Discrete Species of Activated Oxygen Yield Different Cytochrome P450 Heme Adducts from Aldehydes†

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ABSTRACT: Aldehydes are known to inactivate cytochrome P450 in the reconstituted enzyme system containing NADPH and NADPH−cytochrome P450 reductase under aerobic conditions in a mechanism-based reaction involving heme adduct formation [Raner, G. M., Chiang, E. W., Vaz, A. D. N., and Coon, M. J. (1997) *Biochemistry 36*, 4895−4902]. In the study presented here, artificial oxidants were used to examine the mechanism of aldehyde activation by purified P450 2B4 in the absence of the usual O_2-reducing system, and the adducts that were formed were isolated and characterized. With hydrogen peroxide as the oxidant, 3-phenylpropionaldehyde gives an adduct with a mass corresponding to that of native heme modified by a phenylethyl group, presumably arising from the reaction of a peroxy−iron species with the aldehyde to give a peroxyhemiacetal, which upon deformylation yields the alkyl radical. NMR analysis indicated that the substitution is specifically at the γ-meso position. In contrast, with *m*-chloroperbenzoic acid as the oxidant, an adduct is formed from 3-phenylpropionaldehyde with a mass that is consistent with the addition of a phenylpropionyl group, apparently arising by hydrogen abstraction from the aldehyde to give the carbonyl carbon radical. *m*-Chloroperbenzoic acid by itself forms a heme adduct with a mass corresponding to the addition of a chlorobenzoyloxy group apparently derived from homolytic oxygen−oxygen bond cleavage. These and other results with nonanal and 2-*trans*-nonenal support the concept that this versatile enzyme utilizes discrete oxidizing species in heme adduct formation from aldehydes.

Cytochrome P450 (P450)[1] is unmatched among known oxygenases in the breadth of its catalytic activities, which encompass the oxidation or other transformations of compounds as diverse as physiologically important substances, including steroids and retinoids, and xenobiotics, including drugs, procarcinogenic hydrocarbons, and pesticides. These catalysts are subject to various types of endogenous regulation and in addition may be inactivated by the reactive metabolites that they generate. In the latter case, these products may bind to the heme or to the protein of the P450 cytochromes or may even lead to covalent binding of the heme to the protein moiety (*1*−*5*). In a comprehensive review of the possible modifications of the prosthetic group of hemoproteins, Ortiz de Montellano (*6*) noted that reactions of radicals include additions to the iron atom, pyrrole carbons, pyrrole nitrogens, vinyl groups, and meso carbons.

Well-established types of organic compounds that lead to heme adducts of P450 include olefins (*7*, *8*), acetylenes (*9*), dihydropyridines (*3*), hydrazines (*10*), and halogenated compounds (*11*). Our discovery that aldehydes also undergo mechanism-based oxidation by P450, resulting in heme modification with the loss of catalytic activity, grew out of studies with two types of physiologically important substrates, retinoids and products of membrane lipid peroxidation. The oxidation of various isomers of retinal to retinoic acids is catalyzed chiefly by P450 1A1, and the reaction is inhibited by citral, an α,β-unsaturated terpenoid, in a reaction dependent upon NADPH and molecular oxygen (*12*). Similar dependencies were found for the metabolic activation of *trans*-4-hydroxy-2-nonenal, a particularly toxic product of lipid peroxidation that is further oxidized with concomitant inactivation of several P450s, particularly 2B1 and 2B4 (*13*). The adduct formed with P450 2B4 was submitted to mass spectral analysis and found to have a mass consistent with the addition of the presumed carbonyl carbon radical formed from the aldehyde and the loss of a hydrogen atom from the porphyrin (*13*). Thus, adduct formation may occur during oxidation of aldehydes to the carboxylic acids. Another route involves a reaction in which aldehydes undergo loss of the aldehyde carbon as formate, with desaturation of the remaining structure. This pathway is involved in the terminal reaction in which an androgen is converted to an estrogen (*14*), and has been studied extensively in our laboratory

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[1] Abbreviations: P450, cytochrome P450; reductase, NADPH−cytochrome P450 reductase; 3-PPA, 3-phenylpropionaldehyde; *m*-CPBA, *m*-chloroperbenzoic acid; DLPC, dilauroylglyceryl-3-phosphorylcholine; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser spectroscopy.

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with a variety of xenobiotic aldehydes that undergo deformylation with purified liver microsomal P450s (*15*−*17*). This route also leads to heme adduct formation, as shown with a variety of aldehydes, and in the case of 3-PPA, the modified heme was shown to have a molecular weight corresponding to that expected if a peroxyhemiacetal intermediate had undergone homolytic cleavage with the loss of the aldehyde function and formation of a reactive alky radical (*18*).

Hypervalent iron−oxene has long been believed to be the sole oxidant in P450-catalyzed reactions (*19*, *20*). Various laboratories have had partial success in identifying this putative species by spectroscopic methods (*21*, *22*), and recent developments in transient state crytallographic techniques appear to have identified such an oxidant in the P450cam reaction cycle (*23*). However, in addition to the electrophilic iron−oxene species, the occurrence of a functional nucleophilic iron−peroxo species was proposed by Akhtar et al. (*24*) to occur in P450-catalyzed androgen demethylation and by this laboratory to participate in the deformylation of xenobiotic aldehydes (*15*−*17*). Of several agents known to support P450 catalysis in the absence of NADPH and O_2, H_2O_2 functions in the decarbonylation of cyclohexane carboxaldehyde to yield cyclohexene and formate, but iodosobenzene, *m*-CPBA, and cumyl hydroperoxide do not (*16*). Additional evidence for alternative oxygenating agents in catalysis was provided by studies with the T302A mutant of P450 2B4, in which proton delivery is apparently blocked and aldehyde deformylation is greatly enhanced (*25*). This alteration in a microsomal P450 was selected on the basis of earlier evidence that the T252A mutant of P450cam exhibits greatly diminished activity in camphor hydroxylation (*26*, *27*). More recently, we have examined the epoxidation of olefins by mutants of P450 cytochromes and have obtained evidence for a hydroperoxo−iron species as an electrophilic oxidant (*28*). In this paper, we have used artificial oxidants to generate heme adducts of P450 2B4 from aldehydes in the absence of NADPH and the reductase. The results provide additional support for alternative oxygenating agents in P450 catalysis.

EXPERIMENTAL PROCEDURES

Materials. Rabbit liver microsomal cytochrome P450 2B4 and NADPH−cytochrome P450 reductase were purified according to previously published procedures (*29*, *30*). NADPH and DLPC were from Sigma. Hydrogen peroxide, *m*-CPBA, cumyl hydroperoxide, *tert*-butyl hydroperoxide, 3-PPA, nonanal, and nonenal were from Aldrich. Iodosobenzene was prepared, and the purity was determined as described previously (*21*). The aldehydes were converted to the dimethylacetals, which were purified by silica gel column chromatography and judged to be more than 98% pure by GC−MS. The free aldehydes were freshly prepared on the day of use by hydrolysis of the acetals with 1 mM HCl (for nonanal and nonenal) or 10 mM HCl (for 3-PPA) for 30 min at room temperature.

Detection of Heme Adducts Formed from Aldehydes in the Reconstituted P450 System. The reaction mixture contained 2.0 μM P450 2B4, 1.0 μM reductase, 30 μg/mL DLPC, 2.0 mM aldehyde in 50 mM potassium phosphate buffer (pH 7.4), and 1.0 mM NADPH as the last addition in a final volume of 0.5 mL. The mixture was incubated at 30 °C for 10 min. A portion of the mixture (0.1 mL) was then injected directly onto a C4 Vydac column (5 μm, 4.6 mm × 250 mm) and submitted to HPLC at room temperature at a flow rate of 1.0 mL/min. A solvent linear gradient was programmed to deliver acetonitrile and aqueous 0.1% trifluoroacetic acid (pH ∼2.0) from a 40:60 to a 50:50 ratio by volume over the course of 15 min, then to an 80:20 ratio over the course of 15 min, and finally to 100% acetonitrile over the course of 5 min. In typical experiments, the retention time was 11 min for intact heme and between 13 and 28 min for the heme adducts. Before mass spectral analysis, the samples were concentrated as follows. Each sample containing a heme peak was collected, diluted 3-fold with 0.1% trifluoroacetic acid, and loaded onto a C_{18} Sep-Pak cartridge from Waters. The cartridge was washed with 10 column volumes of 0.1% trifluoroacetic acid, and the heme was then eluted with 100% methanol.

Detection of Heme Adducts Formed with Artificial Oxidants. Artificial oxidants, including hydrogen peroxide, iodosobenzene, *m*-CPBA, cumyl hydroperoxide, and *tert*-butyl hydroperoxide, were used to form heme adducts. To a reaction mixture containing 2.0 μM P450 2B4, 30 μg/mL DLPC, and 2.0 mM aldehyde in 50 mM phosphate buffer (pH 7.4) was added an oxidant (70 μM) to initiate the reaction. The final volume was 0.5 mL. The mixture was incubated at 30 °C for 10 min, and heme products were detected by HPLC as described above.

Purification of Heme Adducts for NMR Analysis. The reaction mixture contained 40 μM P450 2B4, 30 μg/mL DLPC, and 2.0 mM 3-PPA or nonenal in 50 mM phosphate buffer (pH 7.4), and hydrogen peroxide or *m*-CBPA (1.4 mM) was then added for the experiment with 3-PPA and nonenal, respectively, in a final volume of 2.0 mL. The reaction was allowed to proceed for 10 min at 30 °C, and the mixture was acidified with 1.0 N HCl to pH 1.0. Two volumes of 2-butanone that had been treated with sodium thiosulfate to remove peroxides was added to extract the hemes. The organic phase was concentrated in a Speed Vac concentrator, and the residue was dissolved in methanol and submitted to HPLC at a flow rate of 3.0 mL/min. A C4 Vydac semipreparative column (10 μm, 10 mm × 250 mm) was used to separate heme from its adducts. The mobile phase was the same as that under the analytical conditions described above. The eluted heme adducts were freeze-dried by lyophilization and then stored at −70 °C. A typical yield of adduct from 3-PPA with hydrogen peroxide was about 30%, while that from nonenal with *m*-CPBA was about 15%. The total amounts of 2B4 used for NMR spectra were 1.5 and 3.0 μmol for the 3-PPA and nonenal adducts, respectively. Other free adducts were not detected, but irreversibly bound adducts would not have been detected, since radioactive aldehydes were not tested.

Identification of Heme Adducts by NMR. Pooled samples of the heme adduct were dissolved in deuterium oxide in the presence of triethylamine to increase solubility, and then freeze-dried overnight, the process being repeated twice to remove remaining water. Dry samples were dissolved in 0.5 mL of [$^2H_5$]pyridine followed by reduction by stannous chloride (*31*). ^{1}H NMR spectra were determined in deuterated pyridine with a Bruker Avance DRX 500 spectrometer,

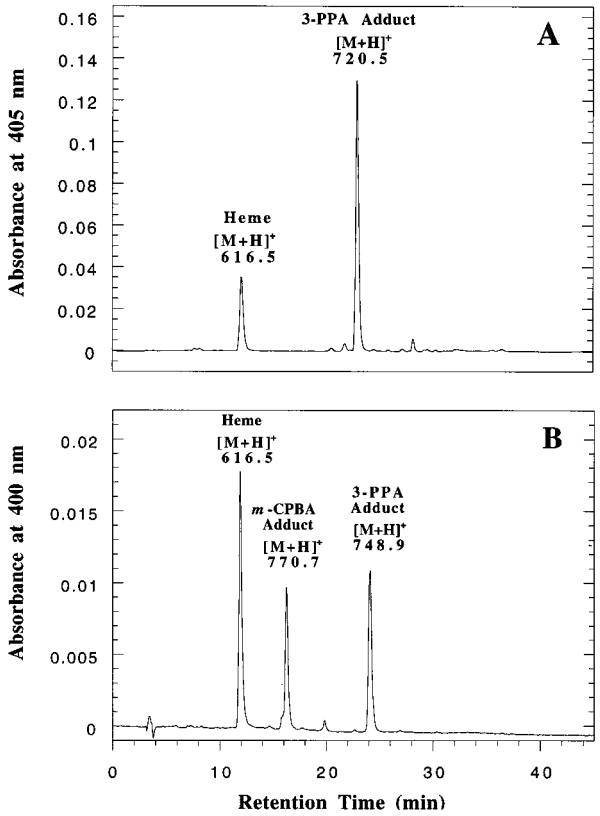

FIGURE 1: HPLC profile of intact heme and heme adducts resulting from the oxidation of 3-PPA by P450 2B4 in the presence of hydrogen peroxide (A) or *m*-CPBA (B). Molecular weights of the native and modified hemes were determined by electrospray mass spectrometry. The conditions for the reaction and for HPLC analysis were as described in Experimental Procedures.

operated at 500 MHz, at The University of Michigan Biochemical NMR Spectrometry Core Facility. ^{1}H NOESY data with water-gate solvent suppression were obtained with use of a delay time of 1.3 s and a mixing time of both 300 and 800 ms and processed on an Indy Silicon Graphics data station. The NOESY spectra were obtained with 1K data points in the t_2 dimension and a lK block of 128 scans each in the t_1 dimension. The free induction decays were zero filled once in both dimensions and apodized with a sine function.

Other Procedures. Absorption spectra were obtained with a Varian Cary 3E UV−visible spectrophotometer. For HPLC analysis, a Waters 996 photodiode array detector was connected to the system to obtain the absorption spectra and peak profiles simultaneously. Molecular weights of heme and heme adducts were determined by electrospray mass spectrometry performed at the Michigan Core Facility with a VG Fisons "Platform" single-quadrupole spectrometer.

RESULTS

Heme Adducts Generated from 3-PPA by P450 with Added Oxidants. To a reaction mixture containing P450 2B4 and 3-PPA was added hydrogen peroxide as described in Experimental Procedures, and after 10 min, the mixture was examined by HPLC with monitoring at 405 nm; the results are shown in Figure 1A. The retention times for the intact heme and the adduct were about 11 and 23 min, respectively. Each heme component was concentrated and submitted to electrospray mass spectrometry, which showed molecular ions $[M + H]^+$ at *m*/*z* 616.5 and 720.5, respectively. The mass of the 3-PPA-generated adduct was 104 units larger than that of intact heme, which suggests that 3-PPA underwent deformylation to form a phenylethyl radical (*m*/*z* 105) that gave an adduct accompanied by the loss of a hydrogen atom from the porphyrin. This finding is consistent with the results of a previous study in which the adduct was generated in the reconstituted system containing NADPH (*18*).

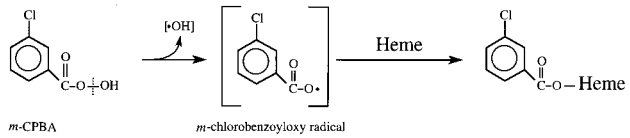

FIGURE 2: Proposed homolysis of *m*-CPBA.

With *m*-CPBA as the oxidant, however, two major heme products were detected. The HPLC profile of this reaction mixture at 400 nm (Figure 1B) exhibited peaks with retention times of 17 and 25 min, as well as the starting heme at 12 min. Mass spectral analysis revealed two product ions, with peaks at *m*/*z* 770.7 and 748.9. The mass of 770.7 for the peak with retention time of 17 min does not involve 3-PPA, since the same product was detected in a control experiment with only *m*-CPBA present (results not shown). This adduct is 154.2 units larger than heme, while the molecular weight of *m*-CPBA is 172.5 units. Thus, the results indicate that *m*-CPBA apparently forms the *m*-chlorobenzoyloxy radical by homolytic cleavage of the oxygen−oxygen bond, followed by addition to the porphyrin (Figure 2). For the peak in Figure 1B with a retention time of 25 min, the mass of 748.9 is 132.4 units larger than that of heme, which is consistent with the addition of a 3-PPA carbonyl carbon radical and the loss of a hydrogen atom from the porphyrin.

Heme Adducts Generated from Nonanal and 2-trans-Nonenal. Our earlier studies showed that heme adduct formation from 3-PPA in the reconstituted P450 enzyme system containing NADPH involves oxidative loss of the formyl group, but adduct formation from *trans*-4-hydroxy-2-nonenal involves loss of only the aldehyde hydrogen atom upon oxidation to the acid (*13*). Because of other structural differences, however, it was not clear whether the pathway was altered by the presence of α,β-unsaturation, and nonanal and nonenal were therefore compared in this investigation. As shown in Figure 3A, the major heme adduct generated from nonanal in the reconstituted system containing NADPH has a retention time of 28 min upon HPLC analysis with monitoring at 405 nm. The molecular ion $[M + H]^+$ was determined to be at *m*/*z* 728.9. The difference in *m*/*z* between this adduct and intact heme is 112.4 units, which is consistent with the deformylation pathway leading to formation of an octyl radical that reacts with the heme. When hydrogen peroxide was added in place of NADPH and the reductase, the same heme adduct was formed as judged by the retention time of 28 min as determined by HPLC analysis and the $[M + H]^+$ value of 728.9 (Figure 3B). *trans*-2-Nonenal was then examined in the reconstituted enzyme system containing P450 2B4, the reductase, and NADPH, and the HPLC profile of the resulting modified heme was determined. As shown in Figure 3C, the adduct monitored at 400 nm had a retention time of 27 min, and the molecular ion $[M + H]^+$ was

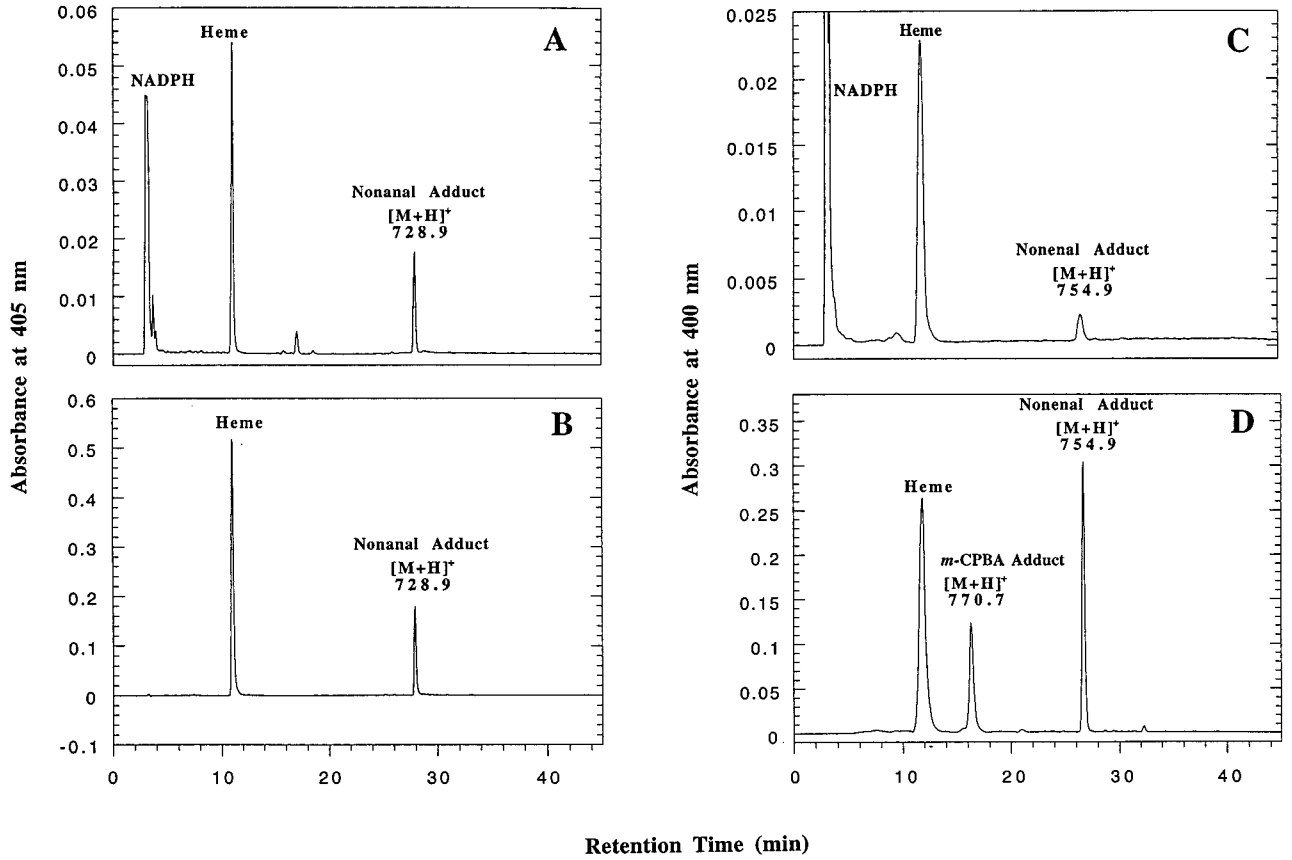

FIGURE 3: HPLC profiles of the intact heme and heme adducts resulting from nonanal oxidation by P450 2B4 in the reconstituted system (A) or in the presence of hydrogen peroxide (B) and of the intact heme and heme adducts resulting from nonenal oxidation by P450 2B4 in the reconstituted system (C) or in the presence of *m*-CPBA (D).

detected at *m*/*z* 754.9. The difference in *m*/*z* between the adduct and intact heme is 138.4 units, which is consistent with the addition of a nonenoyl carbonyl carbon radical and the loss of a hydrogen atom from the porphyrin. With *m*-CPBA as the oxidant, the same nonenal-generated heme adduct was formed, as shown in panel D, with a retention time of 28 min and an ion $[M + H]^+$ of 754.9. Also formed was the *m*-CPBA-generated heme adduct described above as being formed in the absence of an aldehyde, with a retention time of 16 min and an ion $[M + H]^+$ of 770.7. In the experiments whose results are not shown, HPLC analysis demonstrated the formation of the same adduct from nonenal with oxidants other than *m*-CPBA, including iodosobenzene, *tert*-butylhydroperoxide, and cumene hydroperoxide, but no adduct was formed with hydrogen peroxide.

Absorption Spectra of Intact Heme and Adducts. After purification of the modified hemes by HPLC, their absorption spectra in the visible region were determined. Except for the starting heme, the extinction coefficients have not been determined accurately; the concentrations were in the same range but not necessarily identical to that of heme, which was 10 μM. Compared to heme with a Soret peak at 399 nm, the maxima for the adducts are all red-shifted, as shown in Figure 4. The alkylated hemes derived from 3-PPA and nonanal with hydrogen peroxide as the oxygen donor have a maximum at 408 nm, and the acylated hemes derived from 3-PPA and nonenal with *m*-CPBA as the donor have a maximum at 402 nm. The adduct formed directly from *m*-CPBA, with or without an aldehyde present, is presumed to be an ester, and it also absorbs maximally at 402 nm.

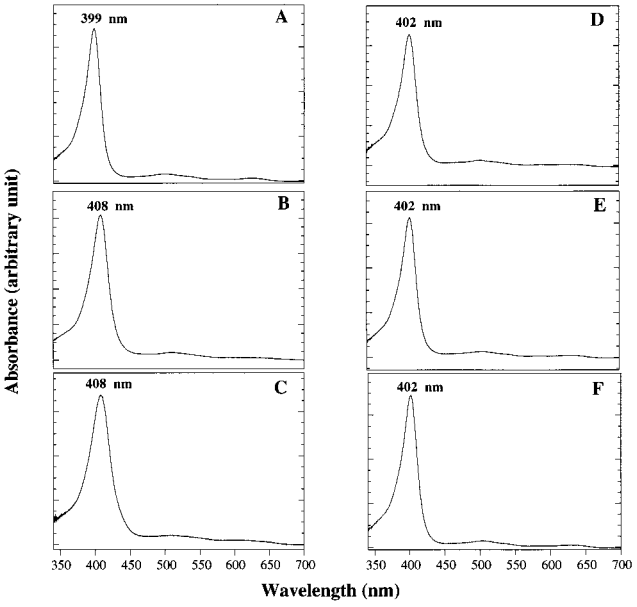

FIGURE 4: Absorption spectra of heme and heme products. The spectra are of the following: (A) intact heme, (B) 3-PPA-generated adduct formed with hydrogen peroxide as the oxidant, (C) nonanal-generated adduct formed with hydrogen peroxide as the oxidant, (D) *m*-CPBA-generated adduct in the absence of an aldehyde, (E) 3-PPA-generated adduct formed with *m*-CPBA as the oxidant, and (F) nonenal-generated adduct formed with *m*-CPBA as the oxidant. Native heme and adducts were isolated from a C4 semipreparative column as described in Experimental Procedures, and the spectra were determined in 50% acetonitrile containing 0.05% trifluoroacetic acid.

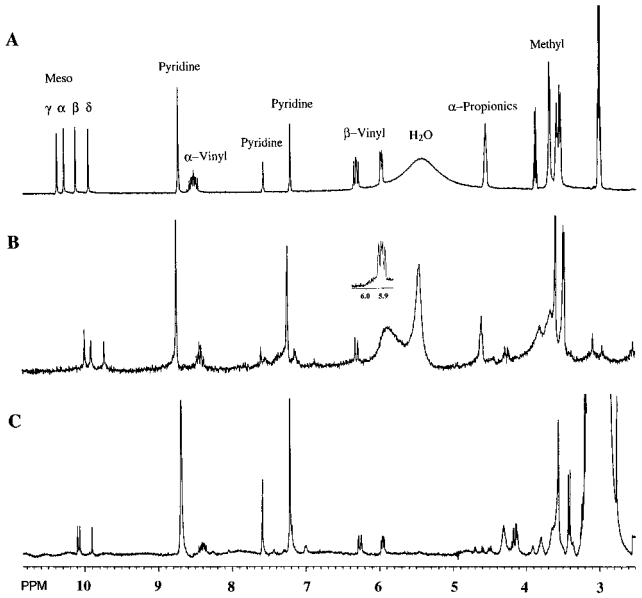

FIGURE 5: ^{1}H NMR spectra at 500 MHz of intact heme (A), the 3-PPA-generated adduct with hydrogen peroxide as the oxidant (B), and the nonenal-generated adduct with *m*-CPBA as the oxidant (C). Samples were dissolved in deuterated pyridine and reduced with stannous chloride. In panel B, no clear peaks are visible from the aromatic hydrogens of the phenylethyl adduct. We are unsure whether they are buried in the pyridine peaks, but in any case, the presence of the phenyl group is indicated by the mass spectrometric results. In the spectrum in panel B, the expected quartet near 6.0 ppm was obscured by a contaminant, but in more typical experiments, this quartet was readily distinguished (see the inset). The multiplet at 6.3 ppm in the intact heme appears to become a doublet in both heme adducts. The alkyl substitution at the meso position may cause distortion of the vinyl group, resulting in the collapse from a distinct quartet to an apparent doublet due to diminished spin coupling of the α- to the *β*-vinyl protons.

Characterization of Heme Adducts by ^{1}H NMR Spectroscopy. The one-dimensional NMR spectrum of intact heme, as shown in Figure 5A, has four singlets near 10 ppm corresponding to the meso protons, multiplets near 8.5 ppm corresponding to the α-proton of the two vinyl groups, two quartets near 6.0 and 6.3 ppm corresponding to the *β*-proton of the two vinyl groups, a broad peak near 4.5 ppm corresponding to the α-methylene protons of the propionic residues, and multiple peaks near 3.5 ppm representing the four methyl groups. The spectrum of the 3-PPA adduct formed in the presence of hydrogen peroxide, as shown in Figure 5B, is similar for the most part except for the loss of one meso proton singlet. Similarly, the spectrum of the adduct formed from *trans*-2-nonenal with *m*-CPBA as the oxidant is similar to that formed from 3-PPA with one meso proton singlet missing (Figure 5C). The absence of one meso proton thus indicates that the phenylethyl or nonenoyl substituent is located at this position.

To determine the specific position at which the meso proton was lost in the 3-PPA adduct, two-dimensional NOESY experiments were performed. The meso proton region of the NOESY map in Figure 6A unambiguously shows that the *γ*-meso position is the site of substitution, since no NOE interaction was seen between the meso protons and the α-methylene protons of the propionic residues (4.55 ppm). In addition, the *δ*-meso proton at 9.71 ppm was identified by NOE interactions with the 1- and 8-methyl

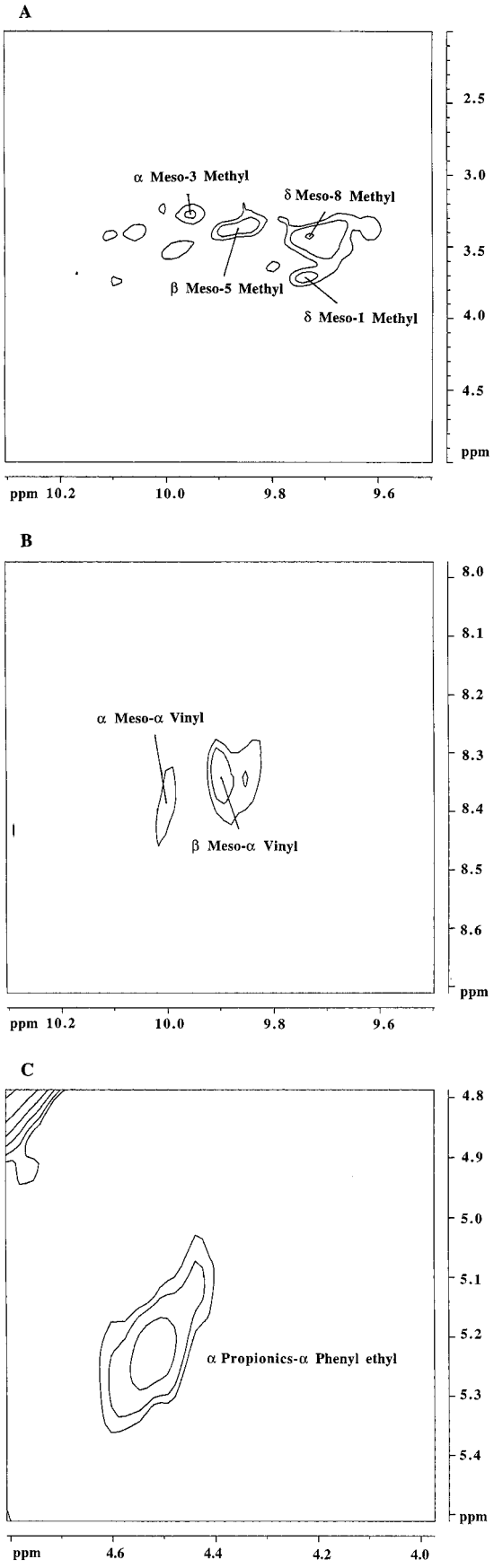

FIGURE 6: NOESY regional maps of the 3-PPA-generated heme adduct formed with hydrogen peroxide as the oxidant. The interactions between meso and methyl protons are shown in panel A, the interactions between meso and vinyl α-protons in B, and the interactions between propionics α- and phenylethyl α-protons in C.

Table 1: NMR Chemical Shifts and NOESY Interactions of the 3-PPA Heme Adduct

proton	chemical shift (ppm)	interaction
α-meso	9.98	3-methyl; α-vinyl
β-meso	9.89	5-methyl; α-vinyl
δ-meso	9.71	1,8-methyl
α-vinyl	8.41, 8.39	α,*β*-meso; *β*-vinyl
β-vinyl	6.33, 6.30; 6.01, 5.97	α-vinyl
α-phenylethyl	5.32	α-propionics
α-propionics	4.55	α-phenylethyl; 5,8-methyl
1-methyl	3.74	*δ*-meso
8-methyl	3.44	*δ*-meso; α-propionics
5-methyl	3.42	*β*-meso; α-propionics
3-methyl	3.29	α-meso

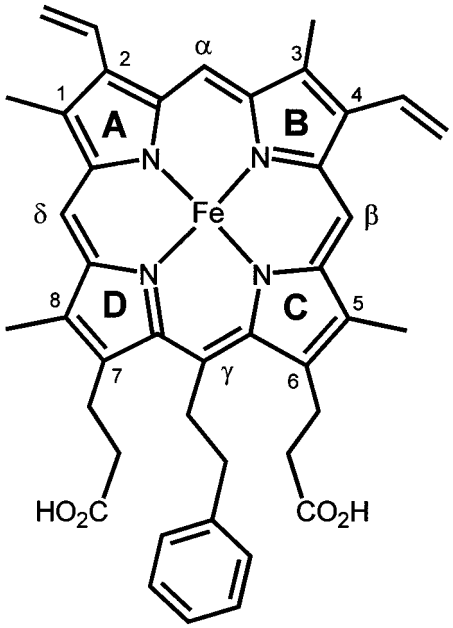

FIGURE 7: Structure of the adduct formed from 3-PPA in the presence of hydrogen peroxide.

protons. Therefore, the two meso protons at 9.98 and 9.89 ppm must be those at the α- and *β*-meso positions, respectively, as confirmed by the NOE interactions with the 3- or 5-methyl protons in panel A and the α-vinyl protons in panel B. The 3- and 5-methyl protons were distinguished because α-methylene protons of the propionics interacted with the 5-methyl but not with the 3-methyl proton (data not shown). Thus, the chemical shifts of the α- and *β*-meso protons must be at 9.98 and 9.89 ppm, respectively. Finally, the phenylethyl α-protons were identified by the NOE interaction with the α-methylene protons of the propionics as shown in panel C. Table 1 summarizes the chemical shifts and NOE interactions of the adduct formed from 3-PPA in the presence of H_2O_2. The structure is shown in Figure 7. The adduct formed from nonenal was also submitted to NOESY analysis, but the results were inconclusive, possibly because of the lability of the preparation.

DISCUSSION

We interpret the results given above, along with our earlier findings on P450 inactivation by aldehydes (*12*, *13*, *18*), in terms of the scheme shown in Figure 8. A variety of aldehydes, represented by RCH_2CH_2CHO, undergo oxidation by either of the two pathways that are shown, one resulting in the loss of the aldehyde group as formate with desaturation in the remainder of the molecule, and the other leading to the corresponding carboxylic acid by the well-accepted mechanism of hydrogen abstraction followed by recombination of the carbon radical and hydroxyl radical to give the product. The structure of the aldehyde influences the deformylation reaction, with branching at the *β*-carbon and particularly at the α-carbon favoring higher activity (*15*). However, α,*β*-unsaturation, as in *trans*-4-hydroxy-2-nonenal (*13*) or nonenal in the study presented here, completely blocks deformylation. Aside from these characteristics of the substrate, the nature of the oxidant determines the reaction catalyzed by P450. Pictured in route A in the figure are the steps by which molecular oxygen is converted to more reactive species by P450 in the reconstituted system containing NADPH and reductase, with the uptake of one proton being necessary for the reversible conversion of peroxo−iron, a nucleophilic species, to hydroperoxo−iron, a weaker nucleophilic species, both of which are presumed to function in aldehyde deformylation by forming the peroxyhemiacetal intermediate. Fe−O_2H, which also has electrophilic properties, is converted irreversibly by uptake of an additional proton with cleavage of the O−O bond to give the strongly electrophilic oxenoid−iron (Fe═O); both species are now believed to function in olefin epoxidation (*28*). Additional strong evidence for separate oxidants in the two pathways is provided in this paper (route B), with added hydrogen peroxide leading to the alkyl heme adduct or added *m*-CPBA to the acyl adduct from the same aldehyde, as shown with 3-PPA and also with nonanal.

Several lines of evidence support the concept that alkyl and acyl hydroperoxides undergo homolytic cleavage by P450, as shown in Figure 2 for *m*-CPBA. The reaction of P450 2B4 with peroxides, including perbenzoic acids, is known to differ in two important respects from that of typical peroxidases; the intermediates are formed reversibly, and the spectra vary with structural differences in the peroxy compounds (*32*). Furthermore, as shown with substituted cumene hydroperoxides and substituted toluenes that yield benzyl alcohols, the rate-limiting decay of the active complex formed by P450 2B4 is dependent on the structure of the oxygen donor as well as on the structure of the hydroxylatable substrate (*33*). These results are compatible with a homolytic mechanism of oxygen−oxygen bond cleavage. In addition, a study of the decarboxylation of perphenylacetic acid with formation of benzyl alcohol by several P450s during hydroxylation of substrates gave support to a homolytic mechanism (*34*). With respect to heme adduct formation by *m*-CPBA and P450, with or without substrate present, the results given here are compatible with homolytic scission to give the acyloxy radical, as shown in Figure 2. In this connection, Lee and Bruice (*35*) examined oxygen−oxygen bond scission accompanying oxygen transfer to iron(III) porphyrin models by percarboxylic acids and hydroperoxides and proposed that if the product alcohol or acid had a pK_a of <11 the cleavage would be heterolytic, but otherwise the process would be homolytic. Since the pK_a of the *m*-CPBA product, *m*-chlorobenzoic acid, is around 3.0, it appears that P450s, and by implication the thiolate ligand, may alter the course of O−O bond cleavage.

Iodosobenzene would be expected to generate the predicted oxenoid−iron intermediate, as shown in Figure 8, but it is

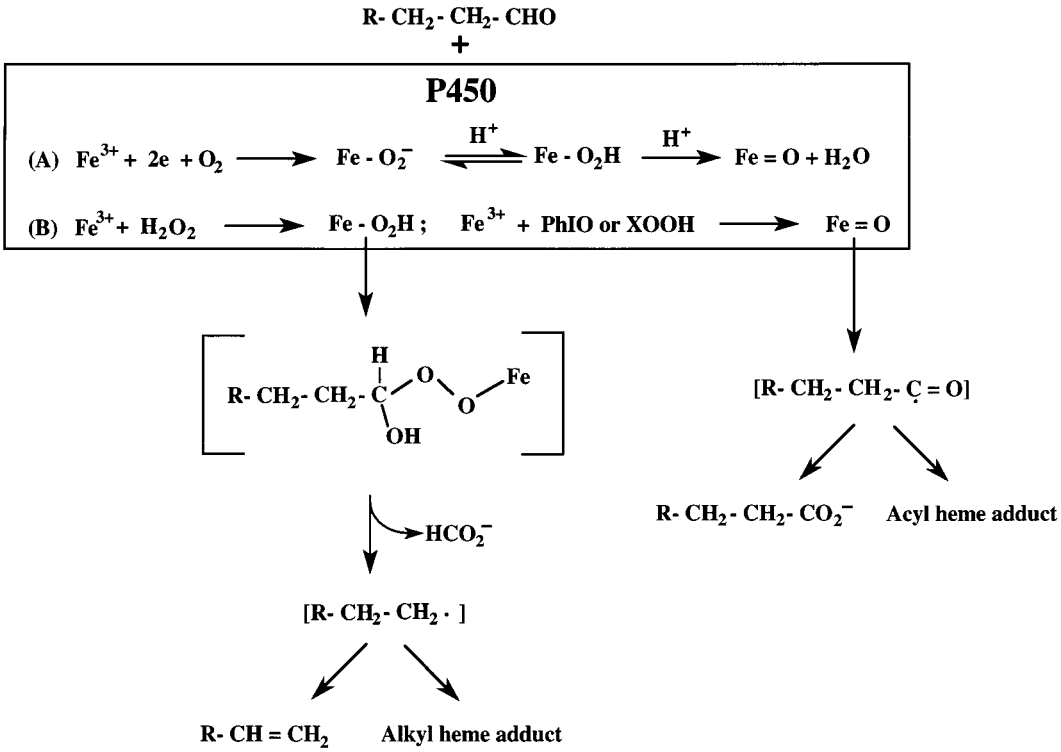

FIGURE 8: Proposed reactions for aldehyde metabolism by P450 accompanied by heme adduct formation, where Fe represents the iron atom at the active site: (A) active oxidants generated by reduction of molecular oxygen in the reconstituted system with NADPH and reductase present and (B) active oxidants generated by the addition of oxygen donors to P450, where PhIO represents iodosobenzene and XOOH an alky or acyl hydroperoxide. The deformylation pathway is believed to involve the formation of a peroxy−hemiacetal intermediate and to yield an olefin as shown, or alternatively by a concerted process that is not shown.

not yet clear whether alkyl and acyl hydroperoxides do the same by undergoing some heterolytic cleavage. In this respect, Egawa et al. (*22*) have obtained evidence for oxene formation from *m*-CPBA by P450cam. An alternative possibility that has been proposed (*19*) is that with a peracid, for example, homolysis yields the acyloxy and hydroxyl radicals (Figure 2), and that the acyloxy radical by hydrogen abstraction generates the substrate carbon radical. Combination with the iron−hydroxyl radical would then yield the expected product, the carboxylic acid derived from the aldehyde.

Kunze et al. (*36*) proposed a structure for the active site of P450 2B4 according to the regiospecificity of prosthetic heme alkylation by olefins and acetylenes. The model, which incorporated the absolute geometry of the prosthetic heme, showed a steric constraint over pyrrole ring B and a lipophilic channel over pyrrole ring C. Our results, in which the 3-PPA-generated heme adduct has a substitution at the γ-meso position, would conform with the proposed model.

The toxicity of lipid peroxidation products generated in mammalian biological membranes is widely attributed to reactive aldehydes, including 2-alkenals and 4-hydroxyalkenals (*37*), and similar products are generated in plants (*38*). As already indicated above, *trans*-4-hydroxy-2-nonenal, a particularly cytotoxic compound (*39*), is known to inactivate certain P450 cytochromes by heme adduct formation. It remains to be established whether aldehydes from dietary sources or those generated in metabolism significantly alter P450 function, an effect which could help account for the wide variation in the human population in response to drugs and other substrates of the P450 oxygenases.

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