Mechanism-Based Inactivation of Cytochrome P450 2B4 by Aldehydes: Relationship to Aldehyde Deformylation via a Peroxyhemiacetal Intermediate†

Gregory M. Raner,* Eric W. Chiang, Alfin D. N. Vaz, and Minor J. Coon

Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109-0606

Received December 12, 1996; Revised Manuscript Received February 18, 1997⊗

ABSTRACT: The inactivation of cytochrome P450 2B4 by aldehydes in a reconstituted enzyme system requires molecular oxygen and NADPH and is not prevented by the addition of catalase, superoxide dismutase, epoxide hydrolase, glutathione, or ascorbic acid. A strong correlation between loss of enzymatic activity and bleaching of the heme chromophore was established, and the inactivation was shown to be irreversible upon dialysis. In general, saturated aldehydes are more inhibitory than those with α,β-unsaturation, as indicated by the k_{inact} values, and primary aldehydes are more potent inactivators than the structurally related secondary and tertiary aldehydes. Consistent with recent studies on catalytic specificity of the T302A mutant of this cytochrome [Vaz, A. D. N., Pernecky, S. J., Raner, G. M., & Coon, M. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4644−4648], the rate of aldehyde deformylation, as determined by formation of the alcohol with one less carbon atom, is greatly stimulated over that of the wild-type enzyme. Of particular interest, the rate of oxidation of aldehydes to carboxylic acids is decreased with the mutant, whereas the rate of inactivation via heme destruction is enhanced. Furthermore, comparative deuterium isotope effects and the relative rates of inactivation and product formation suggest that the mechanism of aldehyde inactivation of P450 2B4 involves the deformylation reaction and is unrelated to carboxylic acid formation. Finally, in the reaction of P450 2B4 with 3-phenylpropionaldehyde, the formation of a heme adduct with a molecular weight corresponding to that of native heme plus 104 mass units confirms the loss of the carbonyl group from the aldehyde prior to reaction with the chromophore. We conclude that inactivation of P450 by aldehydes occurs via homolytic cleavage of a peroxyhemiacetal intermediate to give an alkyl radical that reacts with the heme.

Aldehydes occur very widely in the metabolism of physiologically important compounds and xenobiotics (Marinello et al., 1984; DeMaster et al., 1986; Ji et al., 1989) and are commonly known to be converted to acids and their derivatives by the action of dehydrogenases (Weiner, 1979; Lindahl & Peterson, 1991). More recently, examples have been found in which P450 cytochromes catalyze the oxidation of aldehydes with the incorporation of molecular oxygen into the products; the mechanism of such conversions is not yet well understood. The present paper is concerned with the inactivation of P450[1] by a variety of aldehydes and the question of whether this process may by related to either of the two known oxidative pathways, one giving the corresponding carboxylic acid and the other yielding formate from the aldehyde carbon accompanied by desaturation or hydroxylation of the parent structure.

Some examples of acid formation are as follows: the oxidation of several xenobiotic aldehydes to carboxylic acids by mouse liver microsomes, including evidence for $^{18}O_2$ incorporation and inhibition by CO (Watanabe et al., 1990); the oxidation of acetaldehyde to acetic acid by P450 2E1-containing reconstituted membrane vesicles (Terelius et al., 1991); the conversion of 11-oxo-Δ^8-tetrahydrocannabinol to Δ^8-tetrahydrocannabinol-11-oic acid by P450 MUT-2 purified from hepatic microsomes of male ddN mice (Watanabe et al., 1991); the oxidation of several saturated and unsaturated aldehydes by mouse liver microsomes (Watanabe et al., 1992); and the oxidation of *all-trans*-, 9-*cis*-, and 13-*cis*-retinal to the corresponding retinoic acids by a number of purified rabbit liver microsomal P450s, of which the 1A1 isoform is the most active (Roberts et al., 1992; Raner et al., 1996). In addition, bacterial P450 BM3 catalyzes the oxidation of fatty acids with a terminal aldehyde group to the corresponding ω-diacids (Davis et al., 1996).

From an enzymological viewpoint, P450 aromatase is of much interest because of the unusual mechanism by which, in three sequential oxidative reactions, it effects demethylation of the 10β-methyl group of the androgen nucleus (Cole & Robinson, 1990). In the terminal reaction, oxidative deformylation of the aldehyde occurs to yield the estrogen and formic acid (Skinner & Akhtar, 1969). The mechanism of P450-catalyzed aromatization has been a topic of much debate. Akhtar and colleagues (1982) observed that the pattern of isotope label distribution and the incorporation of an atom from molecular oxygen into the products were inconsistent with an oxenoid oxidant. Further, they hypothesized that the active oxidant in the aromatase-catalyzed carbon−carbon bond cleavage step was a nucleophilic, iron−peroxo species (Akhtar et al., 1982; Stevenson et al., 1988). More recently, xenobiotic aldehydes have been shown in this laboratory to undergo a similar oxidative cleavage with purified liver microsomal P450s. The reactions include the

† This investigation was supported by Grant DK-10339 from the National Institutes of Health.

* Author to whom correspondence should be addressed: tel, (313) 764-9101; fax, (313) 763-4581; e-mail, gmraner@umich.edu.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: P450, cytochrome P450; reductase, NADPH-cytochrome P450 reductase; DLPC, dilauroylglyceryl-3-phosphorylcholine.

S0006-2960(96)03056-5 CCC: $14.00 © 1997 American Chemical Society

conversion of cyclohexanecarboxaldehyde to cyclohexene (Vaz et al., 1991), a series of low molecular weight branched-chain aldehydes to the n-1 alkenes and citronellal to 2,6-dimethyl-1,5-heptadiene (Roberts et al., 1991), and 3-oxo-decalin-4-ene-10-carboxaldehyde, a bicyclic steroid analog, to 3-hydroxy-6,7,8,9-tetrahydronaphthalene (Vaz et al., 1994). Our recent investigation on the effect of site-directed mutagenesis of threonine-302 to alanine on the activities of recombinant P450 2B4 (Vaz et al., 1996) makes use of evidence from others (Martinis et al., 1989; Imai et al., 1989) with bacterial P450cam that the mutation interferes with the activation of dioxygen to the oxenoid species by disrupting proton delivery to the active site. In particular, the deformylation of cyclohexanecarboxaldehyde by the mutant 2B4 P450 to produce cyclohexene was greatly increased, leading us to conclude that the iron-peroxy species is the oxygen donor.

We have recently reported the mechanism-based inactivation of purified P450 isozymes 2B4 and 1A2 by citral, a terpenoid α,β-unsaturated β-methyl aldehyde that is widely used as a flavorant and odorant (Raner et al., 1996). The inhibition was found not to be attributable to the corresponding acid or alcohol. Two earlier studies that are pertinent to our own work on aldehyde inactivation have dealt with the inhibition of P450 in microsomal suspensions. Patel et al. (1978) noted that *p*-xylene administration to rats resulted in a large decrease in pulmonary hydroxylation of this compound. The loss in catalytic activity was traced to the formation of *p*-tolualdehyde, which requires NADPH for the inactivation to occur in rat lung microsomes. White (1982) found that a number of saturated aliphatic aldehydes caused the NADPH-dependent destruction of P450 in liver microsomes with formation of a green fluorescing chromophore similar to the green pigment observed by others in NADPH-dependent inactivation by olefinic and acetylenic compounds (Ortiz de Montellano & Mico, 1980; Ortiz de Montellano & Kunze, 1981).

The present study makes use of the T302A mutant of P450 2B4, along with isotope effects and observations on the influence of aldehyde structure, to examine the inactivation of P450. The results indicate that a free radical species produced upon decomposition of the peroxyhemiacetal intermediate may react with the heme and thus account for the observed inhibition by aldehydes.

EXPERIMENTAL PROCEDURES

Enzymes, Substrates, and Products. NADPH-cytochrome P450 reductase (French & Coon, 1980), full-length rabbit liver cytochrome P450 2B4 (Coon et al., 1978), and truncated recombinant P450 2B4 (2B4 $\Delta 2-27$) (Pernecky et al., 1995) and the T302A mutant (2B4 $\Delta 2-27$ T302A) (Vaz et al., 1996) were purified according to these published procedures. Most of the aldehydes and other compounds used in this study were from Sigma, Aldrich, Fluka, or ICN, and those judged to be greater than 95% pure by GC/MS were used without further purification. Aldehydes that contained significant amounts of the corresponding acids were purified by extraction with 1:1 dichloromethane: ammonium bicarbonate (1.0 M). NADPH and DLPC were from Sigma. Deuterated aldehydes were prepared by reduction of the corresponding acids with $LiAlD_4$ followed by oxidation of the resulting alcohol with pyridinium chlorochromate. Deuterium incorporation was >95% as determined by GC/MS. The 2- and 3-phenylpropionic acid standards, prepared by oxidation of the aldehydes using the Jones reagent, were >95% pure as judged by GC/MS.

Inactivation of P450 by Aldehydes and Heme Determination. P450 2B4 (1.0 μM) was incubated at 30 °C with the reductase (0.5 μM), DLPC (50 μg/mL), potassium phosphate buffer, pH 7.4 (100 mM), and varying amounts of an aldehyde, with NADPH (1.0 mM) as the last addition, in a final volume of 0.2 mL. For the assay of remaining P450 catalytic activity, the mixtures were then diluted 1:20 into a reaction mixture containing 10 mM 1-phenylethanol and NADPH as described by Raner et al. (1996). The acetophenone formed in 20 min at 30 °C was determined by HPLC. At least two independent experiments were carried out with each of the aldehydes examined, and values presented represent the average of these values. Anaerobic inactivation experiments were performed in air-tight vials after repeated purging with argon. For determination of remaining NADPH oxidase activity of the P450, an aliquot was diluted 1:20 into a reaction mixture (final volume, 1.0 mL) containing 1.0 mM benzphetamine, 50 μg/mL DLPC, 100 mM potassium phosphate buffer (pH 7.4), and 0.15 mM NADPH. The decrease in NADPH absorbance at 340 nm at 30 °C was measured with time, and the rates were determined by linear regression analysis. In control experiments the reaction mixtures were immediately diluted upon the addition of NADPH and thus served to detect any non-mechanism-based inactivation. Plots of the ln of the fraction of remaining activity as a function of time were linear. The inverse of the individual rate constants for inactivation (k_{inact} values) determined in these experiments were then plotted vs the inverse of the aldehyde concentrations to yield K_I and maximal k_{inact} values for each aldehyde. For the determination of heme destruction, reactions were quenched with 1/200 volume of 88% formic acid and frozen immediately in a liquid nitrogen bath. Aliquots were then submitted to HPLC for heme determination at 405 nm.

Product Analysis. The products of 2- and 3-phenylpropionaldehyde metabolism were identified by comparison of the GC and HPLC retention times with those of authentic acid and n-1 alcohol standards. The 1- and 2-phenylethanol standards were well resolved by GC with a 6-ft Supelco 60/80 Carbopack B column containing 4% carbowax 20M and 0.8% KOH at 210 °C. The carrier gas was N_2 at a flow rate of 20 mL/min. Quantitation of the alcohols and acids formed was by HPLC analysis with a Waters Nova-Pak C_{18} reverse phase column and a mobile phase consisting of 30:70:0.07 acetonitrile:H_2O:trifluoroacetic acid at a flow rate of 1.0 mL/min. Detection of the products was at 220 nm with a Waters model 490E programmable wavelength detector in combination with a Waters model 600 controlling unit and model 710 autoinjector, all operated by the Millenium software package. *trans*-Cinnamic acid was quantified by HPLC under identical conditions, except that detection was at 240 nm. Samples to be used for heme extraction were prepared by the incubation of 1.0 mM aldehyde with liver microsomes from phenobarbital-induced rabbits (50 mg of protein) (Coon et al., 1978) in the presence or absence of 1.0 mM NADPH for 15 min at 30 °C. The reaction was quenched by the addition of ice-cold 5% H_2SO_4 in methanol as described by White (1982). Heme adducts to be used for mass spectral analysis were generated using

Table 1: Inactivation of P450 2B4 by Various Aldehydes[a]

aldehyde	K_I (mM)	k_{inact} (min^{-1})
p-nitrobenzaldehyde	1.45	2.65
3-phenylpropionaldehyde	0.71	0.31
phenylacetaldehyde	0.50	0.26
citronellal	1.0	0.23
benzaldehyde	0.86	0.15
cinnamaldehyde	0.09	0.10
citral	0.04	0.08
acetaldehyde	ND[b]	0.02
formaldehyde	ND[b]	0.02

[a] Rates of inactivation by aldehydes were determined by the effect on P450-catalyzed 1-phenylethanol oxidation in the reconstituted system as described in Experimental Procedures. Values reflect the mean of two independent determinations for which the standard errors are less than 5%. [b] Not determined; acetaldehyde and formaldehyde were used under saturating conditions.

purified P450 2B4 in a reconstituted system containing 7.5 μM P450, 2.0 μM reductase, 0.1 mM phosphate buffer (pH 7.4), 50 μg/mL DLPC, 1.0 mM 3-phenylpropionaldehyde, 250 units of catalase, and 1.0 mM NADPH in a 1.0-mL reaction mixture at 30 °C for 10 min. Heme adducts were detected by a variation of the procedure described by Osawa et al. (1990, 1994). Absorption at 350, 405, and 412 nm was monitored with the Waters HPLC system described above with a Vydac C_4 column and a solvent gradient from 45:55:0.055 acetonitrile:H_2O:trifluoroacetic acid to 70:30:0.03 acetonitrile:H_2O:trifluoroacetic acid over 20 min. Electrospray and MALDI (matrix assisted laser desorption ionization) mass spectral analyses were performed at The University of Michigan mass spectrometry core facility using a VG Fisons "Platform" single quadrupole mass spectrometer and Perspective Biosystems Vestec Lasertec MALDI linear time-of-flight mass spectrometer with the laser set at 327 nm. Samples were introduced in 50:50:0.05 acetonitrile:H_2O:trifluoroacetic acid.

RESULTS

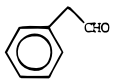
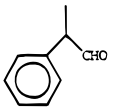
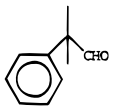
Inactivation of P450 by Various Aldehydes. The reaction of P450 2B4 in a reconstituted system with structurally diverse aldehydes leads to a decrease in catalytic activity. The structure clearly influences the rate of inactivation, as illustrated with a variety of aldehydes in Table 1; of those examined, citral, an α,β-unsaturated β-methyl aldehyde, has the lowest K_I, and *p*-nitrobenzaldehyde has the highest k_{inact} value. The importance of the carbonyl function was shown by experiments not presented in which the alcohols and acids corresponding to citral and cinnamaldehyde were found to be ineffective. As previously reported (Raner et al., 1996), inactivation by citral requires NADPH in the incubation with the cytochrome and the reductase, and catalase and epoxide hydrolase do not protect the P450. The current study shows that inactivation requires molecular oxygen and that superoxide dismutase, ascorbic acid, and glutathione do not protect the P450 (data not shown).

Structural Effects on Inactivation. Table 2 lists the rates of inactivation by a series of aldehydes in which the effect of α,β-unsaturation was determined. For the aliphatic compounds, including β-branched citronellal, as well as the aromatic compounds examined, unsaturation results in a decrease in the inactivation rate at the aldehyde concentrations used. In addition, the effect of branching in the α-position was examined, since it is known to enhance the deformylation reaction (Roberts et al., 1991). As indicated in Table 3, those compounds with α-methyl substituents are relatively poor inactivators of P450 2B4 as shown by both the 5-carbon aliphatic series and the aromatic aldehydes. For example, 2-phenylpropionaldehyde inactivates 2B4 much less effectively than phenylacetaldehyde, and dimethylphenylacetaldehyde does not inactivate at a significant rate.

Table 2: Inactivation of P450 2B4 by the Corresponding Saturated and Unsaturated Aldehydes

aldehyde	P450-saturating conctn (mM)	k_{inact} (min^{-1})
citronellal	2.5	0.24 ± 0.01
citral	1.0	0.08 ± 0.01
3-phenylpropionaldehyde	2.5	0.31 ± 0.02
trans-cinnamaldehyde	1.0	0.10 ± 0.01
nonyl aldehyde	1.0	0.70 ± 0.01
trans-2-nonenal	1.0	0.32 ± 0.01
octyl aldehyde	1.0	0.30 ± 0.03
trans-2-octenal	1.0	0.19 ± 0.01
heptanal	2.0	0.33 ± 0.01
trans-2-heptenal	2.0	0.24 ± 0.01
hexanal	2.0	0.23 ± 0.00
trans-2-hexenal	2.0	0.19 ± 0.01

Table 3: Effects of α-Branching on Inactivation of 2B4 by Aldehydes[a]

expt. no.	aldehyde	structure	k_{inact} (min^{-1})
1	valeraldehyde		0.18 ± 0.02
	2-methyl-butyraldehyde		0.05 ± 0.00
	trimethyl-acetaldehyde		0.05 ± 0.00
2	phenylacetaldehyde		0.25 ± 0.02
	2-phenyl-propionaldehyde		0.04 ± 0.00
	2,2-dimethylphenyl-acetaldehyde		0.01 ± 0.00

[a] The aldehyde concentration was 5.0 mM in experiment 1 and 2.0 mM in experiment 2.

Heme Destruction and Inactivation of P450. The loss of catalytic ability upon exposure to citral was compared to loss of the heme chromophore, as shown in Table 4. In the case of P450 2B4, the decreased activity, determined by the rates of NADPH consumption and 1-phenylethanol oxidation, was nearly identical to the rate of heme loss as determined by HPLC quantitation. In addition, a mutant form of P450 2B4 was examined in which the N-terminal 27 amino acid residues were deleted and the conserved threonine at position 302 was converted to alanine; the 2B4 Δ2−27 T302A showed more rapid loss of heme and more rapid loss of activity, as judged by NADPH consumption, under identical

Table 4: Comparison of Rates of Heme Loss and of P450 Inactivation Caused by Citral[a]

P450 examined	heme loss (min^{-1})	NADPH oxidation (min^{-1})	1-phenylethanol oxidation (min^{-1})
2B4	0.046	0.053	0.057
2B4 Δ2−27 T302A	0.095	0.110	ND[b]

[a] The citral concentration was 0.2 mM. Values given represent the average of two independent determinations. Standard deviations are less than 10% in all cases. [b] ND, not determined.

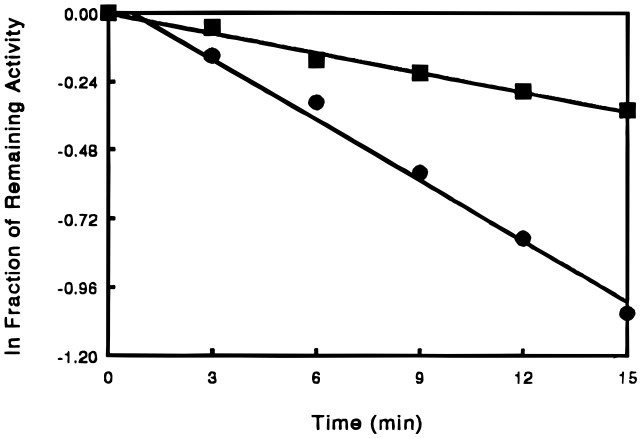


FIGURE 1: Semilog plots for the mechanism-based inactivation of P450 2B4 Δ2−27 (●) and P450 2B4 Δ2−27 T302A (■) by cinnamaldehyde. The reaction mixture contained 1.0 μM P450 and reductase, 1.0 mM cinnamaldehyde, 250 units of catalase, 1 mM ascorbic acid, 50 μg/mL DLPC, 100 mM phosphate buffer (pH 7.4), and 1.0 mM NADPH.

conditions. As with wild-type 2B4, the rate of inactivation of the mutant with respect to NADPH oxidation was similar to the rate of heme destruction. The time course of inactivation of 2B4 Δ2−27 and 2B4 Δ2−27 T302A, as shown in Figure 1, indicates that replacement of Thr-302 with Ala results in a 4−5-fold increase in the rate of inactivation by *trans*-cinnamaldehyde. The loss of heme during the inactivation of P450 by aldehydes may occur in a number of different ways. One possibility is the formation of an unstable heme adduct, which may eventually lead to complete bleaching of the chromophore. We examined this possibility by organic extraction and HPLC analysis of the heme products (data not shown) after limited inactivation of P450 in phenobarbital-induced microsomes with various aldehyde substrates followed by esterification by a published procedure (White, 1981). The chromatographic pattern for each aldehyde used was distinct, apparently indicating that the aldehyde structure influenced the properties of the resulting chromophore.

In addition, with purified P450 2B4 in a reconstituted system, a heme adduct formed during inactivation by 3-phenylpropionaldehyde. A representative HPLC chromatogram, obtained by direct injection of the reaction mixture containing inactivated P450 onto the column, is shown in Figure 2, along with the appropriate control in which NADPH was omitted. The adduct was purified and submitted to electrospray mass spectrometric analysis to determine the molecular weight. The mass of 719.5 for the modified heme is 104 mass units larger than native heme, which has a mass of 615.7. These values are consistent with the addition of a phenylethyl radical accompanied by the loss of a hydrogen atom from the porphyrin.

Mechanistic Studies Based on Product Analysis and Quantitation and on Deuterium Isotope Effects. Since aldehydes are metabolized by P450 to the corresponding carboxylic acids and in many cases also undergo deformylation to produce alcohols and alkenes, the possible relationship of these pathways to the inactivation process was explored. Benzaldehyde and 3-phenylpropionaldehyde were synthesized with deuterium in place of hydrogen in the aldehyde group and examined for isotope effects on the rates of P450 2B4 inactivation and carboxylic acid formation. As shown in Table 5, a V_{max} isotope effect of 2.6 was observed in benzoic acid formation, whereas replacement by deuterium had no effect on the rate of loss of P450 2B4 activity.

In other experiments with 2- and 3-phenylpropionaldehydes, the rates of $n - 1$ alcohol and acid formation were determined and compared to the rates of enzyme inactivation by the two compounds. The rate of acid formation was slower with 3-phenylpropionaldehyde than with 2-phenylpropionaldehyde as substrate, while the reverse was true with alcohol formation (Figure 3). The inactivation of P450 2B4 by the two aldehydes at various concentrations was then determined, as shown in Figure 4. The results clearly demonstrate that 3-phenylpropionaldehyde effects a substantially greater loss in catalytic activity and thus implicate the deformylation pathway rather than that leading to the carboxylic acid. Furthermore, 2B4 Δ2−27 T302A is much more effective than 2B4 Δ2−27 in the formation of 1-phenylethanol from 2-phenylpropionaldehyde, while acid formation is almost completely blocked with the mutant enzyme (data not shown). Taken together with the evidence already presented that the T302A mutant undergoes more rapid inactivation, these results also support our conclusion that alcohol formation is closely related to the inactivation that accompanies heme destruction.

DISCUSSION

The results presented above as well as our earlier report on the effect of citral on retinoid and 1-phenylethanol oxidation (Raner et al., 1996) clearly show that the loss of P450 2B4 activity upon exposure to aldehydes is a mechanism-based reaction. Inactivation of the cytochrome requires the presence of NADPH and molecular oxygen and is time-dependent. The aldehyde function of citral is essential to the inactivation as shown by the ineffectiveness of the corresponding acid and alcohol, and in results not presented, similar observations were made with *trans*-cinnamaldehyde, citronellal, and their acid and alcohol derivatives. The effect of citral was unchanged by catalase, superoxide dismutase, or epoxide hydrolase, indicating that superoxide and H_2O_2 generation is not the cause of the inhibition and that a reactive epoxide intermediate is probably not formed. Furthermore, the addition of ascorbic acid or glutathione as free radical scavengers failed to protect against the inactivation. We conclude that the inactivation process requires oxidative activation of the aldehyde by P450 and that the activated product, probably a radical species as discussed below, does not escape the active site of the cytochrome.

Inactivation of P450 by olefins, as described by Ortiz de Montellano and colleagues (1982, 1983), requires oxidative activation of the double bond followed by addition of the terminal olefinic carbon to a pyrrole nitrogen, resulting in

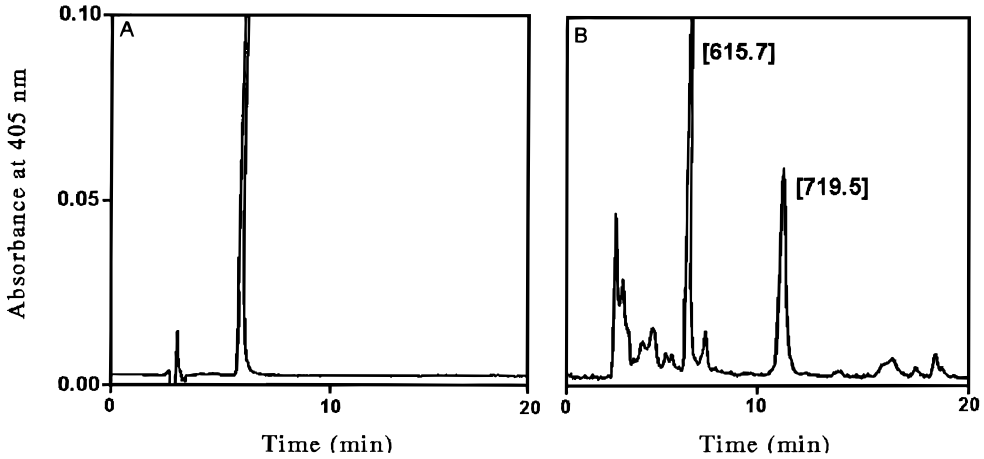


FIGURE 2: HPLC profile of the heme products resulting from the reaction of 1.0 mM 3-phenylpropionaldehyde with P450 2B4 in the absence (A) and presence (B) of 1 mM NADPH. Molecular weights for the native and modified heme as determined by electrospray mass spectrometry are given. Conditions for the reaction and HPLC analysis are given in the Experimental Procedures section.

Table 5: Deuterium Isotope Effects on Benzaldehyde Oxidation and P450 Inactivation[a]

parameter determined	benzaldehyde ^{1}H	benzaldehyde ^{2}H	$(^1H)/(^2H)$
Benzoic Acid Formation			
k_{cat} (nmol/min/nmol of P450)	9.8 ± 0.3	3.8 ± 0.2	2.6
K_m (mM)	1.1 ± 0.1	1.7 ± 0.1	0.7
k_{cat}/K_m	8.7	2.2	4.0
P450 Inactivation			
k_{inact} (min^{-1})	0.15 ± 0.1	0.15 ± 0.2	1.0

[a] Values are the average of two independent determinations.

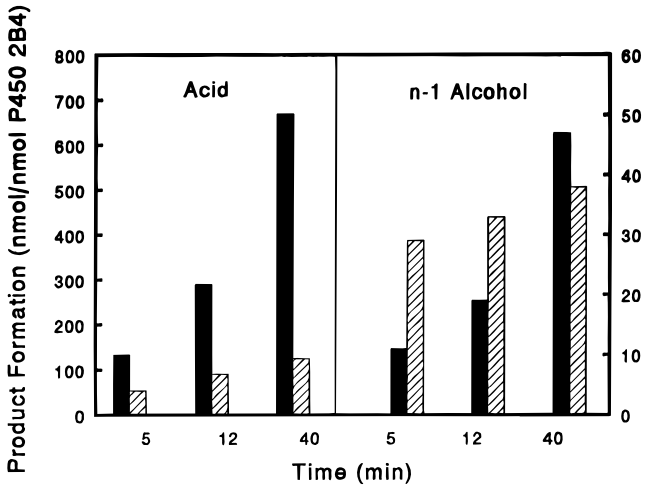


FIGURE 3: Rates of acid and $n-1$ alcohol formation in the P450 2B4-catalyzed oxidation of 2-phenylpropionaldehyde (solid bars) and 3-phenylpropionaldehyde (hashed bars). P450 and reductase concentrations were 0.4 μM, and substrate concentrations were 2.0 mM.

an N-alkylated heme that possesses a red-shifted absorption spectrum. Oxidation of the double bond is thought to generate a free radical at the terminal carbon atom, an sp^3 hybridized orbital which overlaps with the porphyrin π orbitals, thus facilitating adduct formation. White (1982) has reported the formation of a similarly modified heme in mouse liver microsomes as a result of NADPH-dependent octyl aldehyde metabolism by P450, but the identity of the modified heme and the mechanism by which it was formed were not addressed. To determine the extent to which heme destruction contributes to the inactivation of P450 by

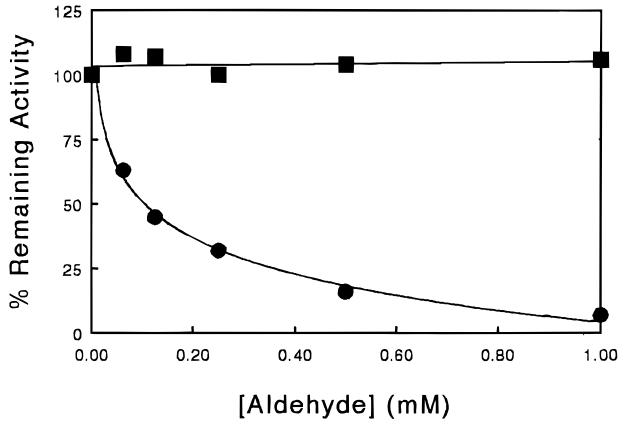


FIGURE 4: Remaining activity of P450 2B4 following a 10-min incubation at 30 °C with varying amounts of 2-phenylpropionaldehyde (■) or 3-phenylpropionaldehyde (●). P450 and reductase concentrations were 1.0 μM, and 1-phenylethanol oxidation was used to assay the remaining catalytic activity.

aldehydes, we have measured the rate of both processes in a reconstituted system under identical conditions. The loss in catalytic activity seen in the presence of citral was measured both by NADPH oxidation and by the oxidation of 1-phenylethanol to acetophenone. A good correlation between heme loss and activity loss was established, indicating that inactivation was apparently due to the loss of intact heme. In regard to the "green heme" reported by White (1982) as resulting from the addition of octyl aldehyde and NADPH to microsomes, we have examined the NADPH-dependent metabolism of a series of saturated and unsaturated aldehydes by P450 in microsomes and have also observed the formation of a red-shifted chromophore. The chromatographic and spectral properties of the heme degradation products formed in these reactions were significantly dependent on aldehyde structure, with each compound giving a unique HPLC profile. In fact, many of the aldehydes gave multiple chromatographic peaks, suggesting that multiple forms of the activated aldehyde may exist, each capable of giving adducts with the heme, or more likely that a single activated product reacts with the heme in several ways. We conclude, therefore, that heme destruction during P450 inactivation by aldehydes proceeds, at least in part, through initial covalent modification of the porphyrin structure by an activated aldehyde species.

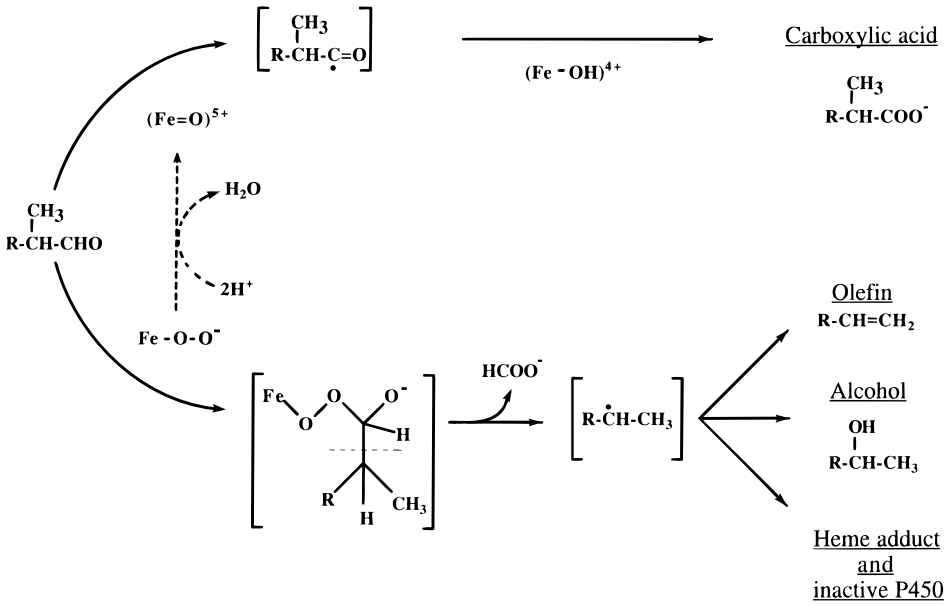


FIGURE 5: Proposed pathways for the P450-dependent conversion of a typical aldehyde to the carboxylic acid with involvement of the oxenoid−iron species or to the n-1 olefin and alcohol with involvement of the peroxo−iron species. Fe represents the iron atom at the active site. Also shown is the role of the alkyl radical generated in the deformylation pathway in heme adduct formation, which apparently leads to P450 inactivation.

Since impairment of the catalytic competence of P450 by aldehydes requires metabolic activation, we have considered the possible relevance of the two known metabolic pathways for aldehyde metabolism by this cytochrome, namely, oxidative carbon-carbon bond cleavage to produce formate and the n-1 product (Vaz et al., 1994) and oxidative conversion to the carboxylic acid (Watanabe et al., 1991). The former pathway has been examined extensively as it relates to the desaturation reaction catalyzed by the steroidogenic P450s, aromatase (Oh & Robinson, 1993), and $P450_{17\alpha}$ (Swinney & Mak, 1994). Akhtar et al. (1982) first proposed that the deformylation reaction occurs via the nucleophilic attack of an iron-bound peroxide on the carbonyl carbon of the aldehyde, resulting in the formation of a peroxyhemiacetal intermediate, which undergoes homolytic O−O bond cleavage and rearranges to give the observed products. In an attempt to provide evidence for peroxo−iron in this reaction, Vaz et al. (1996) made a mutant of P450 2B4 in which the conserved Thr-302 was converted to Ala, thus eliminating the putative proton donor for promotion of the heterolytic O−O bond cleavage step. This mutant was 5−10 times as active as the wild-type enzyme in converting cyclohexanecarboxaldehyde to cyclohexene, while the ability of the mutant to catalyze benzphetamine N-demethylation and cyclohexane hydroxylation was severely diminished. In the present study we have used this T302A mutant to examine the possibility that peroxo−iron may be involved in the inactivation of P450 by aldehydes. The results show that the mutant is inactivated by *trans*-cinnamaldehyde about 4 times faster than the wild type. As with full-length wild-type 2B4, heme loss with the T302A mutant correlates very well with the rate of inactivation. Thus, our results with the mutant enzyme show an apparent role of the deformylation pathway in activity loss and heme destruction.

Watanabe et al. (1992) have examined carboxylic acid formation in microsomes from a number of xenobiotic aldehydes and have made the interesting observation that ^{18}O from molecular oxygen is incorporated into this product from saturated, but not unsaturated, aldehydes. They suggest either the involvement of two distinct enzyme systems (aldehyde dehydrogenases and P450s) or alternative catalytic mechanisms for the same enzyme system. In the present study we have examined the acid-forming reaction with purified P450 2B4 in a reconstituted enzyme system and have found that, in general, the rate of formation of carboxylic acids from aldehydes greatly exceeds that for the formation of the n-1 products. For example, with 1.0 mM 2-phenylpropionaldehyde as a substrate for the full-length 2B4, acid formation is favored nearly 50:1 (data not presented). Furthermore, inactivation of the T302A mutant enzyme by cinnamaldehyde is much more rapid than that of the wild-type enzyme but that cinnamic acid formation by the mutant is nearly abolished.

In an attempt to establish a correlation between product formation and inactivation, we have measured the rates of acid formation, alcohol formation, and P450 inactivation with the primary and secondary aldehydes 3- and 2-phenylpropionaldehyde. At saturating levels of the two compounds, the rate of inactivation of P450 2B4 by the 3-phenyl compound was much higher than with 2-phenylpropionaldehyde. Under identical conditions, the rate of acid formation from the secondary aldehyde was higher than for the primary aldehyde. Alcohol formation, however, was much more rapid with 3-phenylpropionaldehyde. Of further interest, the 2B4 T302A mutant that serves as a marker for peroxo *vs* oxenoid chemistry is dramatically enhanced in its ability to effect the formation of the n-1 alcohol from 2-phenylpropionaldehyde, while the ability to promote acid formation is nearly lost. The data suggest that the mechanism of P450 inactivation by aldehydes is related to n-1 alcohol formation and distinct from carboxylic acid formation.

Additional evidence concerning these reactions was obtained with several aldehydes labeled with a deuterium atom at the aldehyde carbon. A V_{max} isotope effect of 2.6 on acid formation was observed, in keeping with a mechanism involving a partially rate-limiting hydrogen abstraction followed by oxygen rebound; thus, an oxenoid−iron inter-

mediate would account for the observed effect. By comparison, Vaz and Coon (1994) reported intramolecular and steady state isotope effects of 2.6 and 2.8, respectively, in the oxidation of benzyl alcohol to benzaldehyde by P450 2B4, a reaction also thought to occur via hydrogen abstraction and oxygen rebound. Davis et al. (1996) found no deuterium isotope effect on the oxidation of ω-oxo fatty acids by P450 BM3; however, BM3 is distinct from the mammalian forms in that it has a self-contained reductase domain and may have a different rate-limiting step. The observation that deuterium has no detectable effect on the rate of inactivation by the aldehydes used in the present study further suggests a mechanistic distinction between acid formation and inactivation. Given the ability of the T302A mutant to promote enhanced peroxo chemistry, we conclude that the mechanism-based inactivation of P450 by aldehydes occurs through the formation of a peroxyhemiacetal intermediate. The scheme in Figure 5 summarizes our findings and shows the requirement for protons in the active site for cleavage of the oxygen−oxygen bond in the oxenoid route but not for the deformylation pathway that leads to the formation of an olefin or an alcohol with one less carbon atom and also to P450 inactivation.

We have also examined the effects of α-branching and α,β-unsaturation in the aldehyde on P450 inactivation. Under apparent substrate-saturating conditions, α-branching dramatically lowers the k_{inact} of the aldehyde. The proposed pathway for the formation of n-1 alcohol, as shown in Figure 5, involves the intermediate formation of an alkyl radical that combines with an iron-bound hydroxyl radical to form the product. Branching in the α-position of the aldehyde substrate would lead to the formation of a secondary radical that might be less reactive than the corresponding primary radical, and thus the rate of inactivation of the enzyme via heme adduct formation would be expected to decrease. The general reduction in inactivation potency of the unsaturated relative to the saturated compounds could be related to a lack of rotational freedom around the α,β-bond. For example, inactivation by olefins requires the overlap of the sp^3-hybridized terminal carbon orbital with the π-system of the porphyrin (Ortiz de Montellano et al., 1983). After β-scission of the putative peroxyhemiacetal intermediate formed from α,β-unsaturated aldehydes, the orientation of the resulting sp^2-hybridized orbital in which the alkyl radical resides may not be favorable for overlap with the porphyrin π-system. The rotational freedom afforded in the saturated compound about the α,β-bond may contribute, therefore, to the inactivating potential of these compounds. In fact, as the molecular weights of the compounds used in this study decrease, the effect of α,β-unsaturation appears to decrease as well, suggesting that translational motion may compensate for a lack of rotational freedom in the smaller substrates.

That inactivation of P450 2B4 by 3-phenylpropionaldehyde occurs via a free radical process as represented in Figure 5 was indicated by electrospray and laser desorption mass spectrometry analysis of the modified heme. Both methods gave identical values for the net gain in molecular weight for adduct formation relative to the native heme. The value of 104 Da is consistent with addition of an alkyl radical accompanied by loss of a hydrogen atom from the porphyrin. Several additional aldehydes have been examined with respect to adduct formation, and all appear to form similar types of modified chromophores, but mass spectral data are not yet available.

ACKNOWLEDGMENT

We are grateful to Dr. Yoichi Osawa, Department of Pharmacology, The University of Michigan, for helpful discussions.

REFERENCES

Akhtar, M., Calder, M. R., Corina, D. L., & Wright, J. N. (1982) *Biochem. J. 201,* 569−580.
Cole, P. A., & Robinson, C. H. (1990) *J. Med. Chem. 33,* 2933−2942.
Coon, M. J., van der Hoeven, T. A., Dahl, S. B., & Haugen, D. A. (1978) *Methods Enzymol. 52,* 109−117.
Davis, S. C., Sui, Z., Peterson, J. A., & Ortiz de Montellano, P. R. (1996) *Arch. Biochem. Biophys. 328,* 35−42.
DeMaster, E. G., Shirota, F. N., & Nagasawra, H. T. (1986) *Biochem. Pharmacol. 35,* 1481−1489.
French, J. S., & Coon, M. J. (1979) *Arch. Biochem. Biophys. 195,* 565−577.
Imai, M., Shimada, H., Watanabe, Y., Matsushima-Hibiya, Y., Makino, R., Koga, H., Horiuchi, T., & Ishimura, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 7823−7827.
Ji, C., Mirvish, S. S., Nickols, J., Ishizaki, H., Lee, M. J., & Yang, C. S. (1989) *Cancer Res. 49,* 5299−5304.
Lindahl, R., & Peterson, D. R. (1991) *Biochem. Pharmacol.* 41, 1583−1587.
Marinello, A. J., Bansal, S. K., Paul, B., Koser, P. L., Love, J., Struck, R. F., & Gurtoo, H. L. (1984) *Cancer Res. 44,* 4165−4621.
Martinis, S. A., Atkins, W. M., Stayton, P. S., & Sligar, S. G. (1989) *J. Am. Chem. Soc. 111,* 9252−9253.
Oh, S. S., & Robinson, C. H. (1993) *J. Steroid Biochem. Mol. Biol. 44,* 389−397.
Ortiz de Montellano, P. R., & Mico, B. A. (1980) *Mol. Pharmacol. 18,* 128−135.
Ortiz de Montellano, P. R., & Kunze, K. L. (1981) *Biochemistry 20,* 7266−7271.
Ortiz de Montellano, P. R., Kunze, K. L., Beilan, H. S., & Wheeler, C. (1982) *Biochemistry 21,* 1331−1339.
Ortiz de Montellano, P. R., Mangold, B. L. K., Wheeler, C., Kunze, K. L., & Reich, N. O. (1983) *J. Biol. Chem. 258,* 4208−4213.
Osawa, Y., Martin, B. M., Griffin, P. R., Yates, J. R., III, Shabanowitz, J., Hunt, D. F., Murphy, A. C., Chen, L., Cotter, R. J., & Pohl, L. R. (1990) *J. Biol. Chem. 265,* 10340−10346.
Osawa, Y., Fellows, C. S., Meyer, C. A., Woods, A., Castoro, J. A., Cotter, R. J., Wilkins, C. L., & Highet, R. J. (1994) *J. Biol. Chem. 269,* 15481−15487.
Patel, J. M., Harper, C., & Drew, R. T. (1978) *Drug Metab. Dispos. 6,* 368−374.
Pernecky, S. J., Olken, N. M., Bestervelt, L. L., & Coon, M. J. (1995) *Arch. Biochem. Biophys. 318,* 446−456.
Raner, G. M., Vaz, A. D. N., & Coon, M. J. (1996) *Mol. Pharmacol. 49,* 515−522.
Roberts, E. S., Vaz, A. D. N., & Coon, M. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 8963−8966.
Roberts, E. S., Vaz, A. D. N., & Coon M. J. (1992) *Mol. Pharmacol. 41,* 427−433.
Skinner, S. J. M., & Akhtar, M. (1969) *Biochem. J. 114,* 75−81.
Stevenson, D. E., Wright, J. N., & Akhtar, M. (1988) *J. Chem. Soc. Perkin Trans. I* 2043−2052.
Swinney, D. C., & Mak, A. Y. (1994) *Biochemistry 33,* 2185−2190.
Terelius, Y., Norsten-Hoog, C., Cronholm, T., & Ingelman-Sundberg, M. (1991) *Biochem. Biophys. Res. Commun. 179,* 689−694.
Vaz, A. D. N., & Coon, M. J. (1994) *Biochemistry 33,* 6442−6449.
Vaz, A. D. N., Roberts, E. S., & Coon, M. J. (1991) *J. Am. Chem. Soc. 113,* 5886−5887.

Vaz, A. D. N., Kessell, K. J., & Coon, M. J. (1994) *Biochemistry 33,* 13651−13661.

Vaz, A. D. N., Pernecky, S. J., Raner, G. M., & Coon, M. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93,* 4644−4648.

Watanabe, K., Narimatsu, S., Yamamoto, I., & Yoshimura, H. (1990) *Biochem. Biophys. Res. Commun. 166,* 1308−1312.

Watanabe, K., Narimatsu, S., Yamamoto, I., & Yoshimura, H. (1991) *J. Biol. Chem. 266,* 2709−2711.

Watanabe, K., Matsunaga, T., Narimatsu, S., Yamamoto, I., & Yoshimura, H. (1992) *Biochem. Biophys. Res. Commun. 188,* 114−119.

Weiner, H. (1979) in *Biochemistry and Pharmacology of Ethanol* (Majchrowicz, E., Noble, E. P., Eds.), Vol. 1, pp 107−124, Plenum Press, New York.

White, I. N. H. (1982) *Chem.-Biol. Interact. 39,* 231−243.

BI9630568