Mechanism-based inactivators serve as probes of enzyme mechanism, function, and structure. Koshland’s Reagent II (2-methoxy-5-nitrobenzyl bromide, KR-II) is a potential mechanism-based inactivator of enzymes that perform O-dealkylations. The major phenobarbital-inducible form of cytochrome P-450 in male rat liver microsomes, CYP2B1, is capable of catalyzing O-dealkylations.

The interactions of KR-II with purified CYP2B1 in the reconstituted system containing P-450, NADPH: P-450 oxidoreductase, and sonicated dilaurylphosphatidyl choline were studied. The benzphetamine N-demethylase activity of CYP2B1 was inactivated by KR-II in a time- and NADPH-dependent manner, and the loss of activity followed pseudo-first-order kinetics. The inactivation also required KR-II, and the rate of activity loss was dependent on the concentration of KR-II in a saturable fashion. The inactivator concentration required for the half-maximal rate of inactivation ($K_a$) was approximately 0.1 mM. The inactivation was not prevented by the addition of the nucleophiles dithiothreitol and glutathione, nor was it reversed by gel filtration. The present results demonstrate that KR-II is a mechanism-based inactivator of rat CYP2B1.

In many cases, oxidative metabolism by the cytochromes P-450 (P-450) critically determines the balance between detoxification and the formation of toxic products from xenobiotics (Porter and Coon, 1991). The common catalytic function of these enzymes involves a two-electron reduction of molecular oxygen to form water and a reactive oxygen species. Because the insertion of the activated oxygen into substrate is a common feature of all P-450s, the balance between metabolic activation and detoxification must depend on how a given substrate binds to a specific form of P-450, which in turn is determined by the structure of the active site of that P-450. One way to find which peptide regions of a given form of P-450 define its active site is through identification of the peptides labeled during the inactivation of that P-450 by mechanism-based inactivators that bind to the apoprotein.

Mechanism-based inactivation occurs when metabolism of the substrate results in the formation of a highly reactive intermediate, which reacts with a moiety in the active site before leaving the active site and covalently modifies that amino acid residue, leading to inactivation of the enzyme. This phenomenon has been reviewed in detail by Abeles (1983) and Waley (1980). For several classes of mechanism-based inactivators of P-450, the general reaction sequences proposed to explain the modification of the active site and the subsequent activity loss have been reviewed by Ortiz de Montellano and Correira (1983), Osawa and Pohl (1989), and Murray and Reidy (1990). Several classes of compounds that inactivate P-450s have been identified including alkenes, alkynes, dichloromethylenes, and dihydropyridines (Ortiz de Montellano et al., 1981; Augusto et al., 1982; Kunze et al., 1983; Ortiz de Montellano et al., 1983; Halpert et al., 1986; Caliaco et al., 1988).

An alternative to the previously characterized pathways for the formation of a reactive intermediate leading to active site labeling involves starting with 2-methoxy-5-nitrobenzyl bromide (Koshland’s Reagent II, KR-II), which would be expected to undergo P-450-catalyzed O-demethylation. The desmethyl analog of KR-II, 2-hydroxy-5-nitrobenzyl bromide, is Koshland’s Reagent I (KR-I). KR-I is a highly reactive, electrophilic reagent that has been used to label tryptophan residues (Barman and Koshland, 1967; Loudon and Koschid, 1970). As noted by Horton and Koschid (1967), alklylation of the phenolic group of KR-I greatly reduces the susceptibility of the benzyl bromide to nucleophilic attack. Therefore, the resultant esters and ethers of this phenolic hydroxy group can serve as precursors of reactive intermediates. KR-II and KR-I are analogs of the P-450 substrate $p$-nitroanisole and $p$-nitrophenol, the product formed during P-450-catalyzed $O$-demethylation of $p$-nitroanisole. Metabolism of KR-II might occur by several alternate routes, including hydroxylation of the aromatic ring or the benzylic carbon. Conversion of KR-II to a phenol by either P-450-catalyzed $O$-demethylation or ring hydroxylation would generate an electrophile at the enzyme active site, where it could then react with active site nucleophiles. Generation of an electrophile would be expected to produce mechanism-based inactivation of the enzyme if there were one or more critical nucleophilic residues in the active site with which it could react. Because we are interested in identifying active site peptides rather than heme adducts, it was hoped that the electrophilic carbon thus generated...
would not label the heme. Investigation of the enzyme activity levels before and after incubation of P-450 with KR-II demonstrated that KR-II acted as a mechanism-based inactivator of P-450 in liver microsomes from phenobarbital-induced rats and in the reconstituted system containing CYP2B1.

Materials and Methods

Preparation of Enzymes. The major phenobarbital-inducible form of liver microsomal cytochrome P-450 (CYP2B1) was purified from liver microsomes of male Long Evans rats obtained from Harlan-Sprague-Dawley (Indianapolis, IN). Microsomes were prepared from the livers of rats induced with 0.1% phenobarbital in the drinking water for 10 to 13 days as described by Coon et al. (1978). The CYP2B1 was purified from liver microsomes using the procedure of Imai et al. (1980). The purified P-450 gave a single band on SDS-polyacrylamide gel electrophoresis and was electrophoretically pure. Cytochrome P-450 concentrations were determined from the reduced CO difference spectra by the method of Omura and Sato (1964a,b), using a micromolar extinction coefficient of 0.091. The specific contents were 11 to 16 nmol/mg protein.

Rat and rabbit NADPH/cytochrome-P-450 oxidoreductases (reductase) were purified from liver microsomes of phenobarbital-treated male Long Evans rats and male New Zealand White rabbits (Langshaw, Augusta, MI) by the single-column procedure of Shephard et al. (1963) using an adenosine 2’,5’-diphosphate agarose (ADP-agarose, Sigma A3515; Sigma Chemical Co., St. Louis, MO) affinity column. Rat and rabbit reductases functioned equally well in the reconstituted system with rat CYP2B1. Flavin mononucleotide (1.0 μM) was added to the flavoprotein preparations to ensure full reconstitution of the reductase protein with the flavin. The protein was concentrated to at least 10 nmol/ml in an Amicon (Danvers, MA) stirred-cell concentrator and then dialyzed against 50 mM potassium phosphate buffer, pH 7.4. The purified reductase proteins in the absence of detergents, assay volumes were reduced 5-fold. Otherwise, the precipitating modification of Bensadoun and Weinstein (1976), with 5- or 10-fold reduced volumes, was used. BSA was used for standard concentrations of 1.0 nmol/ml. All other chemicals used for these studies were reagent grade or better and were purchased from several suppliers.

To gain a better understanding of the active site structure and the mechanism of action of the P-450s, we investigated the inactivation of rat CYP2B1 by KR-II. Horton and Koshland (1967) developed a clever approach for modifying hydrolytic enzymes such as the serine proteases that catalyze esterolytic reactions. They discovered that acetylation of the phenolic hydroxyl group of 2-hydroxy-5-nitrobenzyl bromide results in a marked decrease in the reactivity of the benzyl bromide function. Subsequently, it was demonstrated that methylation of the phenolic hydroxyl to give the methoxy derivative greatly reduced the chemical reactivity of the benzyl bromide when compared with the unsubstituted form (Lundblad and Noyes, 1984). The major reactions predicted for the P-450-catalyzed metabolism of the methoxy derivative KR-II, either O-demethylation or ring hydroxylation, would both be expected to regenerate the reactivity of the benzyl bromide. By analogy with p-nitroanisole, a well-characterized P-450 substrate that is structurally similar to KR-II, the demethylation route would be expected to predominate (Fig. 1). This route of metabolism would generate 2-hydroxy-5-nitrobenzyl bromide, also known as Koshland’s Reagent I. Koshland’s Reagent I has been used to selectively label tryptophan residues at low pH (4.0), but at neutral pH it will react with other nucleophiles in proteins (tyrosine, cysteine, or methionine).
Reaction with one of these nucleophilic amino acid residues in the P-450 active site could lead to inactivation of the enzyme. Figure 1 also shows that ring hydroxylation might possibly occur at any of the three ring hydrogens, leading to the subsequent reaction of the activated benzyl bromide with a protein nucleophile. Based on the chemical reactivity of the ring carbon atoms, the most likely site for ring hydroxylation would be at the 3 position adjacent to the methoxy substituent.

Requirement for NADPH for Inactivation of P-450 by Koshland’s Reagent II. P-450-catalyzed metabolism of substrates normally requires NADPH and molecular oxygen. Because mechanism-based inactivation requires the initial metabolism of the inactivator, a mechanism-based inactivation would be expected to exhibit the same cofactor requirements as seen for the metabolism of normal substrates. Therefore, the requirement for NADPH during the inactivation of CYP2B1 by KR-II was investigated (Table 1). Incubation with methanol, the solvent for KR-II, resulted in no significant loss of activity. The slight decrease in activity (5–10%) seen with KR-II in the absence of metabolism was interpreted as due to competitive inhibition by KR-II carried over into the benzphetamine assay with the enzyme. The activity loss (22%) observed following incubation with NADPH in the absence of KR-II is due to the auto-inactivation of CYP2B1 in the reconstituted system, which has previously been described (Loosemore et al., 1981). In the presence of both KR-II and NADPH, however, significant (67%) inactivation was observed (Table 1).

Dependence on Koshland’s Reagent II Concentration for P-450 Inactivation. Because mechanism-based inactivation is an enzyme-catalyzed phenomenon, it would be expected to display a concentration dependence for inactivation similar to that observed for the metabolism of normal substrates. Thus, the rate of activity loss should parallel the rate of substrate metabolism. The initial rates for substrate metabolism normally exhibit saturation kinetics with respect to substrate concentration, as demonstrated by a hyperbolic approach of the initial rate of the reaction to a limiting maximum rate at saturating substrate concentrations. The inactivation of CYP2B1 by KR-II was determined at different concentrations of KR-II to see whether this pattern was observed. As shown in Fig. 2, the inactivation of CYP2B1 by KR-II exhibited the expected approach to saturation kinetics. The data are plotted as percent activity lost after a 10-min incubation for a range of concentrations of KR-II. From these data, a $K_i$ value of 0.1 mM was estimated for KR-II for the mechanism-based inactivation of CYP2B1.

Protection of CYP2B1 Against Mechanism-Based Inactivation by Addition of Nucleophiles. For mechanism-based inactivation, the inactivating intermediate must be generated in the active site and react there without leaving the active site and diffusing back in or inactivating by reacting elsewhere on the protein. Because mechanism-based inactivators generated by cytochrome P-450 would be expected to be highly electrophilic, a strong nucleophile added to the reaction medium should serve as a trapping reagent to prevent back diffusion of any electrophile(s) escaping into the incubation medium from the enzyme active site. Thus, the rate of inactivation would be decreased in the presence of such a trapping agent if the reactive intermediate responsible for the inactivation were leaving the active site before inactivating the enzyme.

Because neither dithiothreitol nor glutathione exhibited any significant effects on the rate of CYP2B1-catalyzed benzphetamine N-demethylation when added to the incubation mixtures at 1 mM concentrations (data not shown), these strong nucleophiles were investigated as potential trapping agents for reactive electrophiles released following metabolism at the P-450 active site. As shown in Table 2, mechanism-based inactivation by KR-II resulted in a 70% loss of the benzphetamine N-demethylase activity. However neither
was examined for its ability to inactivate benzphetamine activity in the reconstituted system and in liver microsomes. KR-II, a P-450 enzyme with several different isoforms of P-450, it is a good measure of CYP2B1 activity in those preliminary studies (data not shown), the hypothesis that KR-II inactivation of CYP2B1 is a mechanism-based inactivation, the loss of enzymatic activity would be irreversible. Gel filtration of the primary incubation mixtures was used to separate the reconstituted enzymes from the other reactants and products before testing for enzymatic activity. If product inhibition is involved, enzymatic activity should be recovered, whereas with mechanism-based inactivation, the loss of enzymatic activity would be irreversible.

**Failure to Reverse Koshland’s Reagent II Inactivation of CYP2B1 by Gel Filtration.** Mechanism-based inactivation involves covalent modification(s) of the enzyme. It can be differentiated from product inhibition, which can exhibit many of the same properties, by separating the enzyme from the substrate and products of the reaction and assaying for catalytic activity. If product inhibition is involved, enzymatic activity should be recovered, whereas with mechanism-based inactivation, the loss of enzymatic activity would be irreversible. Gel filtration of the primary incubation mixtures was used to separate the reconstituted enzymes from the other reactants and products before testing for enzymatic activity. The data shown in Table 3 demonstrate that CYP2B1 inactivated by KR-II did not recover any activity after separation from the substrate and metabolites. The ratio of activity in the preparation incubated with NADPH to that of the preparation incubated with water is the same (0.65) both before and after gel filtration. Thus, as expected for mechanism-based inactivation, the loss of activity could not be reversed by gel filtration.

**Kinetics of CYP2B1 Inactivation by Koshland’s Reagent II.** Enzyme inactivation by mechanism-based inactivators should exhibit pseudo-first-order kinetics with respect to the enzyme concentration (Abeles, 1983). As shown in Fig. 3, the inactivation of CYP2B1 by KR-II exhibited a first-order loss of activity when plotted as the log of the percentage of activity remaining versus time, consistent with the hypothesis that KR-II inactivation of CYP2B1 is a mechanism-based inactivation.

**Discussion**

Although benzphetamine N-demethylase activity is expressed by several different isoforms of P-450, it is a good measure of CYP2B1 activity in the reconstituted system and in liver microsomes. KR-II was examined for its ability to inactivate benzphetamine N-demethylase activity in liver microsomes from phenobarbital-pretreated rats and then further tested to see whether the loss of activity was time dependent. Because KR-II produced a time-dependent inactivation of P-450 activity in those preliminary studies (data not shown), the inactivation was subsequently characterized in the reconstituted system containing CYP2B1, reductase, and DLPC to see whether it was a mechanism-based inactivator.

Loss of CYP2B1 activity in the reconstituted system required the addition of NADPH, as had been observed in microsomes. The values for the percentage of activity remaining were determined at various concentrations of KR-II and were plotted versus the concentration to see whether the inactivation exhibited saturation kinetics. The concentration dependence for the inactivation of rat CYP2B1 by KR-II showed, as does normal substrate metabolism, a hyperbolic approach to a limiting maximum rate. More rigorous methods of kinetic analysis such as those of Waley (1980, 1985) or Tatsunami et al. (1981) were not used because this treatment adequately demonstrated the nature of the inactivation. Loss of CYP2B1 activity during the metabolism of KR-II was first-order with respect to time. The absence of a lag period in the inactivation kinetics showed that inactivation was an immediate result of the primary metabolism of KR-II by CYP2B1.

The absence of protection by the exogenous nucleophiles glutathione and dithiothreitol reinforced the conclusion that this was mechanism-based inactivation. Thus, the inactivation took place when the reactive intermediate was generated in the active site and before the intermediate was diffused out of the active site. The inability to reverse the loss of activity by gel filtration showed the permanence of the inactivating modification, presumably the result of the formation of a covalent adduct. The characteristics of the inactivation, especially the pseudo-first-order nature of the KR-II inactivation kinetics, suggest that inactivation by KR-II fits a simple model where metabolism leads either to free products or an inactivating modification of the CYP2B1.

Selectivity for protein rather than heme modification during the mechanism-based inactivation of P-450s has been observed with

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**TABLE 2**

Effects of added nucleophiles on Koshland’s Reagent II inactivation of CYP2B1

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Turnover Number</th>
<th>Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>min⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>Time 0 Control</td>
<td>55 ± 1⁺</td>
<td>100</td>
</tr>
<tr>
<td>Water</td>
<td>17 ± 6</td>
<td>30</td>
</tr>
<tr>
<td>Glutathione</td>
<td>19 ± 2</td>
<td>34</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>14 ± 9</td>
<td>25</td>
</tr>
</tbody>
</table>

⁺Mean ± S.D., n = 3.

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**TABLE 3**

Inability to reverse Koshland’s Reagent II inactivation of CYP2B1 by gel filtration

<table>
<thead>
<tr>
<th>Addition to the Primary Reaction Mixtures</th>
<th>Turnover Number</th>
<th>Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>min⁻¹</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Before gel filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>54 ± 1⁺</td>
<td>100</td>
</tr>
<tr>
<td>NADPH</td>
<td>37 ± 3</td>
<td>68</td>
</tr>
<tr>
<td>After gel filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>50 ± 8</td>
<td>92</td>
</tr>
<tr>
<td>NADPH</td>
<td>34 ± 4</td>
<td>62</td>
</tr>
</tbody>
</table>

⁺Mean ± S.D., n = 3.
some, but not all, mechanism-based inactivators. Selectivity is dependent in part upon the position of the activatable group when bound in the active site. For example, Ortiz de Montellano and Komives (1985, 1987) proposed that the regiospecificity of alkyne oxidation determines whether the inactivating intermediate reacts with the heme or protein. They observed that oxidation of internal carbons leads to heme alkylation and destroys the P-450 spectrum, whereas oxidation of terminal carbons produces an intermediate that, after rearrangement to a ketene, can react with protein nucleophiles or be hydrolyzed to carboxylic acid products. Consistent with this, CaJacob et al. (1988) demonstrated that mechanism-based inactivation of P-450 4A1 (the (1993, 1994, 1995) demonstrated that protein labeling occurs during the polycyclic aromatic ring system, were all critically important in microsomes by aryl acetylenes. They found that the position of modifications of the P-450 protein by a ketene intermediate. More recently, Hopkins et al. (1992) looked at the structure-activity relationships for mechanism-based inactivation of either ethoxyresorufin O-deethylase (1A1) or pentoxyresorufin O-depentylase (2B1) activity in microsomes by aryl acetylenes. They found that the position of attachment of the acetylenic function, along with the size and shape of the polycyclic aromatic ring system, were all critically important in determining the selectivity and efficacy of the arylenes for the mechanism-based inactivation of these P-450 activities. Roberts et al. (1993, 1994, 1995) demonstrated that protein labeling occurs during mechanism-based inactivation of CYP2B1 by 2-ethylmethylphene. The activity loss observed is independent of heme destruction and is due to modification of the protein with an approximate stoichiometry of 1.3 mol of [1H]2-ethylmethylphene incorporated per mole of P-450 inactivated. Although the labeled residue has not yet been unequivocally identified, it is within an 11-amino acid segment of the CYP2B1 sequence from Phe-297 to Leu-307 (FAGTETSTTL) (Roberts et al., 1995). The role of Thr-302 in the mechanism-based inactivation of CYP2B4 by 2-ethylmethylphene has been demonstrated by site-specific mutagenesis (Roberts et al., 1996). HPLC analysis of CYP2B1 inactivated using 14C-labeled KR-II demonstrated that all of the counts were associated with the apoprotein and that there were no counts associated with the heme peak (data not shown). These results suggest that the inactivation of CYP2B1 by KR-II was due to protein modification rather than heme modification.

Having shown here that KR-II acts as a mechanism-based inactivator of CYP2B1, we are further characterizing this inactivation. The mechanism(s) in inactivation are being investigated by isolating and identifying the metabolites produced during the metabolism of KR-II by P-450. The studies described here demonstrate an alternative strategy for the design of a mechanism-based inactivator of P-450. It is hoped that these studies will not only contribute a useful new mechanistic tool but also aid in the identification of specific portions of the P-450 protein that participate in the formation of the active site.

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References
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