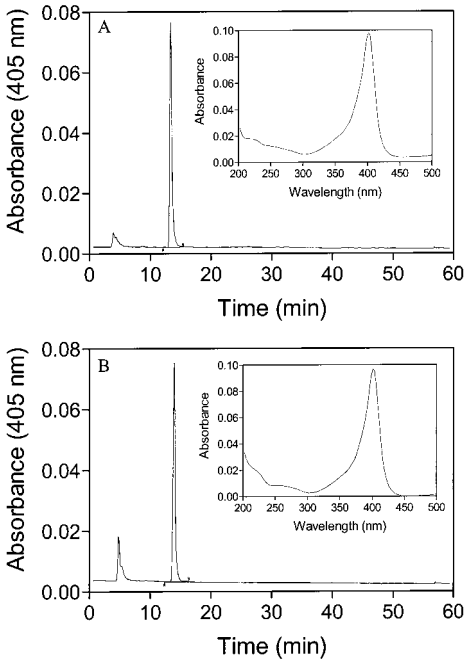

FIGURE 1: HPLC profile and diode array spectrum of the heme moiety of reconstituted P450 2E1 after incubation with tBITC. Incubation conditions and HPLC analysis were as described in Experimental Procedures. (A) Control sample incubated without NADPH. (B) Inactivated sample incubated with NADPH. The insets show the diode array spectra of the heme peak.

Table 1: Effect of tBITC on the Heme Prosthetic Group of P450 2E1[a]

heme assay	control[b]	inactivated[b]	% of control[c]
absolute spectra	1.1 μM[d]	0.9 μM[d]	90 ± 3
pyridine hemochrome	0.38 μM	0.31 μM	85 ± 7
HPLC	1.99×10^6 AU[e]	1.64×10^6 AU	86 ± 4

[a] Assay conditions were as described in Experimental Procedures. [b] The data shown is from a representative experiment. [c] The data shown represents the mean and standard deviation from the combined results of at least three separate experiments. [d] The concentration was determined from the absorbance at 413 nm using an E_m of 72.1 mM^{-1} cm^{-1}(*12*). [e] AU, absorbance units.

acidic conditions that were used during chromatography, P450 2E1 and tBITC-inactivated P450 2E1 that had lost 70% of its 7-EFC activity were chromatographed without acid on a C4 reverse phase column. The heme moiety of both the control and the inactivated sample eluted at the same retention time (14 min) (Figure 1). The insets of Figure 1 show the diode array spectra of the heme peak of the control (Figure 1A) and the inactivated (Figure 1B) P450 2E1. These spectra were virtually identical. The area under the heme peak of the inactivated P450 2E1 was 86 ± 4% as compared to that of the control sample (Table 1). Table 1 also shows that treatment of P450 2E1 with tBITC resulted in the retention of approximately 85−90% of the P450 2E1 Soret peak measured either by absolute visible spectroscopy or as the pyridine hemochrome. Under these conditions, only 31 ± 7% of the CO spectrum was observed compared to control non-inactivated samples (data not shown). Together, these observations indicate that although the inactive enzyme was severely compromised in its ability to form a CO complex, this was not due to significant heme destruction or to a modification of the heme.

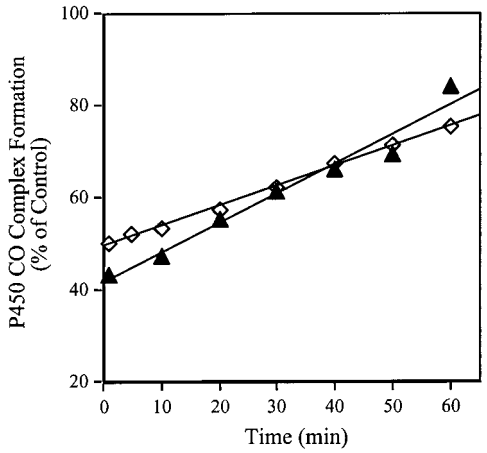

FIGURE 2: Time-dependent recovery of the P450 CO complex. P450 2E1 was inactivated with tBITC and incubated with CO and dithionite as described in Experimental Procedures. Samples were scanned at the indicated times, and the amount of CO complex formed under aerobic (▲) or anaerobic (◇) conditions by the tBITC-inactivated samples was calculated as a percent of the CO complex formed by the non-inactivated sample at 0 min.

Table 2: Recovery of 7-EFC Activity and of the Reduced CO Spectrum of tBITC-Inactivated P450 2E1[a]

sample	% activity remaining	% P450 remaining
with tBITC and without NADPH	100	100
with tBITC and NADPH	24 ± 2	21 ± 2
with tBITC, NADPH, and dithionite (for 1 h)	65 ± 10	62 ± 10

[a] Assay conditions were as described in Experimental Procedures. The data shown represent the means and standard deviations from three to five separate experiments.

CO Complex Recovery with Time. The inability of the tBITC-inactivated P450 2E1 to form a CO complex was reversible. Figure 2 shows that up to 80% of the CO complex was recovered as compared to the non-inactivated enzyme by incubations with dithionite for 60 min. A similar rate of recovery was observed if the tBITC-inactivated P450 2E1 was treated with dithionite under anaerobic conditions [Figure 2 (◇)]. The combined observations from at least 14 separate experiments using different P450 2E1 and tBITC concentrations all showed similar results. Incubations with dithionite and CO of the inactivated sample resulted in an initial 34 ± 13% of CO complex formation within the first minute after addition of dithionite and led to a recovery over the course of 1 h of 65 ± 13% of the CO complex that could be formed compared to control non-inactivated samples measured during the first minute.

Prolonged dithionite treatment of tBITC-inactivated P450 2E1 was also able to partially restore the activity of the enzyme. Samples incubated with tBITC and NADPH had a residual activity of 24 ± 2% as compared to control samples (Table 2). The same tBITC-inactivated sample was able to form 21 ± 2% of the reduced CO complex at the initial scan. When these samples were treated with dithionite for 1 h, both the P450 2E1 activity and the immediate ability of the enzyme to form a CO complex were partially restored to 65 ± 10 and 62 ± 10%, respectively, when compared to the control sample.

Effect of Dithionite Pretreatment on the Rate of CO Binding. The failure to observe a reduced CO spectrum for

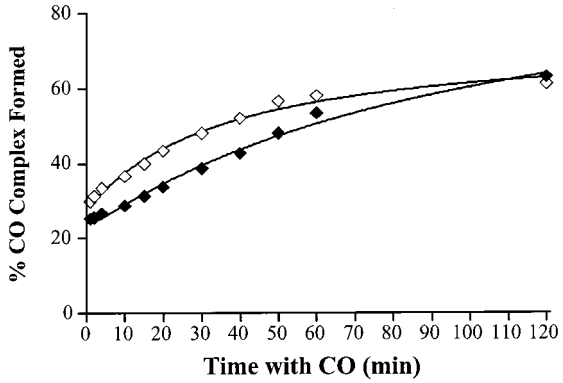

FIGURE 3: Effect of dithionite pretreatment on the rate and extent of CO binding. Reconstituted P450 2E1 was incubated with tBITC and treated with CO and dithionite as described in Experimental Procedures. Samples were scanned at the indicated times, and the amount of CO complex formed by the tBITC-inactivated samples was calculated as a percent of the CO complex formed by the non-inactivated sample at 0 min. (◆) Inactivated sample bubbled with CO and then treated with dithionite for the indicated times. (◇) Inactivated sample pretreated first with dithionite for 1 h and then bubbled with CO and observed for the indicated times.

the tBITC-inactivated P450 2E1 could be brought about by a decreased ability of dithionite to reduce the inactivated P450. This question was addressed by comparing the rate of CO complex recovery of a tBITC-inactivated sample that was first pretreated with dithionite for 1 h to the rate of complex formation of a non-dithionite-pretreated duplicate sample. Incubations of P450 2E1 with 100 μM tBITC in the presence of NADPH resulted in a loss of 92% of the 7-EFC activity compared to the control incubation in the absence of NADPH. One-half of the sample was immediately analyzed for its ability to bind CO. This inactivated sample was unable to form 75% of its reduced CO complex compared to the control sample incubated with tBITC in the absence of NADPH. However, as seen before, prolonged incubations with dithionite in the presence of CO resulted in a gradual recovery of the CO complex of the inactivated sample [Figure 3 (◆)]. The duplicate tBITC-inactivated sample that was first reduced with dithionite for 1 h at room temperature exhibited an initial inability to form 70% of the reduced CO complex. The CO complex of this dithionite-pretreated sample also recovered over the course of 1 h at a rate which was a similar to that of the non-dithionite-pretreated sample [Figure 3 (◇)]. After incubation for 2 h with dithionite, 61−63% of the CO complex was recovered with both samples. In contrast, treatment of the control sample with dithionite and CO for 2 h resulted in the destruction of the P450 2E1 heme, and only 27% of the total complex observed at time zero was still measurable (data not shown). These results suggest that the principle defect in the tBITC-inactivated samples was an inability to bind CO rather than a decreased rate of reduction. Interestingly, in stark contrast to the control sample, the tBITC-inactivated samples not only were able to withstand prolonged incubations with dithionite but even recovered their ability to form a CO complex. The leveling off in the rate of complex formation after 1 h suggested that once the sample was "reactivated" by dithionite it could also undergo destruction of the heme, like the control sample.

Iodosobenzene-Supported Activity. Inactivated P450 2E1 was incubated with iodosobenzene to test if an alternate oxidant was able to restore catalytic activity to the tBITC-treated enzyme. Table 3 shows that only 11% of the 7-ethoxycoumarin activity was regained with iodosobenzene when compared to samples incubated with NADPH.

Table 3: Iodosobenzene-Supported 7-EFC Activity[a]

sample	% NADPH-supported activity	% iodosobenzene-supported activity
control	100	100
inactivated	19 ± 9	30 ± 2

[a] Assay conditions were as described in Experimental Procedures. The data shown represent the average of four samples from two separate experiments.

Table 4: Comparison of P450 2E1 Concentrations Using Reduced CO Spectroscopy and EPR Spectroscopy[a]

sample	[P450] via reduced CO complex (μM)	% of control[b]	[P450] via EPR spectroscopy (μM)	% of control
control (sample 1)	37	100	33	100
inactivated (sample 1)	22	59	25	76
control (sample 2)	28	100	28	100
inactivated (sample 2)	11	39	18	64

[a] Assay conditions were as described in Experimental Procedures. [b] The % of total spectrally detectable tBITC-inactivated P450 2E1 was determined by comparison to the non-inactivated control sample in each group.

EPR Spectral Analysis. EPR spectra were obtained for control or tBITC-inactivated P450 2E1 to determine if the portion of the inactivated P450 2E1 that was unable to form a CO complex was locked in the Fe^{2+} state and unable to transfer the first electron or to bind oxygen. Table 4 shows that significant amounts of heme were no longer detectable in the inactivated samples when the P450 2E1 concentrations were measured either by EPR spectroscopy or by reduced CO spectroscopy after the initial reduction with dithionite. These results demonstrate that a fraction (24−36%) of the inactivated samples was unable to give rise to an EPR signal and was therefore presumably locked in the Fe^{2+} state.

Figure 4 shows the EPR scans of the control sample incubated with tBITC in the absence of NADPH (A) and the EPR scans of the tBITC-inactivated P450 2E1 (B). Table 5 shows the experimental parameters from these EPR spectra of P450 2E1 as well as the theoretically predicted values derived from the currently accepted model for heme spectra (*31*). The choice for the g value signs and the directional assignments for the theoretical values resulted from choosing the largest distortion to octahedral symmetry along the z-axis (*32*). The g value signs are a result of the theory that was used to interpret the data. The determination of the signs is therefore a special property of the EPR specta determined from the theoretical low-spin ferric ions and is not normally available to the spectroscopist because the sign of the g value is usually not an experimentally determined parameter. The control sample (C) gave signals with typical high-spin heme g values ($g_x = 3.5$, $g_y = 7.9$, and $g_z = 1.7$). The high-spin heme signals did not change with the tBITC-inactivated P450 2E1 samples, again giving the same typical signals as those seen in the control sample ($g_x = 3.5$, $g_y = 7.9$, and $g_z = 1.7$). An exact match to the experimental g values was obtained by choosing the parameter values ($g\beta H/D$ and η).

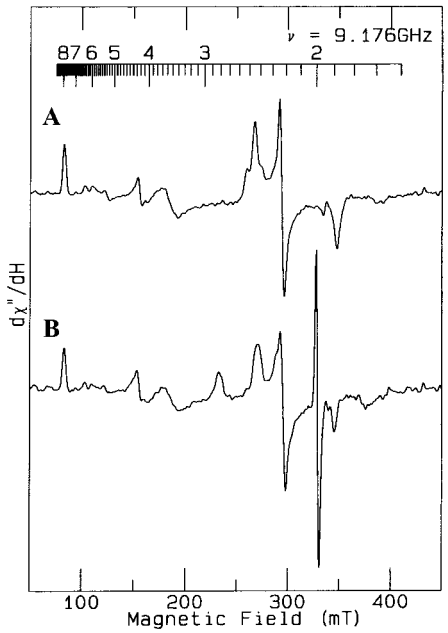

FIGURE 4: EPR spectra of reconstituted P450 2E1. The inactivation and EPR spectral conditions were as described in Experimental Procedures. (A) P450 2E1 incubated with tBITC and without NADPH. (B) P450 2E1 incubated with tBITC and NADPH.

Table 5: Low- and High-Spin *g* Values of Control and tBITC-Inactivated P450 2E1[a]

	low-spin *g* values						distortion	
	experimental *g* values			theoretical *g* values			axial	rhombic
	g_x	g_y	g_z	g_x	g_y	g_z	Δ/λ	ν/λ
C and I[b]	2.218	2.428	1.883	−2.220	2.433	−1.897	3.3	1.7
I only	2.425	2.815	1.735	−2.352	2.727	−1.646	6.2	3.3

	high-spin *g* values							
	experimental *g* values			theoretical *g* values				
	g_x	g_y	g_z	g_x	g_y	g_z	$g\beta H/D$	η
C and I	3.5	7.9	1.7	3.5	7.9	1.7	0.022	0.098

[a] Samples were subjected to EPR spectroscopy and analyzed as described in Experimental Procedures. [b] C, control sample incubated with tBITC in the absence of NADPH; I, tBITC-inactivated sample incubated with tBITC in the presence of NADPH.

These paramaters refer to the Hamiltonian spin equation $H = D[S_z^2 - S(S+1)/3] + E[S_x^2 - S_y^2]$, where $\eta = E/D$. The control P450 2E1 sample also exhibited three *g* values indicative of a low-spin heme iron (C; $g_x = 2.218$, $g_y = 2.428$, and $g_z = 1.883$) (Figure 4 and Table 5). Approximately 45% of the tBITC-inactivated P450 2E1 also gave rise to an unusual low-spin heme (I; $g_x = 2.425$, $g_y = 2.815$, and $g_z = 1.735$) in addition to the same low-spin heme signals that were observed in the control samples (C and I; $g_x = 2.218$, $g_y = 2.428$, and $g_z = 1.883$). The data show that the axial and rhombic distortion to the octahedral crystal field surrounding the heme iron atom (Δ/λ and ν/λ) were twice as large for I as for C. In other studies, an increase in the axial and rhombic distortion was interpreted to be the result of stress on the heme iron sixth ligand H_2O molecule (*32*). Therefore, such an increase in the distorion could be obtained if tBITC binding to the apoprotein resulted in the displacement of a water molecule from the sixth ligand position and direct interaction of the tBITC adduct with the heme iron.

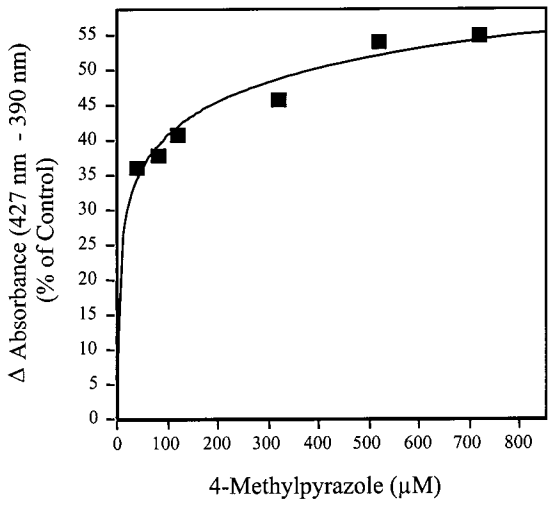

FIGURE 5: Effect of 4-methylpyrazole binding to tBITC-inactivated P450 2E1. P450 2E1 was reconstituted and incubated with tBITC. Spectra were recorded as described in Experimental Procedures.

4-Methylpyrazole Binding Spectra. The tBITC-inactivated P450 2E1 was tested for its ability to bind 4-methylpyrazole (4-MP) when compared to the control sample that had been incubated with tBITC without NADPH. Both samples were dialyzed extensively to remove all free tBITC. As had been previously observed with P450 2E1 isolated from phenobarbital-induced rabbit livers, incubations of 4-MP with the bacterially expressed P450 2E1 control sample resulted in a spectrum with an absorbance maximum at 427 nm and a trough at 390 nm (ref *29* and data not shown). No further increase in the absorbance difference between 427 and 390 nm could be obtained if the 4-MP concentration was increased above 20 μM with the control sample. The same type II binding spectrum was observed with the tBITC-inactivated P450 2E1. In contrast, only 36% of the expected absorbance change between 427 and 390 nm could be obtained with 20 μM 4-MP and the tBITC-inactivated P450 2E1 compared to the non-inactivated sample (Figure 5). Maximally, 55% of the binding spectrum relative to the control, non-inactivated P450 2E1 was obtained when the 4-MP concentration was increased to 720 μM with the tBITC-inactivated P450 2E1. The estimated K_D for 4-MP binding to the tBITC-inactivated P450 2E1 derived from the nonlinearized analysis of the binding data in Figure 5 was 27 μM, which is approximately 36-fold higher than the published K_D for 4-MP and P450 2E1 (*29*).

ESI-LC−MS Analysis of the tBITC-Inactivated P450 2E1. ESI-LC−MS analysis was used to determine if a tBITC adduct was covalently attached to the tBITC-inactivated P450 2E1. P450 2E1 and tBITC-inactivated P450 2E1 were separated from the other components of the reconstituted system by reverse phase chromatography prior to mass analysis. Clipped reductase eluted at 12.7 min; active reductase eluted at 16.2 min, and P450 2E1 eluted at 18.4 min (data not shown). Figure 6 shows the deconvoluted mass spectrum of the inactivated P450 2E1 peak with a mass of 53 922 ± 2 Da. The control sample that was incubated with tBITC [$(CH_3)_3CNCS$, 115 Da] in the absence of NADPH had a mass of 53 803 ± 2 Da (inset of panel B). The mass spectrum of the inactivated protein also contains some unmodified protein with a mass of 53 804 ± 2 Da. The

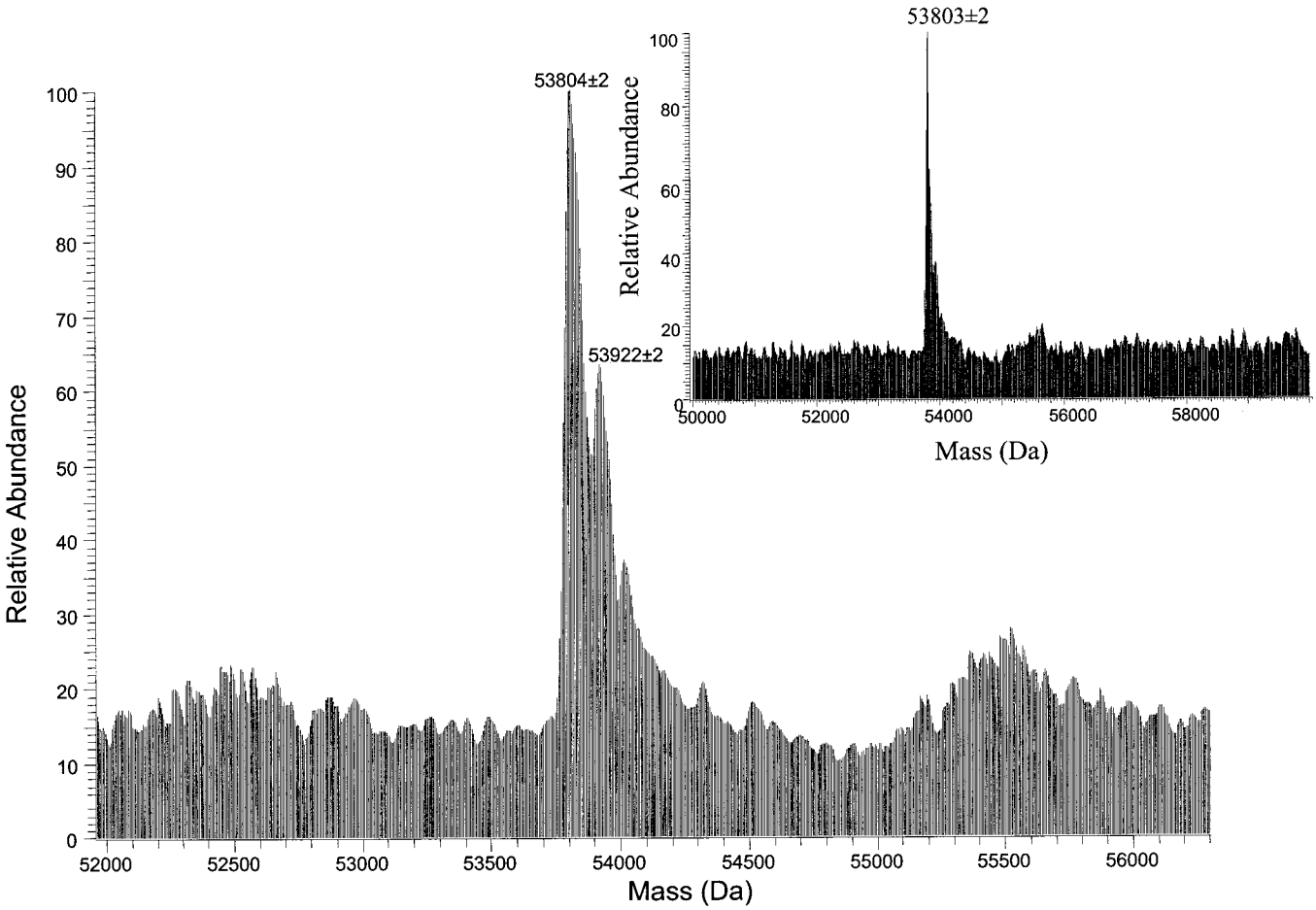

FIGURE 6: ESI-LC−MS analysis of P450 2E1 inactivated with tBITC. Samples were incubated with tBITC in the presence or absence of NADPH and analyzed as described in Experimental Procedures. Deconvoluted spectrum of the inactivated apo-P450 2E1 (M_r = 53 922 ± 2 Da, n = 6). The inset shows a deconvoluted spectrum of apo-P450 2E1 from a control incubation (M_r = 53 803 ± 2 Da, n = 6).

difference in mass (118 Da) between the control and tBITC-inactivated P450 2E1 was similar to the mass of a tBITC molecule (115 Da). These observations indicate that the entire tBITC molecule was covalently attached to the P450 apoprotein presumably through a disulfide bond to one of the four Cys residues of P450 2E1. Alternatively, rearrangement and loss of the isothiocyanate sulfur to a *tert*-butyl isocyanate [$(CH_3)_3CNCO$, 99 Da] with the addition of an additional oxygen (16 Da) could also give rise to a similarly sized adduct.

DISCUSSION

In a previous report, tBITC was shown to be an effective mechanism-based inactivator for P450 2E1 (*17*). The loss in catalytic activity was accompanied by a similar loss in the P450 carbon monoxide spectrum. A loss in the P450 CO spectrum is generally taken as an indication of heme modification or heme destruction (*33*). The inability of the majority of the P450 2E1 to bind carbon monoxide was, however, not due to heme alkylation or destruction. Alternate methods such as absolute spectra, pyridine hemochrome spectra, and HPLC separation methods that measured the recovery of heme indicated that approximately 84% of the heme from the tBITC-inactivated P450 2E1 samples was still intact.

Interestingly, we also observed an up to 60−80% recovery in the CO spectrum with prolonged (1−2 h) incubations of inactivated samples in the presence of dithionite and CO. This recovery was striking since control samples were not stable to the same treatment of dithionite and CO. After a 2 h incubation, only 27% of the initial CO complex could be observed (data not shown). This loss of the CO complex in the control samples has been observed previously and was attributed to the formation of hydrogen peroxide and other radicals that in turn destroy the heme (*33*). The rate of recovery of the CO complex from the tBITC-inactivated samples started to level off after 1 h, but the extent of CO complex formation after 2 h was still considerably higher (60%) compared to samples that were not inactivated (27%). Dithionite treatment presumably resulted in the removal of a tBITC blocking group, and as the CO complex was recovered, the fully functional P450 2E1 heme could then undergo radical destruction similar to that observed in the control sample. This is probably the reason we observed some variability in the extent of CO complex recovery and why none of the samples exhibited 100% recovery of the CO complex after dithionite treatment. The rate and extent of CO complex development were similar under aerobic or anaerobic conditions, suggesting that oxygen did not interfere with the formation of the CO spectrum in the inactivated samples.

Treatment of the tBITC-inactivated P450 2E1 with dithionite for 1 h also resulted in the recovery of a significant amount of catalytically active enzyme (65%). This recovery

could only be observed in tBITC-inactivated samples since, as mentioned above, the control P450 2E1 was not stable to a 1 h treatment with dithionite. Therefore, the tBITC modification also appeared to result in a protective effect by decreasing the level of production of oxygen radicals of P450 2E1. These observations again suggest that incubations with dithionite resulted in the release of a blocking group that prevented the oxygen or substrate from binding in the appropriate orientation at the P450 active site. The recovery of both activity and CO spectrum with strong reducing agents could suggest that a tBITC reactive intermediate may have reacted with a critical sulfhydryl group on the apoprotein of P450 2E1 (*34*). The reactivity of isothiocyanates is similar to those of other well-known SH modifiers such as iodoacetamide, *n*-ethylmaleamide, and DTNB (*34*). Isothiocyanates have been shown to react at neutral pH with sulfhydryl groups in preference (approximately 2000-fold) to amino or hydroxyl groups (*34*). In the case of alcohol dehydrogenase, a loss in enzymatic activity after incubation with isothiocyanates was observed and the activity of alcohol dehydrogenase could be restored following incubations of the inactivated enzyme with 20 mM β-mercaptoethanol or DTT. In the case of P450 2E1, enzymatic turnover was essential for tBITC modification of P450 2E1 as evident from the absolute requirement of NADPH to obtain a loss in activity. No enzymatic or spectral recovery was observed when the tBITC-inactivated P450 2E1 was dialyzed against buffer containing 0.5 mM DTT or 0.5 mM β-mercaptoethanol (data not shown). However, enzymatic activity could be recovered, although to a lesser extent than with dithionite, when tBITC-inactivated samples were incubated with 6 mM β-mercaptoethanol at room temperature for 2 h (data not shown).

Enzymatic activity could not be restored when iodosobenzene was used as an alternate oxidant, suggesting that one or more steps following reduction of P450 2E1 were not blocked. EPR spectral analyses indicated that the inactivated P450 2E1 samples contained an EPR silent Fe^{2+} population. The same proportion of P450 2E1 that was unable to elicit a CO spectrum also was unable to give rise to an EPR signal. Therefore, in the catalytic cycle of P450 2E1, either the first electron could not be transferred or CO could not bind. In addition, a significant fraction of the tBITC-inactivated sample gave rise to an EPR spectrum with an unusual low-spin heme signal. The differences in the low-spin heme signal between the control and tBITC-inactivated P450 2E1 samples suggest that inactivation by tBITC caused changes in the exogenous neighbors to the sixth ligand to the heme iron, namely, a water molecule. These changes are such that the water molecule is released to lower the strain on the heme iron crystal field. One possibility is that a hydrogen bonding network to the sixth ligand is sterically hindered by the *tert*-butyl group of the tBITC adduct. Inactivation of P450 2E1 samples with tBITC also resulted in the disappearance of the charge transfer band at 650 nm (data not shown). This observation has been inferred to be a result of the binding of a strongly interacting ligand that acts as a π electron acceptor such as CN^- or N_3 (*35*).

These combined spectral and activity observations raised the question of whether the defect in the tBITC-inactivated samples was due to a decrease in the rate of reduction or an inability of CO to bind. The loss in activity did not appear to be the result of a decreased ability of P450 2E1 to become reduced. Samples incubated first for 1 h with dithionite before addition of CO showed a similar rate and extent of complex recovery as samples that were not preincubated with dithionite. If reduction had been affected, the 1 h dithionite pretreatment should have resulted in an initial extent of CO complex formation of approximately 60%, similar to the extent of complex formation of the non-pretreated sample. Further, addition of methyl viologen in combination with dithionite did not result in faster formation of a CO complex (data not shown).

In a previous study, 3-amino-1,2,4-triazole was shown to inactivate P450 2E1 (*29*). The 3-amino-1,2,4-triazole-inactivated enzyme exhibited a reduced ability to bind CO and an inability to bind 4-MP, although the pyridine hemochrome was unchanged. Binding spectra with 4-MP, a tight binding P450 2E1 substrate, showed that only 55% of the tBITC-inactivated P450 2E1 was able to bind 4-MP. In addition, the estimated K_D for 4-MP of the tBITC-inactivated sample was approximately 27 μM, a 36-fold increase compared to that of the non-inactivated P450 2E1 (*29*). The 4-MP binding curve obtained for the tBITC-inactivated P450 2E1 suggested that the inactivated sample was heterogeneous, containing inactivated and non-inactivated P450 2E1. This was also apparent from the activity, the CO recovery, and the EPR studies. Residual activity in the inactivated sample could indicate either that some proportion of the non-inactivated P450 2E1 was still present or that all the molecules were affected but only "wounded". The CO recovery studies also indicated that a certain proportion of the P450 2E1 in the inactivated sample was immediately able to form a CO complex. The EPR studies further suggested that the inactivated sample consisted of three populations: a non-inactivated fraction, a fraction that was EPR silent, and a fraction that was able to elicit an EPR signal but was unable to form a CO complex. The inactivation by 3-amino-1,2,4-triazole was thought to be due to a modification of the P450 2E1 apoprotein at an amino acid residue near the heme, although no covalent radiolabel could be observed with this compound. With the tBITC-inactivated P450 2E1, a protein modification was observed. ESI-LC−MS analysis of the tBITC-inactivated P450 2E1 samples indicated that the loss in enzymatic activity was accompanied by the formation of a protein adduct. The increase in mass of the adducted P450 2E1 was consistent with the binding of one molecule of tBITC, presumably through a disulfide with a critical cysteine residue. Such a modification would be expected to be reversed by reducing agents such as dithionite. Alternatively, a mass difference of 118 Da could also be obtained if a loss of the isothiocyanate sulfur and rearrangement to the isocyanate had occurred followed by the addition of an additional oxygen. Such an adduct would not be expected to be cleaved by reducing agents. This possibility in addition to the ones mentioned above may also account for the $<$100% recovery of the CO spectrum or the activity of the dithionite-treated samples. Studies are underway to characterize the metabolites of tBITC. Similar studies were carried out with benzyl isothiocyanate and P450 2E1 where the observed metabolites were consistent with two different routes of metabolism (*36*). The results of such metabolite studies in addition to the isolation of the tBITC-modified peptide(s) and identification of the modified amino acid(s) should allow us to identify the reactive intermediate(s).

The spectral and activity studies presented in this report indicate that the mechanism-based inactivation of P450 2E1 by tBITC is the result of the binding of a tBITC reactive intermediate to the P450 apoprotein. The tBITC adduct then presumably interferes with substrate or oxygen binding by blocking key active site residues. Studies are currently underway to identify these key amino acid residues. These results also show for the first time that a loss in the CO spectrum and activity can be partially reversed in certain instances by incubation of the inactivated P450 with reducing agents.

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