Mechanism-Based Inactivation of Cytochrome P450 2B1 by 8-Methoxypsoralen and Several Other Furanocoumarins†

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ABSTRACT: Of several furanocoumarins [5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), 5-hydroxypsoralen (5-OH-P), 8-hydroxypsoralen (8-OH-P), 4′,5′-dihydro-8-MOP (DH-8-MOP), and psoralen (P)] tested as mechanism-based inactivators (MBIs) of purified reconstituted cytochrome P450 (P450) 2B1, 8-MOP was found to be the most potent (K_I, k_{inact}, and partition ratio of 2.9 μM, 0.34 min^{-1}, and 1.3, respectively). The inactivation was not prevented by reactive oxygen species scavengers or nucleophilic trapping agents and proceeded with a decrease in P450 spectral content. Liquid chromatography (LC) separation of the reconstituted enzyme mixture, followed by liquid scintillation counting, indicated that [^{14}C]-8-MOP binding was specific to the apoprotein of P450 2B1 with a binding stoichiometry of 0.7:1. The major metabolites formed by P450 2B1 from the furanocoumarins that were MBIs were characterized by LC electrospray ionization tandem mass spectrometry (ESI-MS/MS) as dihydro diols. Results from $H_2{}^{18}O$ incorporation experiments supported initial oxidation of 8-MOP and P to an epoxide which can react with some nucleophilic active site residue and inactivate the enzyme or partition to a dihydro diol metabolite by hydrolytic ring opening. On the other hand, 5-MOP was converted to an epoxide or γ-keto enal intermediate prior to inactivation or dihydro diol formation. Comparison of the ESI mass spectra of P450 2B1 and furanocoumarin exposed P450 2B1, indicated a mass difference consistent with the covalent addition of a furanoepoxide to P450 2B1.

In the absence of an X-ray crystal structure for any mammalian cytochrome P450 (P450),[1] indirect methods offer the only means for obtaining structural information on the active sites of these hemoproteins (*1*). Homology modeling of mammalian P450s based on the available crystal structures of prokaryotic P450s appears to be a powerful approach, particularly when coupled with COMFA modeling (*2*). However, methods by which active site residues can be identified and confirmed experimentally will be required in order to validate computer-derived models. One such method involves the use of mechanism-based ("suicide") inactivators (MBIs). Mechanism-based inactivation of P450s is known to proceed via three pathways: (a) alkyation of the heme, (b) covalent modification of the apoprotein, and (c) MBI induced heme fragmentation to products which alkylate the apoprotein (*3*). Since this metabolic event takes place at the active site, it is probable that the amino acid residue that will be covalently modified is the one that is the most susceptible and the closest to the inhibitor as it undergoes activation.

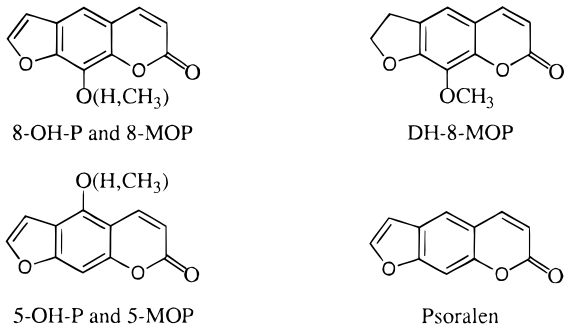

FIGURE 1: Chemical structures of the furanocoumarins tested as potential MBIs of P450 2B1.

Furanocoumarins are present in many foodstuffs such as celery, parsley, figs, parsnips (*4*), and grapefruit juice (*5, 6*) and have been found to inhibit the metabolism of xenobiotics in vivo (*7*−*12*) and P450-mediated activities in vitro (*5, 6, 13*−*23*). The most widely studied furanocoumarin, 8-methoxypsoralen (8-MOP) (Figure 1), has been found to be a potent MBI of human liver microsomal P450 2A6 (*15*). The reactive intermediate that is the likely cause of inactivation was shown to be a furanoepoxide (*24*) by liquid chroma-

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[1] Abbreviations: ACN, acetonitrile; b_5, human cytochrome b_5; BSA, bovine serum albumin; CID, collisionally induced dissociation; CuOOH, cumene hydroperoxide; DH-8-MOP, 4′,5′-dihydro-8-methoxypsoralen; DLPC, L-α-dilauroylphosphatidylcholine; EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; 8-MOP, 8-methoxypsoralen; 8-OH-P, 8-hydroxypsoralen; 5,8-diOH-P, 5,8-dihydroxypsoralen; 5-MOP, 5-methoxypsoralen; 5-OH-P, 5-hydroxypsoralen; 4′-OH-DH-8-MOP, 4′-hydroxy-4′,5′-dihydro-8-methoxypsoralen; GSH, reduced glutathione; HFC, 4-(trifluoromethyl)-7-hydroxycoumarin; LC/ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; MBI, mechanism-based inactivator; MeOH, methanol; MM, molecular mass; MOA, methoxylamine; NAC, *N*-acetylcysteine; NaCN, sodium cyanide; P, psoralen; PEG, poly(ethylene glycol); P450, cytochrome P450; P450 reductase, recombinant rat NADPH cytochrome P450 oxidoreductase; SCA, semicarbazide hydrochloride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, superoxide dismutase.

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tography electrospray ionization tandem mass spectrometric (LC/ESI-MS/MS) and UV characterization of the metabolites.

8-MOP is also known to inactivate (*19*−*23*) rat liver microsomal P450s, and the major metabolites formed from 8-MOP result from initial oxidation at the furan ring (*16*, *25*). Since pretreatment of rats with phenobarbital results in an increase in the amount of liver microsomal P450 activity inactivated (*20*−*22*) and the amount of radiolabeled 8-MOP covalently bound to microsomal protein (*21*, *23*), it seemed likely that P450 2B1 would be susceptible to 8-MOP (and other furanocoumarin) mediated inactivation. Thus, the purpose of the present study was (a) to determine if 8-MOP and other furanocoumarins could act as MBIs of P450 2B1 and (b), if they could, to characterize the inactivation in terms of mechanism and type(s) of covalent adduction.

MATERIALS AND METHODS

Materials. 8-MOP, 5-methoxypsoralen (5-MOP), psoralen (P), L-α-dilaurylphosphatidylcholine (DLPC), catalase, reduced glutathione (GSH), *N*-acetylcysteine (NAC), bovine serum albumin (BSA), superoxide dismutase (SOD), and NADPH were purchased from Sigma (St. Louis, MO). 5-Hydroxypsoralen (5-OH-P) was purchased from Indofine (Somerville, NJ), and 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) and 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) were purchased from Molecular Probes (Eugene, OR). Sodium cyanide (NaCN), H_2O_2, semicarbazide hydrochloride (SCA), and methoxylamine hydrochloride (MOA) were purchased from Aldrich (Milwaukee, WI). [^{14}C]-8-MOP (50 Ci/mol) was synthesized from 8-OH-P, and [^{14}C]-CH_3I, as previously described (*24*), and the specific activity of the preparation was confirmed by HPLC-UV (Hewlett-Packard Series 1050, Avondale, PA) analysis using a standard curve comprised of known concentrations of unlabeled 8-MOP and a liquid scintillation counter (Packard 2200CA Tricarb liquid scintillation counter, Downers Grove, IL). Cumene hydroperoxide (CuOOH) was from Pfaltz and Bauer (Waterbury, CT). Centricon 30 concentrators were from Amicon (Bedford, MA), and Cytoscint ES liquid scintillation cocktail and hyamine hydroxide were from ICN (Costa Mesa, CA). $H_2{}^{18}O$ was from Cambridge (Andover, MA). HPLC solvents were of the highest grade commercially available and were used as received. All other reagents were analytical grade.

Enzymes. Adult male specific pathogen-free Sprague−Dawley rats (225−250 g each, Charles River Laboratories) were induced with 0.1% sodium phenobarbital in their drinking water for 14 days, and a liver microsomal fraction was prepared by standard methods (*26*). P450 2B1 was purified from rat liver microsomes according to published procedures (*27*), except that an octyl sepharose CL-4B chromatography step was substituted for the initial poly(ethylene glycol) (PEG) 6000 fractionation step. The purified P450 2B1 was judged to be >95% homogeneous by sodium dodecyl sufate−polyacrylamide gel electrophoresis (SDS−PAGE). Recombinant rat NADPH cytochrome P450 oxidoreductase (P450 reductase) was purified according to published procedures (*28*), with minor modifications. Human cytochrome b_5 (b_5) was expressed and purified from bacterial cultures according to previously published procedures (*29*). Experimental data are presented as the average of duplicate determinations which did not vary by greater than 10%. Each experiment was repeated at least twice, and consistent results were obtained.

Purified Reconstituted P450 2B1 Inactivation Assays. P450 2B1, P450 reductase, and b_5 were combined in that order in a 1:2:1 molar ratio and dialyzed against 100 volumes of potassium phosphate buffer (50 mM, pH 7.4) for at least 3 h at 4 °C. DLPC was added (50 μg/nmol P450), and this mixture was allowed to reconstitute on ice for at least 1 h. The inactivation assays contained the reconstituted enzyme mixture (50 pmol P450) and catalase (200 U) in potassium phosphate buffer (50 mM, pH 7.4). Preliminary experiments were performed with various furnaocoumarin concentrations and exposure times to determine appropriate values for obtaining reliable kinetic inhibition constants. Different concentrations of each furanocoumarin were used in order to determine inactivation kinetics: 8-MOP (0−10 μM), 5-MOP (0−100 μM), P (0−100 μM), DH-8-MOP (0−100 μM), 5-OH-P (0−100 μM), and 8-OH-P (0−200 μM). All furanocoumarins were added as methanolic solutions but in a manner so that the final concentration of MeOH in each incubation was always less than 1%. The exposure times were for 0−9 min for each furanocoumarin except 8-MOP, which was incubated with P450 2B1 and NADPH for 0−4.5 min. At various time points after initiation of the inactivation reaction with NADPH (100 μM) (final incubation volume, 500 μL), a small aliquot (10%) was transferred to a tube containing EFC (100 μM), BSA (40 μg), and NADPH (100 μM) in potassium phosphate buffer (50 mM, pH 7.4) (final incubation volume, 1 mL). In some instances, additional P450 reductase (7-fold greater than the amount of P450 transferred) was added to the activity assay, and in others, H_2O_2 or CuOOH (250 μM) was substituted for NADPH. After 15 min, the reaction was quenched with 6 N $HClO_4$ (50 μL) and set on ice. With the addition of exogenous reductase to the activity assay, the EFC O-deethylase activity was approximately 1.25-fold greater than when it was absent. Substitution of CuOOH or H_2O_2 for NADPH in the activity assay resulted in the formation of 30 and 100% of HFC, respectively, relative to the NADPH control.

Insoluble material was pelleted by centrifugation for 10 min at 2500 rpm (HNS II Centrifuge, International Equipment Co., Needham HTS, MA), and an aliquot (50 μL) of the supernatant was injected onto an HPLC equipped with a reverse-phase C8 column (Econosphere 5 μm, 150 mm × 4.6 mm). Fluorescence detection (Hewlett-Packard 1046A programmable fluorescence detector, Avondale, PA) coupled to the HPLC was used to measure 7-HFC formation: excitation, 338 nm; emission, 463 nm. Buffer A (2.5% glacial acetic acid), buffer B (ACN), and a gradient elution of 39−60% buffer B from 0 to 7 min was used to separate 7-HFC and 7-EFC, with retention times 3.2 and 6.3 min, respectively. For DH-8-MOP experiments, a gradient elution of 20−60% buffer B over 0−8 min was used to separate the 7-HFC, DH-8-MOP, and 7-EFC, with retention times 5.7, 6.3, and 7.5 min, respectively. Experiments involving nucleophilic trapping agents and reactive oxygen species scavengers were carried out in exactly the same manner but with only two time points (0 and 5 min).

A standard curve composed of four concentrations of HFC that bracketed both the minimum and maximum amounts of metabolite formed indicated the method had a detection limit

of less than 1 pmol. The slopes obtained from the natural log percent remaining activity versus time plots were replotted as 1/slope (i.e. 1/rate) versus 1/(furanocoumarin concentration). K_I and k_{inact} were determined by nonlinear regression analysis of the rate data using the statistical package SYSTAT 5.0 (*30*) and the mechanism-based inactivation equation (*31*). The reconstituted P450 2B1 EFC deethylase activity was 0.81, 0.56, 0.48, and 0.43 nmol/nmol P450/min in the presence of 100, 250, 500, and 1000 μM NADPH, respectively, and no detectable product was formed if an NADPH-generating system was used. The activity of the reconstituted 2B1 mixture did not decrease upon storage for up to 24 h at 4 °C.

Effect of 8-MOP on P450 2B1 Spectral Content. P450 2B1 (407 pmol) was reconstituted as described above and then preincubated with catalase (200 U) at 30 °C for 3 min in the absence or presence of 8-MOP (25 μM) and in the absence or presence of NADPH (100 μM) in potassium phosphate buffer (50 mM, pH 7.4) (final incubation volume, 550 μL). At 0, 2, 5, 10 and 20 min, aliquots (100 μL) were removed and diluted 1:6 into a chilled potassium phosphate buffer (100 mM, pH 7.4) solution containing EDTA (0.1 mM), DTT (0.1 mM), sodium cholate (1% w/v), and glycerol (20% v/v) and set on ice. These mixtures were assayed for cytochrome P450 content (Hewlett-Packard 8451A diode array spectrophotometer, Avondale, PA) using the method of Omura and Sato (*32*).

Partition Ratio Determinations and Furanocoumarin Metabolite Analysis. A partition ratio is one measure of the efficiency of a MBI and is defined as the number of all metabolic events that the inhibitor undergoes divided by the number of inactivating events (*31*). P450 2B1 (200 pmol) was reconstituted and preincubated as described above with various amounts of furanocoumarin: 8-MOP (5 nmol), 5-MOP (10 nmol), and DH-8-MOP (5 nmol) in the absence or presence of exogenous GSH, NAC, and MOA in potassium phosphate buffer (50 mM, pH 7.4) (final incubation volume, 100 μL). Preliminary experiments were performed to determine appropriate amounts of P450 and furanocoumarin to use in order to avoid substrate depletion. The reaction was initiated by NADPH (100 μM), allowed to proceed for 45 min, terminated by the addition of $HClO_4$ (0.2 N final concentration, final volume, 200 μL), and set on ice. After centrifugation for 10 min at 2500 rpm, an aliquot (50 μL) was injected onto an HPLC (Shimadzu LC10AD, Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with the reverse-phase C8 HPLC column described above and operated in tandem with a mass spectrometer fitted with an ESI source. The solvent system consisted of buffer A (2.5% glacial acetic acid) and buffer B (ACN), the flow rate was 1 mL/min with 5% (50 μL) of the flow directed to the ion source using a splitter, and variable wavelength detection was at 320 nm (Shimadzu SPD-10AV module UV−vis spectrophotometer, Shimadzu Scientific Instruments, Inc., Columbia, MD). An HPLC gradient of 0−45% buffer B from 0 to 20 min was used to separate metabolites, conjugates, and parent compound. A partition ratio for the furanocoumarin mediated inactivation of P450 2B1 was calculated as the ratio of the amount of furanocoumarin consumed (relative to minus NADPH control) to the amount of spectrally detectable P450 2B1 present in the incubation. Since significant degradation did occur if the furanocoumarins were stored at room temperature as MeOH solutions for a few days, only freshly prepared solutions were used. LC/ESI-MS/MS instrumentation for the analysis of the furanocoumarin metabolites has been previously described (*24*).

$H_2{}^{18}O$ Experiments. Purified P450 2B1 (250 pmol) was exposed to 8-MOP, 5-MOP, or P (100 μM) as described for the partition ratio experiments in a total volume (210 μL) that was 33% $H_2{}^{18}O$. The incubations were carried out at 30 °C for 45 min and terminated by the addition of $HClO_4$ (0.2 N final concentration) (final volume, 240 μL). An aliquot (75 μL) was injected onto the LC/ESI-MS system described above and analyzed by selected ion monitoring (multiple ion detection): $m/z = 250.1-260.1$ for 5-MOP and 8-MOP dihydrodiols and $m/z = 220.1-230.1$ for the P dihydrodiol. The percent ^{18}O incorporated into each dihydrodiol product from $H_2{}^{18}O$ was calculated by correcting for the theoretical isotopic abundance (^{18}O, $^{13}C + {}^{2}H$, etc.) occurring at a mass of M + 2 and the percent $H_2{}^{18}O$ present in the incubation.

Determination of Binding Stoichiometry. P450 2B1 (100 pmol) was reconstituted as described above and preincubated with catalase (400 U) and [^{14}C]-8-MOP (4 nmol) in the presence or absence of GSH or MOA (2 mM) in potassium phosphate buffer (50 mM, pH 7.4) for 3 min at 30 °C. The reaction was initiated by the addition of NADPH (100 μM) and allowed to proceed for 30 min (final incubation volume, 1 μL per pmol P450 2B1). Incubations in which potassium phosphate buffer (50 mM, pH 7.4) alone was the final additive served as control. This solution was concentrated and washed at least 10-times with $\approx$ 2.5 mL potassium phosphate buffer (50 mM, pH 7.4) containing glycerol (20%) and EDTA (1 mM) using a Centricon 30 concentrator. An aliquot of the protein mixture was injected onto an HPLC equipped with a POROS R1 perfusive particle column (4.6 $\times$ 100 mm) from Perseptive Biosystems (Cambridge, MA) operating at a flow rate of 3 mL/min. The solvent gradient consisted of buffer A (0.05% CF_3CO_2H) and buffer B (0.05% CF_3CO_2H in 95:5 ACN/H_2O). The components of the reconstituted enzyme mixture were separated using a stepwise gradient elution of 20%-50% buffer B from 0 to 5 min, hold at 50% buffer B from 5 to 6.5 min, and 50−85% buffer B from 6.5 to 8 min. Under these conditions, 8-MOP dihydrodiol eluted at 0.5 min, 8-MOP eluted at 2.1 min, heme eluted at 3.3 min, catalase eluted at 4.4 min, b_5 eluted at 5.0 min, P450 reductase eluted at 5.5 min, and P450 2B1 eluted at 8 min. Fractions (0.5 min = 1.5 mL) were collected into glass scintillation vials (20 mL) to which Cytoscint ES liquid scintillation cocktail (2.5 mL) was then added, and the radioactivity of each fraction was measured using a liquid scintillation counter. The total amount of P450 2B1 injected was determined using a standard curve made up of unmodified P450 2B1.

Protein Analysis by LC/ESI-MS. P450 2B1 (1 nmol) was reconstituted with 8-MOP (100 nmol) as described above. After incubation, the reconstituted protein mixture was injected onto the POROS HPLC column and $\approx$2% ($\approx$50 μL/min) of the flow (3 mL/min) was diverted to the ESI mass spectrometer. The components of the mixture were separated as described above. Data acquisition was carried out from m/z 200−2000 Da in 4.5 s, and the scans across the HPLC peak were summed to give an ESI mass spectrum that was

subsequently deconvoluted using the MaxEnt computer program. These experiments were performed on at least three different days and were found to be highly reproducible. The intraday variability in the determination of the molecular masses (MMs) of intact P450s and P450 reductase was found to range from ±0.002 to 0.035% while the standard deviation for the experimentally determined MM, calculated on the basis of the charge state distribution, was found to be less than ±0.09% using this method.[2]

Covalent Binding of [^{14}C]-8-MOP to Protein Determined by SDS−PAGE. P450 2B1 (30 pmol) was reconstituted as described above and preincubated in the absence or presence of catalase (100 U) and in the absence or presence of [^{14}C]-8-MOP (2 nmol) for 3 min at 30 °C in potassium phosphate buffer (50 mM, pH 7.4) (final incubation volume, 25 μL). The reaction was initiated by the addition of NADPH (100 μM), CuOOH (500 μM), or H_2O_2 (500 μM), allowed to proceed for 30 min, and terminated by the addition of SDS−PAGE sample buffer (10 μL). Incubations in which potassium phosphate buffer (50 mM, pH 7.4) served as the final additive were used as controls. The sample was boiled for 3 min, and the proteins were separated by 9% SDS−PAGE and visualized by Coomassie blue staining. The destained gels were sliced into sections (5 mm) that were extracted into hyamine hydroxide (75%) by incubating them in glass scintillation vials (20 mL) for 3 h at 60 °C. After cooling of the samples to room temperature, Cytoscint ES liquid scintillation fluid (10 mL) was added to the samples. These mixtures were allowed to equilibrate in the dark for at least 24 h prior to liquid scintillation counting.

RESULTS

Inhibition of P450 2B1 Activity by Furanocoumarins. Reconstituted P450 2B1 was exposed to several concentrations of furanocoumarins. The furanocoumarins were selected on the basis of their potential for providing mechanistic information about the P450 2B1 inactivation process. At various time periods, a small aliquot of this inactivation reaction was transferred to a tube containing EFC to assay for residual activity. 8-MOP was characterized as the most potent MBI of P450 2B1 of all the furanocoumarin analogues tested based on its K_I, k_{inact}, and partition ratio of 2.9 μM, 0.34 min^{-1}, and 1.3, respectively (Figure 2a,b). The relatively insignificant amount of inhibition observed at 0 min of the inactivation assay indicated that the concentration of EFC present in the activity assay was sufficient to minimize competitive inhibition by the 8-MOP transferred to the tube. Addition of various nucleophilic trapping agents or reactive oxygen species scavengers to the inactivation assay failed to prevent inactivation (Figure 3). Nor could activity be restored by the presence of exogenous reductase, CuOOH, or H_2O_2 in the activity assay, although these additives could support EFC O-deethylation. P450 2B1 inactivation by 8-MOP was accompanied by a loss in the characteristic 450 nm chromophore of the reduced CO bound form of the enzyme (Figure 4). At 5 min, when approximately 80% of the EFC O-deethylase activity was destroyed, 50% of the CO binding capacity had been lost.

5-MOP and P also caused time-dependent inhibition of P450 2B1 activity. After a 9 min exposure to 100 μM 5-MOP or psoralen 67% and 31% of P450 2B1 activity was lost, respectively, whereas a 4.5 min exposure to 10 μM 8-MOP resulted in an 80% loss of activity. The partition ratios calculated for 5-MOP and P (14.9 and 14.5, respectively) were approximately 10-fold greater than that determined for 8-MOP. DH-8-MOP was also able to mediate P450 2B1 inactivation and was found to have a K_I, k_{inact}, and partition ratio of 32.9 μM, 0.13 min^{-1}, and 5.2, respectively. In contrast, 5-OH-P and 8-OH-P did not result in time-dependent inhibition of P450 2B1 activity at concentrations up to 100 μM and exposure times of 9 min.

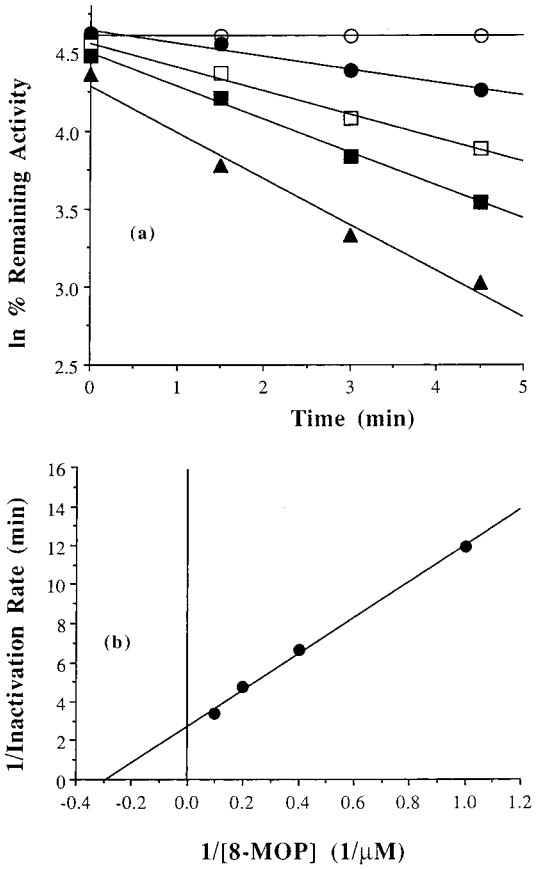

FIGURE 2: (a) 8-MOP-mediated inactivation of purified reconstituted P450 2B1 in the presence of NADPH. The concentrations of 8-MOP present in the inactivation assay were 0 μM (○), 1 μM (●), 2.5 μM (□), 5 μM (■), and 10 μM (▲). (b) Double-reciprocal plot of the relationship between inactivation rate and 8-MOP concentration. The reversible binding constant (K_I) and the rate constant for inactivation (k_{inact}) associated with P450 2B1 and 8-MOP were calculated (*30, 31*) using nonlinear regression to be 2.9 μM and 0.34 min^{-1}, respectively. The rate of turnover for the uninhibited reaction was 0.80 nmol/nmol P450/min. The reported kinetic values are the average of duplicate determinations which did not vary by greater than 10%. Duplicate experiments were performed and consistent results were obtained.

LC/ESI-MS/MS Analysis of the Furanocoumarin Metabolites Generated by P450 2B1. Exposure of P450 2B1 to 5-MOP, 8-MOP, and P resulted in the formation of a single major metabolite from each inhibitor. The metabolite possessed an $[M + H]^+$ ion indicating the addition of two oxygen and two hydrogen atoms. Formation of a monohydroxylated or demethylated product from these furanocoumarins was not detected. These results are consistent with earlier findings using P450 2A6 (*24*) and suggested that the structures of the metabolites of the MBIs are dihydro diols.

[2] Koenigs, L. L., and Trager, W. F., unpublished observations.

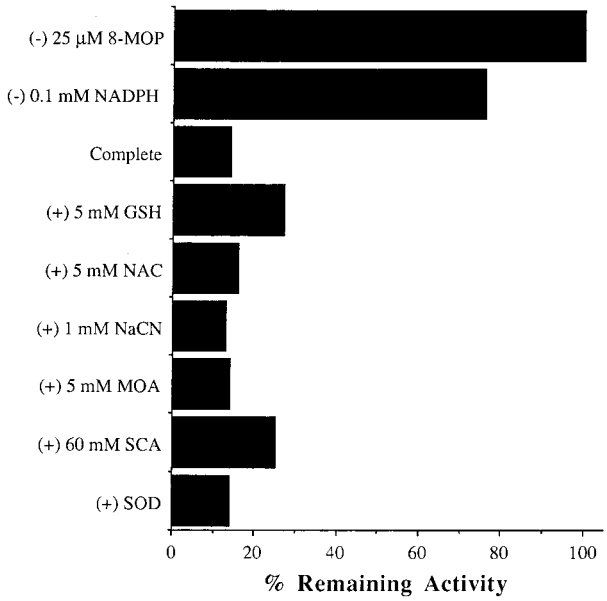

FIGURE 3: Effect of NADPH, various nucleophilic trapping agents, and reactive oxygen species scavengers on the 8-MOP-mediated inactivation of purified reconstituted P450 2B1. The complete system represents P450 2B1 exposed to 8-MOP (25 μM) and NADPH (100 μM) for 5 min at 30 °C. The turnover values are the average of duplicate determinations which did not vary by greater than 10%. Duplicate experiments were performed and consistent results were obtained.

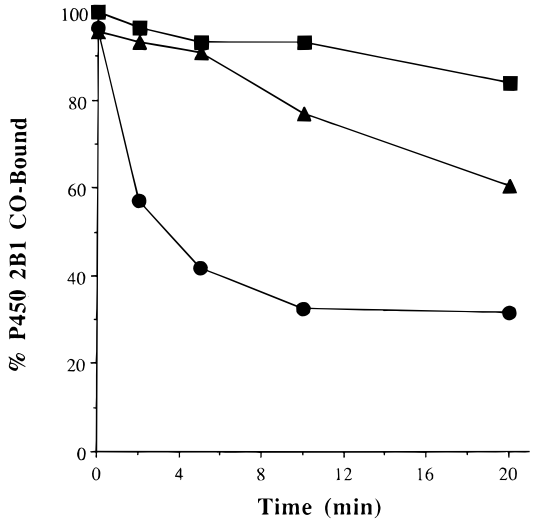

FIGURE 4: Destruction of P450 2B1 spectral content following incubation with 8-MOP (■), NADPH (▲), or 8-MOP and NADPH (●) in a reconstituted system. P450 2B1 was incubated with 8-MOP (25 μM) and/or NADPH (100 μM) in the presence of catalase (200 U) for various time periods (0−20 min) at 30 °C, and the reaction was quenched by diluting an aliquot 1:6 with a chilled buffer solution containing 1% cholate (w/v) and 20% glycerol (v/v). Spectral P450 content was determined by the method of Omura and Sato (*32*), and 100% spectral P450 content represents 73.5 pmol P450 2B1. The spectral P450 content is the average of duplicate determinations which did not vary by greater than 10%. Duplicate experiments were performed and consistent results were obtained.

Inclusion of high concentrations of GSH, NAC, or MOA (10 mM) resulted in the formation of conjugates that exhibited $[M + H]^+$ ions corresponding to the addition of the nucleophile plus an oxygen atom. GSH was the most effective nucleophilic trapping agent tested. Under CID conditions, the dihydro diols and conjugates formed by P450

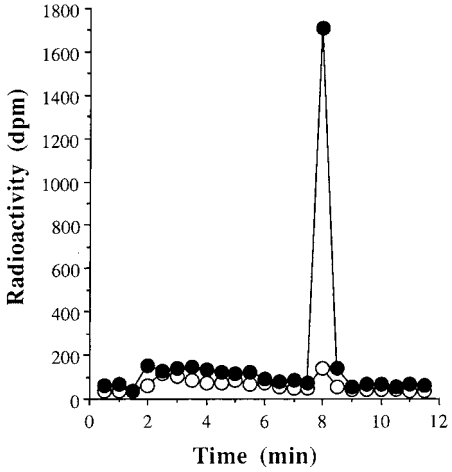

FIGURE 5: Binding profile of [^{14}C]-8-MOP to the reconstituted P450 2B1 enzyme mixture in the absence (○) or presence (●) of NADPH after separation of the proteins by HPLC and liquid scintillation counting of ≈ 1.5 mL fractions (every 0.5 min). Of the 22.7 pmol of P450 2B1 injected, 15.6 pmol was associated with radiolabel (111 dpm/pmol) which corresponds to a binding stoichiometry of 0.7:1. The amount of covalent binding to P450 2B1 was unaltered in the presence of GSH or MOA (2 mM). Under these conditions, 8-MOP dihydrodiol eluted at 0.5 min, 8-MOP eluted at 2.1 min, heme eluted at 3.3 min, catalase eluted at 4.4 min, b_5 eluted at 5.0 min, P450 reductase eluted at 5.5 min, and P450 2B1 eluted at 7.8 min. The amount of radiolabeled 0P450 is the average of duplicate determinations which did not vary by greater than 10%. Duplicate experiments were performed and consistent results were obtained.

2B1 were observed to fragment in patterns essentially identical to those observed for the same species formed from these furanocoumarins and P450 2A6 (*24*). The major metabolite of DH-8-MOP was characterized by an $[M + H]^+$ ion of 235 Da consistent with the formation of a monohydroxylated product; however, species with identical LC/ESI-MS/MS characteristics to 8-MOP and the 8-MOP dihydro diol were also detected. There were no detectable metabolites generated from 5-OH-P and 8-OH-P by P450 2B1 under the experimental conditions used. Incubations performed in the presence of $H_2{}^{18}O$ revealed that >99.5% of the dihydro diol formed from 8-MOP and P was the result of incorporation of one ^{18}O atom. In contrast, 87% of the 5-MOP dihydro diol formed was a result of incorporation of one ^{18}O atom while the remaining 13% arose via incorporation of two ^{18}O atoms.

Covalent Binding of 8-MOP to P450 2B1. Exposure of reconstituted P450 2B1 to [^{14}C]-8-MOP, separation of the components by HPLC, and liquid scintillation counting of the eluting fractions revealed that binding of the MBI was specific to the apoprotein of P450 2B1 (Figure 5). No radioactivity was associated with the heme which was stripped from P450 2B1 under the HPLC conditions employed. On the basis of a standard curve, generated using known amounts of purified P450 2B1 and the specific activity of the MBI, a binding stoichiometry of 0.7:1 was calculated for the association of spectrally detectable P450 2B1 and [^{14}C]-8-MOP. The amount of covalent binding to P450 2B1 was not altered in the presence of GSH or MOA (2 mM); however, in the absence of b_5, only 60% of P450 2B1 was labeled relative to incubations in its presence. This result indicated a distinct requirement for b_5 in the reconstituted enzyme mixture for maximal activity. H_2O_2 could also substitute for NADPH (but not in the absence of P450

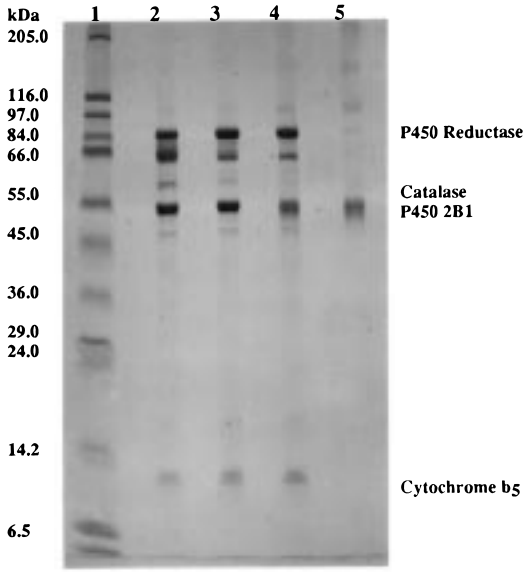

FIGURE 6: SDS−PAGE separation of the components of the reconstituted P450 2B1 enzyme mixture followed by Coomassie blue staining. All incubations contained catalase unless otherwise noted. Lane 1 contains the MM markers. Reconstituted P450 2B1 was exposed to [^{14}C]-8-MOP and NADPH (lane 2), H_2O_2 minus catalase (lane 3), and CuOOH (lane 4). Purified P450 2B1 (in the absence of P450 reductase) was exposed to CuOOH (lane 5).

reductase or b_5), albeit very poorly, to produce ≈ 27% of the covalently adducted protein relative to the NADPH supported control pathway. In experiments where CuOOH was substituted for NADPH, determination of the extent of covalent binding of [^{14}C]-8-MOP to P450 2B1 was complicated by problems associated with protein recovery after the Centricon 30 washing steps performed prior to injection onto the HPLC. Therefore, the relative amount of P450 2B1 bound after exposure to NADPH, CuOOH, or H_2O_2 was determined by liquid scintillation counting of the gel slices after separation of the proteins by SDS−PAGE. Relative to the NADPH dependent pathway, exposure of the reconstituted enzyme system to CuOOH resulted in 100% of the P450 2B1 bearing radiolabel. SDS−PAGE analysis revealed that exposure of the reconstituted P450 2B1 system to CuOOH resulted in the conversion of P450 2B1 to species that gave bands upon Coomassie blue staining that were much more diffuse than those obtained from P450 2B1 that had been exposed to NADPH or H_2O_2 (Figure 6). This conversion was not prevented by the addition of catalase nor was it dependent on P450 reductase or b_5. We have observed the same phenomenon with other P450s exposed to CuOOH (in the presence and absence of reductase and b_5) upon SDS−PAGE and Coomassie blue staining.[2]

Finally, LC/ESI mass spectrometric analysis of intact P450 2B1 that had been exposed to NADPH or NADPH and 8-MOP produced spectra with charge state distributions of +29 to +60 and +30 to +65, respectively. Using the results from three separate experiments performed on separate days, the average MMs (±SD) calculated for P450 2B1 and P450 2B1 covalently modified by 8-MOP were 55925.7 ± 2.2 and 56163.6 ± 7.4 Da, respectively (Figures 7 and 8). These values are in good agreement with the predicted MMs of P450 2B1 (55933.8 Da) and covalently modified P450 2B1 (56166.8 Da) and give a mass shift of 237.9 ± 9.6 Da for the adducted P450. In addition, the mass differences between P450 2B1 and P450 2B1 that had been covalently modified by 5-MOP or P (Figure 9) were 240.9 ± 6.2 and 204.8 ± 11.8 Da, respectively. The standard deviation of the calculated MM based on the charge state distribution observed was never greater than ±0.09%. Furthermore, deconvolution of the ESI mass spectra indicated the presence of one or two major P450 2B1 species (insets to Figures 7−9) corresponding to P450 2B1 or adducted P450 2B1.

DISCUSSION

The metabolic conversion of EFC to HFC has been successfully used as a marker for rat liver microsomal P450 activities (*33*). To enhance its usefulness as a measure of mechanism-based inactivation of P450 2B1 by furanocoumarin analogues, we developed a rapid throughput HPLC-fluorescence assay. Exposure of reconstituted P450 2B1 to the set of furanocoumarins that had been used previously to elucidate the mechanism-based inactivation of P450 2A6 (*24*) gave similar results. Time-dependent inactivation was observed, and the most potent MBI, based on its K_I, k_{inact}, and partition ratio (Figure 2), was again found to be 8-MOP. Furthermore, the results demonstrated that the inactivation (a) is dependent on catalysis, (b) takes place at the active site of P450 2B1, (c) is not a result of the generation of reactive oxygen species (Figure 3), (d) is not due to a disruption of the P450 2B1 and P450 reductase electron-transfer pathway, and (e) is accompanied by a corresponding loss in CO-binding capacity (Figure 4). Interestingly, 30% of both P450 2B1 and P450 2A6 (*24*) could still bind CO after a 20 min exposure to 8-MOP indicating that even after 8-MOP had completely eliminated enzyme catalytic activity, a significant amount of P450 is still capable of binding CO. These findings are consistent with an earlier rat liver microsomal study in which 25−30% of the total P450 was resistant to heme destruction after a 40 min exposure to NADPH in the absence of substrate (*34*).

The low K_I and partition ratio of 8-MOP for P450 2B1 imply that specific binding interactions at the active site lead to a high commitment to mechanism-based inactivation. Similarly, the higher K_I and 10-fold greater partition ratios for 5-MOP and P toward P450 2B1 suggest that the binding interactions between these two compounds and the enzyme are much less effective in producing the reactive intermediate that leads to inactivation. Conversion of the methoxyl group to a hydroxyl group to give 8-OH-P or 5-OH-P resulted in compounds that were not metabolized by P450 2B1 and thus were unable to effect inactivation. Since these compounds are inhibitors but not substrates, it is probable that the phenolic hydroxyl groups reorient their binding topography at the P450 2B1 active site such that effective contact with the active oxygenating species cannot occur. Similar to the earlier studies with P450 2A6, reduction of the furan ring of 8-MOP to generate DH-8-MOP did not result in complete loss of inactivation potency. Again, inactivation presumably results from the oxidation of 8-MOP that is formed as a minor metabolite of DH-8-MOP. This interpretation is consistent with the detection of 8-MOP and 8-MOP dihydrodiol in the mass spectra of extracts of incubations of DH-8-MOP and P450 2B1 (see following).

LC/ESI-MS/MS analysis of the metabolites generated from the furanocoumarins that acted as MBIs of P450 2B1 were

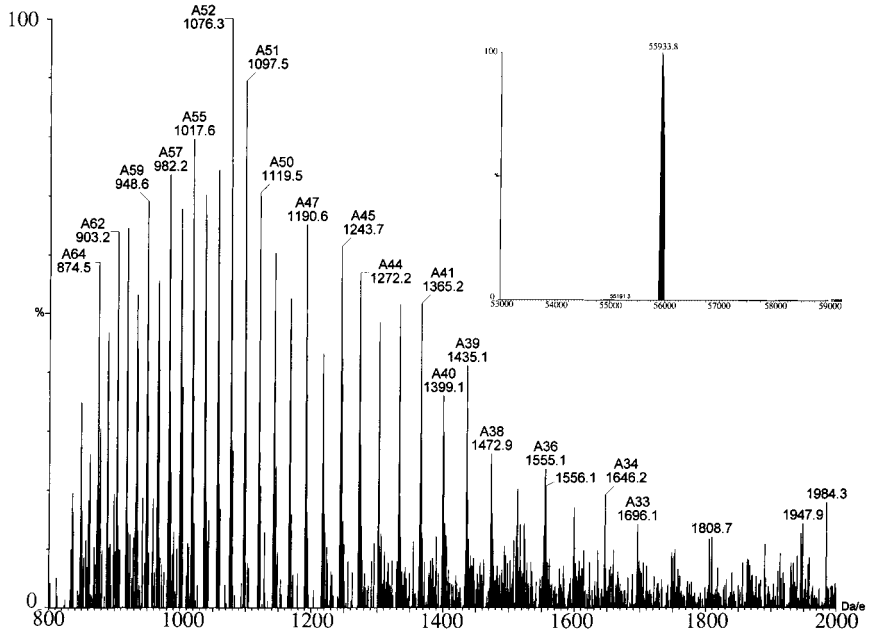

FIGURE 7: ESI mass spectra of P450 2B1 exposed to NADPH (100 μM) in the absence of substrate. The peaks are labeled with the protonation state (An) and the number of protons (*n*) attached to the protein molecule. The average molecular mass of P450 2B1 was calculated to be 55925.7 ± 2.2 after analysis on three different days from three separate experiments. Inset: Deconvolution of the ESI mass spectral data which gives a representative profile of the P450 2B1 species present.

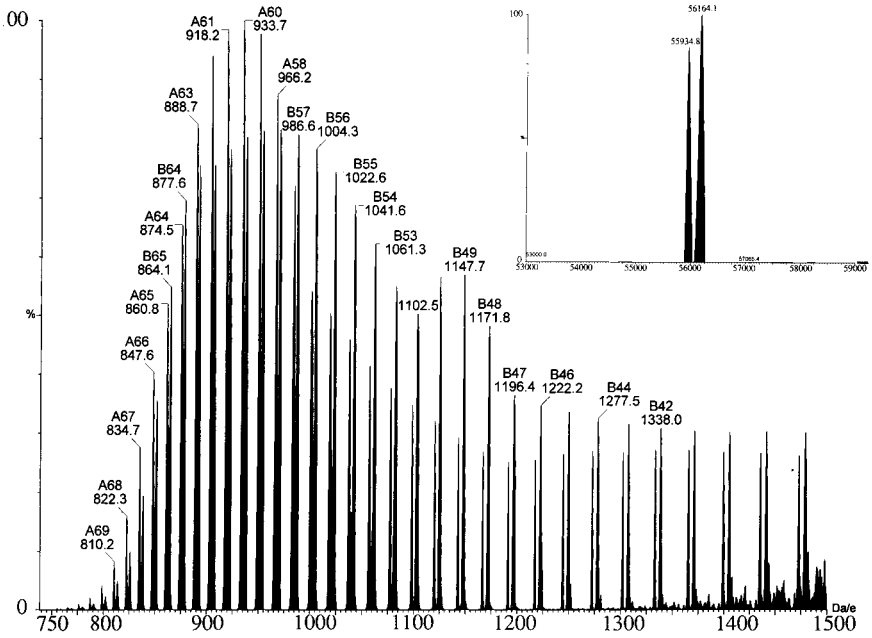

FIGURE 8: ESI mass spectra of P450 2B1 exposed to NADPH (100 μM) and 8-MOP (100 μM). The peaks are labeled with the protonation state (An) and the number of protons (*n*) attached to the protein molecule. The average molecular mass of P450 2B1 covalently modified by 8-MOP was calculated to be 56163.6 ± 7.4 Da after analysis on three different days from three separate experiments. The difference between the molecular masses of P450 2B1 and covalently modified P450 2B1 (237.9 ± 9.6 Da) corresponded to the addition of the postulated reactive 8-MOP epoxide intermediate (232.2 Da). Inset: Deconvolution of the ESI mass spectral data which gives a representative profile of the P450 2B1 species present.

found to be identical to the furanocoumarin dihydro diols generated by P450 2A6 (*24*). Under the CID conditions employed, the initial fragmentation pathway for the furanocoumarin dihydro diols was loss of H_2O followed by either loss of 15 mass units ($-CH_3$, when present) or consecutive losses of 28 mass units (expulsion of CO). A dihydro diol was the major metabolite in all cases except for DH-8-MOP where the monohydroxylated product, 4′-OH-DH-8-MOP was tentatively identified as the major metabolite. Inclusion of GSH, NAC, and MOA in the incubations resulted in the formation of conjugates with $[M + H]^+$ ions and MS/MS characteristics consistent with the addition of the nucleophile plus an oxygen atom, suggesting the intermediacy of an epoxide.

Studies using $H_2{}^{18}O$ indicated that >99.5% of the dihydro diol formed from 8-MOP and P involved the incorporation of a single atom of ^{18}O (Figure 10, pathway a) whereas only 87% of the 5-MOP dihydro diol arose via the incorporation of one ^{18}O atom. The remaining 13% of the 5-MOP dihydro diol was a result of the incorporation of two ^{18}O atoms.

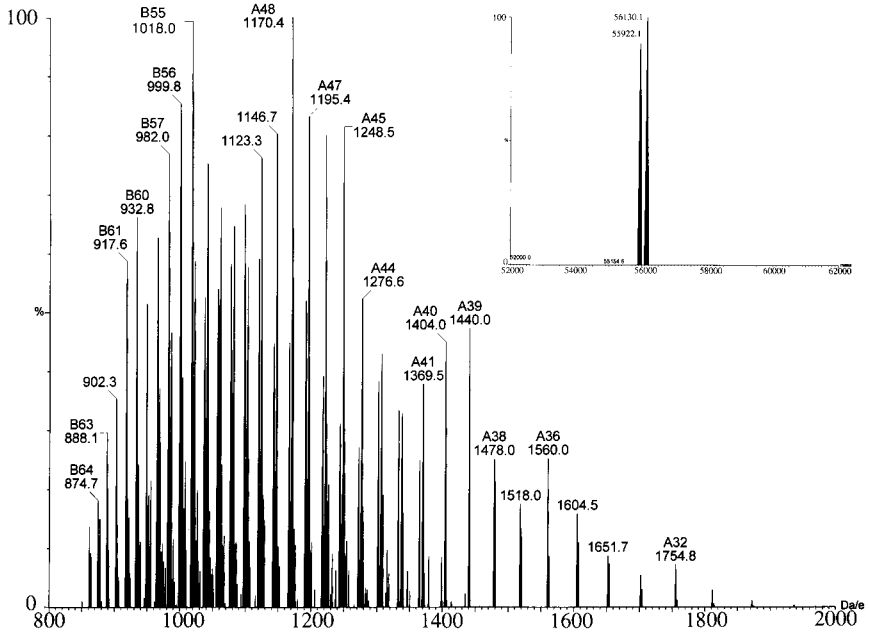

FIGURE 9: ESI mass spectra of P450 2B1 exposed to NADPH (100 μM) in the presence of P (100 μM). The peaks are labeled with the protonation state (An) and the number of protons (*n*) attached to the protein molecule. The average molecular masses of P450 2B1 and P450 2B1 covalently modified by P were calculated to be 55919.1 $\pm$ 8.6 and 56123.9 $\pm$ 3.4 Da, respectively, after injection of a sample of each on three different days. The difference between the molecular masses of these two species (204.8 $\pm$ 11.8 Da) corresponded with the addition of the postulated reactive P epoxide intermediate (202.2 Da). Inset: Deconvolution of the ESI mass spectral data which gives a representative profile of the P450 2B1 species present.

Exactly analogous results were previously obtained from P450 2A6 and were attributed to an additional resonance effect available to 5-MOP which would favor formation of an $H_2{}^{18}O$ exchangeable γ-keto enal intermediate (*24*) (Figure 10, pathway(s) b and/or c). Thus, it can be concluded that the structure of the reactive intermediates generated from these furanocoumarins is primarily dictated by the electronic character of the substrate and not the active site of the P450.

Exposure of reconstituted P450 2B1 to [^{14}C]-8-MOP, followed by separation of the components of the enzyme mixture by HPLC or SDS−PAGE, indicated that binding was specific to the apoprotein of P450 2B1 (Figure 5). This finding is in accord with earlier results where no green pigments (corresponding to N-alkylation of the heme moiety of P450) were observed in the whole livers of phenobarbital-pretreated rats after administration of 8-MOP (*22*). Similar to the inhibition of P450 2B1 activity, the binding of [^{14}C]-8-MOP to P450 2B1 could not be prevented by the presence of GSH or MOA in the incubations, further supporting indications that covalent modification takes place in the active site of the enzyme. Substitution of CuOOH for NADPH did result in the covalent modification of 100% of P450 2B1 by 8-MOP relative to control; however, only minimal amounts of the adducted P450 were recoverable from the Centricon 30 concentrators. It has been reported that CuOOH-mediated P450 2B1 inactivation leads to the formation of heme fragmentation products that covalently bind to the apoprotein (*35*). Thus, it seems likely that the diffuse P450 2B1 band observed upon SDS−PAGE analysis (Figure 6) indicates the presence of a number of P450 2B1 species with different MMs, resulting from the attachment of various heme breakdown products to the enzyme. The detection of several minor species by LC/ESI-MS after exposure of P450 2B1 to CuOOH is consistent with this hypothesis.[2]

A binding stoichiometry of 0.7:1 between covalently bound 8-MOP and spectrally detectable P450 2B1 was calculated on the basis of a standard curve generated from known amounts of purified P450 2B1 injected onto the HPLC. The lack of 1:1 stoichiometry between MBI and P450 has been noted previously (*36*) and could be due to one or more of a number of possible factors. For example, reactive oxygen species (H_2O_2 and/or superoxide anion) produced by uncoupling of P450 2B1 upon exposure to NADPH could inactivate the P450 directly without involving inhibitor activation. Such an effect has been implicated by the observed loss in CO-binding capacity when P450 2B1 was exposed to NADPH in the absence of substrate (Figure 4). It is also possible that all of the spectrally detectable P450 2B1 (measured as amount of $Na_2S_2O_4$–reduced CO-bound P450 2B1) is not capable of NADPH- (or CuOOH-) mediated catalysis.[3] It has been shown that some purified reconstituted human P450s, which were completely reduced by $Na_2S_2O_4$, were only partially reduced by P450 reductase (*37*). The lack of 1:1 stoichiometry found in this study does not appear to be an aberrant result as under similar conditions a binding stoichiometry of only 0.3−0.4:1 between 8-MOP (and 5-MOP) and reconstituted purified and GENTEST P450 2A6 was observed (*24*), presumably for similar reasons. Unlike the P450 2B1 system, binding of 8-MOP (and 5-MOP) to P450 2A6 and P450 reductase was observed in the reconstituted purified P450 2A6 system. On the basis of the results from this laboratory and others, it appears as though the protein interactions among the various purified reconstituted P450s are very different (*37*) and that the escape

[3] $Na_2S_2O_4$ (10 mM) did not support reconstituted P450 2B1 EFC O-deethylase activity which may be due to an inability of $Na_2S_2O_4$ to supply the second electron to the P450 in its catalytic sequence.

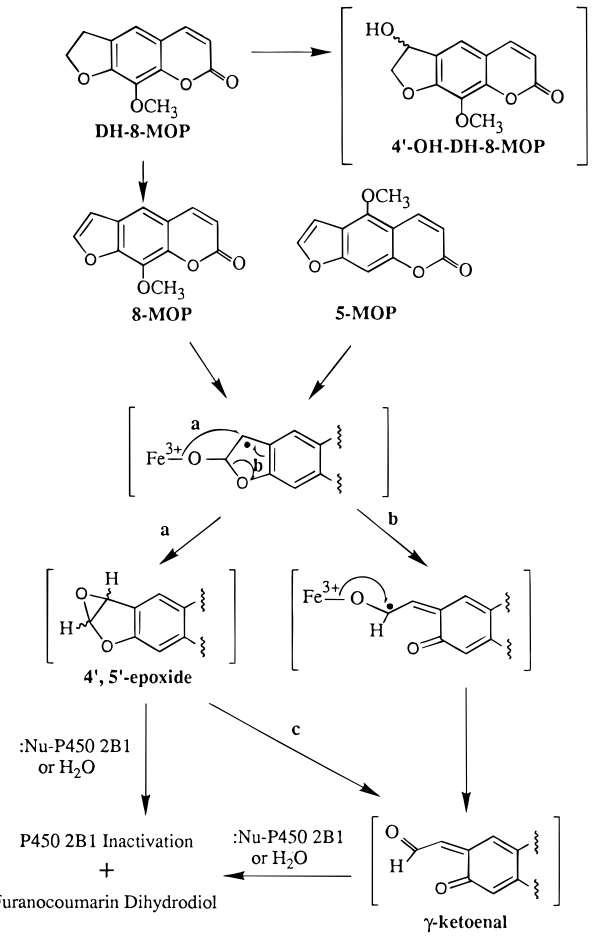

FIGURE 10: Postulated scheme of mechanism-based inactivation of reconstituted P450 2B1 by furanocoumarins. DH-8-MOP is converted to 8-MOP in a P450 2B1 and NADPH dependent process in order to cause inactivation. Pathway a accounts for >99.5% of the formation of the 8-MOP and psoralen dihydro diols and 87% of the 5-MOP dihydrodiol. Pathways b and/or c account for the remaining 13% of the 5-MOP dihydro diol formation. This scheme was based on results from $H_2{}^{18}O$ experiments and LC/ESI mass spectral analysis of the metabolites produced by 8-MOP from the furanocoumarins used in this investigation. The position of the methoxyl group is omitted for clarity.

of reactive intermediates from the P450 active site is strictly P450 dependent.

ESI-MS analysis of P450 2B1 after exposure to NADPH in the absence and presence of 8-MOP and separation of the components of the reconstituted enzyme mixture revealed the presence of a single major species (Figures 7 and 8). On the basis of three separate experiments performed on different days, the average MM (±SD) calculated for the major species present in the P450 2B1 sample not exposed to 8-MOP was 55925.7 ± 2.2. The average MM (±SD) calculated for a P450 2B1 sample covalently modified by 8-MOP was 56163.6 ± 7.4 Da with a mass difference between the two species of 237.9 ± 9.6 Da. This value agrees quite well with that predicted for addition of the putative 8-MOP furanoepoxide (232.2 Da) to P450 2B1. Not only does this finding support the fact that only one 8-MOP molecule is bound to P450 2B1, but it also suggests that the mechanism of inactivation is by direct covalent modification of the apoprotein by 8-MOP furanoepoxide rather than by binding of some reactive species formed from 8-MOP induced heme fragmentation. In addition, P450 2B1 that had been covalently modified by 5-MOP or P (Figure 9) exhibited a mass difference from unmodified P450 2B1 of 240.9 ± 6.2 and 204.8 ± 11.8, respectively. These values agree well with the predicted mass shifts of 232.2 or 202.2 Da for addition of a 5-MOP or P furanoepoxide, respectively. To our knowledge, this is the first successful demonstration of the use of mass spectral techniques to identify the adduction of small molecules to an intact P450.

In this investigation, we have shown that purified reconstituted P450 2B1 is susceptible to mechanism-based inactivation by furanocoumarins and that, of the compounds tested, 8-MOP was the most potent. On the basis of metabolic, $H_2{}^{18}O$, and mass spectrometry experiments, covalent modifiation of the enzyme is thought to proceed predominantly via initial oxidation at the furan ring to form a furanoepoxide. In addition, the mechanism of inactivation proceeds exclusively through covalent modification of the apoprotein by the reactive intermediate(s) of the furanocoumarins. Studies to elucidate which active site amino acid residue(s) of P450 2B1 is covalently bound are currently in progress.

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