Characterization of the Enzymatic and Nonenzymatic Peroxidative Degradation of Iron Porphyrins and Cytochrome P-450 Heme†

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ABSTRACT: Both purified cytochrome P-450 (P-450) and free ferriprotoporphyrin IX are destroyed by NADPH-P-450 reductase in the presence of NADPH and O_2. The process appears to be mediated by H_2O_2 generated by reduction of O_2. Six major products were identified from the reaction of H_2O_2 with ferriprotoporphyrin IX—hematinic acid, methylvinylmaleimide, and four dipyrrolic propentdyopents. The structures of the propentdyopents were elucidated by mass spectrometry and ^1H NMR methods. Both free ferriprotoporphyrin IX and P-450 yielded these same products in similar relative ratios. P-450 heme in rat liver microsomes was degraded in the presence of O_2 and NADPH and either NaN_3 (a catalase inhibitor) or Fe·ADP (which promotes lipid peroxidation); the products were primarily hematinic acid, methylvinylmaleimide, and small quantities of one propentdyopent. Only the two maleimides were detected in the destruction of microsomal P-450 heme by cumene hydroperoxide and iodosylbenzene. On the basis of the reaction of H_2O_2 with several metal–octaethylethylporphyrin complexes and free octaethylporphyrin, the iron chelated in ferriprotoporphyrin IX is required for degradation by H_2O_2. Biliverdin is not an intermediate in the formation of maleimides and propentdyopents from heme. Experiments using the tetraethylpropentdyopent produced from ferrioctaethylporphyrin suggest that propentdyopents are not further cleaved to form the maleimides. A mechanism for oxidative heme destruction consistent with these observations is proposed.

Cytochrome P-450[1] has been shown to be destroyed by NADPH-P-450 reductase in the presence of NADPH and O_2 and absence of any added substrate (Guengerich & Strickland, 1977; Guengerich, 1978). Loss of FePPIX parallels the loss of P-450. Under similar conditions, free FePPIX is also destroyed by NADPH-P-450 reductase (Guengerich & Strickland, 1977; Guengerich, 1978), as well as by the proteolytically cleaved form, NADPH-cytochrome *c* reductase (Yoshinaga et al., 1982).

This mechanism of heme degradation is distinct from that of the heme oxygenase system, oxidation by ascorbate and oxygen, and the suicide inactivation of P-450 by many olefin-containing substrates (Guengerich, 1978). Heme oxygenase cleaves the FePPIX ring to form biliverdin IXα exclusively with release of the α-methene bridge carbon as CO (Tenhunen et al., 1969). Ascorbic acid also oxidizes FePPIX to biliverdin but can attack at any of the methene bridge carbons (Bonnett & McDonagh, 1973). Thus, a mixture of biliverdin isomers is produced. Destruction of P-450 FePPIX by various olefin-containing compounds has been studied extensively [for a review, see Ortiz de Montellano & Correia (1983)]. Evidence suggests that the olefinic substrate is activated by P-450 to an electrophilic species that becomes covalently bound to a P-450 FePPIX nitrogen to inactivate the enzyme.

NADPH-P-450 reductase mediated destruction of both free and P-450-bound FePPIX requires NADPH and O_2. Catalase inhibits loss of FePPIX in microsomes and reconstituted systems, suggesting involvement of H_2O_2. Superoxide anion, however, does not appear to be involved, as superoxide dismutase had no effect on heme destruction (Guengerich & Strickland, 1977). H_2O_2 is known to destroy FePPIX (Fischer & Mueller, 1937). Both the oxidized and reduced forms of FePPIX and P-450 are degraded, but the ferrous forms react much faster. CO is capable of inhibiting reductase-mediated destruction of both free and P-450-bound FePPIX (Guengerich, 1978). These results suggest a role of the FePPIX iron in the reaction.

PDPs have been shown to be formed from FePPIX degraded by NADPH-P-450 reductase in the presence of NADPH (Guengerich, 1978; Yoshinaga et al., 1982), as well as from FePPIX destroyed by H_2O_2 (Fischer & Mueller, 1937). Similarly, evidence supporting PDP formation from purified and microsomal P-450 has been presented (Guengerich, 1978). In the studies presented here we have further characterized the heme products produced in these systems as well as the mechanism of heme breakdown. The structures of four PDPs

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[1] Abbreviations: NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; FePPIX, iron protoporphyrin IX; OEP, octaethylporphyrin; PDP, propentdyopent (PDP1, PDP2, PDP3, and PDP4 refer to methylated heme-derived propentdyopents); BHT, butylated hydroxytoluene; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MVM, methylvinylmaleimide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; FAB, fast-atom bombardment; CI, chemical ionization; P-450, liver microsomal cytochrome P-450; Et_4-PDP, tetraethylpropentdyopent; di-12-GPC, L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine; NaDOC, sodium deoxycholate.

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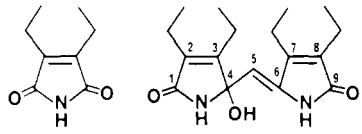

a b

FIGURE 1: Diethylmaleimide (a) and Et_4-PDP (b) produced from reaction of H_2O_2 with Fe(III) OEP.

have been characterized. In addition, the two previously unreported products, hematinic acid and MVM, were identified (Figure 1).

Experimental Procedures

Hemin (bovine), bilirubin (from bovine gall stones), and biliverdin dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). $(CH_3)_3OBF_4$ was purchased from Fluka (Hauppauge, NY). 5-Amino[4-^{14}C]levulinic acid hydrochloride was obtained from New England Nuclear (Boston, MA). All OEP derivatives were purchased from Aldrich (Milwaukee, WI).

Preparation of Diethylmaleimide, Et_4-PDP, and Octaethylbilitriene from Fe(III) OEP. Fe(III) OEP (50 mg) was dissolved in 100 mL of acetone and added to a mixture of 750 mL of 20 mM NaOH and 150 mL 30% H_2O_2 (5% final concentration). After 1–2 h at 37 °C, the mixture was filtered and extracted with 3 × 300 mL of ether; the ether fraction was taken to dryness in vacuo. The residue was dissolved in acetone and applied to a Whatman (Whatman Chemical Separation, Inc., Clifton, NJ) $PLKC_{18}F$ preparative reverse-phase TLC plate (CH_3OH/H_2O, 60:40). The band at R_f 0.5 was scraped and extracted with acetone. Both diethylmaleimide and Et_4-PDP were identified and further purified by reverse-phase HPLC (Altex Scientific, Inc., Berkeley, CA, Ultrasphere-octyl column, 10 × 250 mm at 3 mL min^{-1} for preparative-scale and 4.6 × 250 mm at 1 mL min^{-1} for analytical determinations; 35% CH_3CN, 65% H_2O, isocratic). The structures for the two compounds were confirmed by mass spectrometry and NMR as discussed under Results. Octaethylbilitriene was prepared and characterized by the method of Bonnett & Dimsdale (1972).

Preparation of Propentdyopents from Hemin. PDPs were produced by reaction of H_2O_2 with hemin on the basis of the method of Fischer & Mueller (1937). FePPIX-Cl (100 mg) was dissolved in 8 mL of water with the addition of NH_4OH. H_2O_2 [1.7 mL of a 30% solution (w/v) to yield a 5% (w/v) final concentration] was added slowly, and the reaction mixture was swirled constantly to reduce foaming. The mixture was then heated at 60 °C for 30 min. The mixture was lyophilized, and the products were extracted from the residue with CH_3OH and filtered. Aliquots of the extract were methylated as needed with excess $(CH_3)_3OBF_4$ to derivatize the acidic FePPIX degradation products for chromatography (Dean et al., 1976). Following methylation, 4 volumes of H_2O were added to the mixture, and the products were extracted 3 times into CH_2Cl_2. The CH_2Cl_2 phase was reduced to dryness under N_2, and the residue was resuspended in a small volume of CH_3OH. The methylated PDPs were purified from this mixture by preparative reverse-phase HPLC [Altex Ultrasphere-octyl 10 × 250 mm column; flow 3 mL min^{-1}; 25% CH_3OH, 75% H_2O (v/v) initial, linear gradient to 55% CH_3OH, 45% H_2O (v/v) over 35 min, and held at 55% CH_3OH, 45% H_2O (v/v) for 15 min]. peaks corresponding to methylated PDPs were identified as those which when concentrated gave a positive Stokvis reaction (i.e., turned pink in the presence of 2 N KOH and excess $Na_2S_2O_4$) (Bonnett & Stewart, 1975). PDP-positive peaks were collected and taken to dryness in vacuo.

Preparation of Hematinic Acid and MVM. MVM was prepared from chromic acid oxidation of bilirubin as described by Bonnett & McDonagh (1969). After extraction into ether, MVM was purified by preparative TLC on Whatman PLK5F silica TLC plates: CCl_4/ethyl acetate/cyclohexane (5/3/1 v/v/v) (Rüdiger, 1970). The identity of MVM was confirmed by mass spectrometry (M^+ at m/z 137) and ^{1}H NMR with $CDCl_3$ as a solvent [methyl, 2.05 ppm (3 H); vinyl 5.68 ppm (1 H); vinyl, 6.44 ppm (2 H); –NH, 7.22 (1 H, br)].

Hematinic acid was isolated from the same reaction as MVM. After the MVM had been extracted, the aqueous reaction mixture was acidified to pH 2 with concentrated H_2SO_4 and extracted 5 times with ether. The combined ether fractions were dried with Na_2SO_4 and concentrated in vacuo. A yellow oil remained that was hematinic acid, as judged by ^{1}H NMR [–CH_2–CH_2–, 2.77 ppm (4 H); –CH_3, 2.04 ppm (3 H); –NH and –COOH exchanged with the solvent, D_2O, and were not visible]. Hematinic acid methyl ester was formed from reaction with $(CH_3)_3OBF_4$ as above. Characterization by mass spectrometry and ^{1}H NMR was in agreement with literature data (Lightner & Quistad, 1972). Extinction coefficients at 280 nm were measured for both maleimides for purposes of quantitation by HPLC: hematinic acid methyl ester, ϵ_{280} 0.51 mM^{-1} cm^{-1}; MVM, ϵ_{280} 1.01 mM^{-1} cm^{-1}.

Analysis of Heme Degradation Products. Heme products formed in microsomal and purified enzyme incubations were methylated and analyzed by HPLC. Following incubation, microsomes were precipitated by addition of $CaCl_2$ (to 50 mM final concentration), and the resulting supernatant was lyophilized. Incubations containing purified enzymes were lyophilized directly. The resulting lyophilized residues were extracted twice with CH_3OH containing 1% concentrated NH_4OH (v/v) and then methylated with two aliquots of $(CH_3)_3OBF_4$ added at 5-min intervals. The mixture was diluted with 4 volumes of H_2O, and the methylated products were extracted into CH_2Cl_2 3 times. The combined CH_2Cl_2 fractions were taken to dryness under N_2, and the residue was dissolved in 25 μL of CH_3OH for HPLC. Methylated maleimides and PDPs were separated on an Altex 4.6 × 250 mm Ultrasphere-octyl HPLC column with the following solvent gradient: flow 1 mL min^{-1}, 25% CH_3OH, 75% H_2O (v/v) initial, linear gradient to 55% CH_3OH, 45% H_2O (v/v) over 35 min and held at 55% CH_3OH, 45% H_2O (v/v) for 10 min. With samples containing ^{14}C-labeled FePPIX products, radioactivity was measured either with a Radiomatic Model Flo-One radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL) connected to the outlet of the HPLC column or by collecting 0.5-mL aliquots of eluent from the HPLC, mixing with ACS scintillation cocktail (Amersham, Arlington Heights, IL), and counting in a Beckman Model LS7000 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker (Billerika, MA) AM-400 spectrometer using argon-flushed samples in [$^2H_6$]acetone. Spectra were recorded with a 4000-Hz sweep width, 16K data points (2.04-s acquisition time), a 30° pulse, and a 0.8-s relaxation delay. Fourier transformation was carried out with 0.5-Hz line broadening. Sufficient scans were taken to give satisfactory signal to noise. The residual [^{2}H]acetone signal at 2.05 ppm was used to standardize the spectra. A residual water signal was observed at 2.85 ppm along with an unidentified impurity at 2.87 ppm. Decoupling experiments were carried out with irradiation power set as low as possible to observe

decoupling. NOEs were observed by subtraction of free-induction decays, one with the decoupler frequency on the signal of interest and the other with the irradiation point beyond the end of the spectrum, prior to transformation. A low decoupler power (40-dB attenuation of 0.2 W was used in a gated mode such that the decoupler was on for 6 s and then turned off during data acquisition). All NOEs observed were positive; however, the recorded difference spectra were inverted such that the NOEs appear as negative signals (Figures 2 and 6–9). NOE intensities were calculated on the basis of peak intensities relative to the intensity of the irradiated signal, normalized by their relative intensities in an unirradiated spectrum.

Rat Liver Microsomes and Purified Enzymes. Male Sprague-Dawley rats (200–250 g) were treated with 0.1% (w/v) sodium phenobarbital in drinking water for 5 days. Liver microsomes were prepared according to van der Hoeven & Coon (1974). For preparation of microsomes containing radiolabeled FePPIX, phenobarbital-pretreated rats were administered 500 μCi of 5-amino[4-^{14}C]levulinic acid ip 5 h before sacrifice (Bissell & Hammaker, 1976). This heme precursor results in labeling in the pyrrole rings but not at the methene bridges between rings. NADPH-P-450 reductase and P-450$_{PB-B}$ were prepared from phenobarbital-pretreated rats as described elsewhere (Guengerich et al., 1982).

Assays. FePPIX and P-450 were assayed according to Omura & Sato (1964). Protein was estimated according to Lowry et al. (1951).

Results

Products from Reaction of H_2O_2 with Fe(III) OEP. The symmetrical porphyrin Fe(III) OEP was seleted as a model for FePPIX, as the identical ethyl side chains were expected to simplify product identification. In 5% (w/v) H_2O_2, >95% of the added Fe(III) OEP was destroyed in less than 15 min at 37 °C to yield a colorless solution. Only two major products (Figure 1) were produced as judged by HPLC. The most abundant was 2,3-diethylmaleimide: the structure was confirmed by CH_4 CI mass spectrometry (M^+ at m/z 154), ^{1}H NMR in $CDCl_3$ (–CH_3, 1.16 ppm, 6 H, –CH_2–, 2.43 ppm, 4 H, q; –NH, 7.45 ppm, 1 H, br), and ^{13}C NMR (–CH_3, 13.13 ppm; –CH_2–, 16.87 ppm; vinyl, 142.82 ppm; carbonyl, 171.86 ppm). The other major product was Et_4–PDP, which produced the characteristic pink color upon mixing with 20% (w/v) KOH and excess $Na_2S_2O_4$ (Bonnet & Stewart, 1975). The UV spectrum of Et_4–PDP showed a λ_{max} at 270 nm. Mass spectrometry (CH_4 CI) showed an M^+ ion at m/z 287, consistent with a dehydrated PDP. The ^{1}H NMR spectrum shown in Figure 2, however, revealed that the hydrated PDP is actually formed. The singlet at 5.70 ppm corresponds to the hydroxyl, and the signals at 7.56 ppm and 8.52 ppm represent the amides. The resonances of the methyl and methylene hydrogens comprising each of the ethyl side chains were well resolved. Assignments of the methyl and methylene pairs were determined by decoupling: CH_2 (2.35 ppm) and CH_3 (1.14 ppm); CH_2 (2.31 ppm) and CH_3 (1.07 ppm); CH_2 (2.21 ppm) and CH_3 (1.04 ppm); CH_2 (2.47 ppm) and CH_3 (1.10 ppm). The methylene at 2.35 ppm showed extensive splitting. This pattern was attributed to the asymmetric center at the hydroxylated carbon, and the position of the 2.35 ppm methylene was assigned to C-3.

NOE experiments were done to further determine which ethyl group is at C-7 (Figure 1). NOEs yield information concerning the spatial relationship of atoms, hydrogen atoms in the present case (Sanders et al., 1978; Graden & Lynn, 1984). The effect is based upon spin–lattice transfer of energy between nuclei and depends upon the distance through space between them independent of bond connectivity. When two protons are sufficiently close, irradiation of nucleus A, using the decoupler at a low power setting, disturbs the Boltzman distribution of spin states of nucleus B such that the intensity (and area) of signal B is altered. Both positive and negative changes can be observed, although with ^{1}H spectra of small, rapidly tumbling molecules, such as are the subject of this paper, all NOEs are positive. An NOE was observed between the methylene at 2.47 ppm and the hydrogen on the methene bridge (C-5). NOEs were not found for any of the other methylenes or methyls. A space-filling model of Et_4-PDP supports this observation. Only the methylene on C-7 is in close proximity to the methene bridge. The methylene on C-3 is more distant, and thus, no NOE was observed. Generalizing, an NOE will only be observed for a side chain on the pyrrole ring of a PDP, which is connected to the methene bridge by a double bond; the side chain must also be attached at the position closer to the bridge. This characteristic was important for determining the FePPIX-derived PDPs described below.

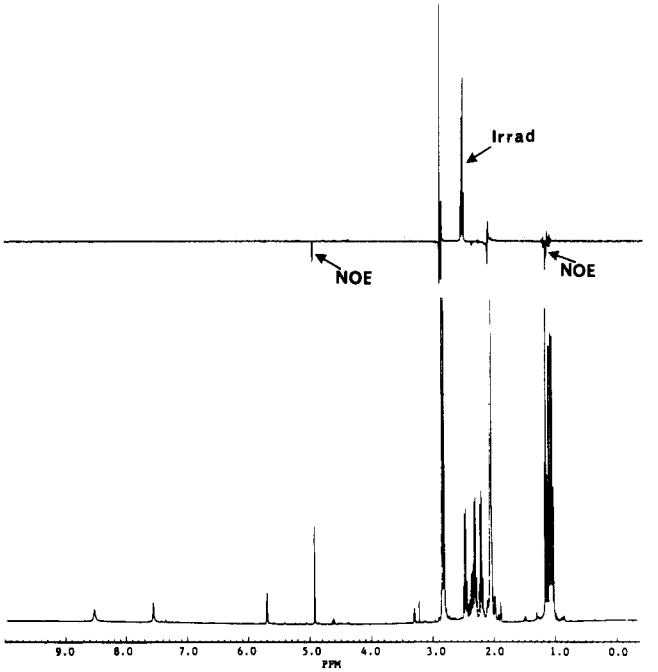

FIGURE 2: NMR spectra of Et_4-PDP. The ^{1}H NMR spectrum of Et_4-PDP is shown at the bottom. The top panel shows the NOE on the vinyl bridge proton at C-5 upon irradiating at the methylene proton at C-7. "Irrad" denotes the site of irradiation.

Products from Reaction of FePPIX with H_2O_2. FePPIX is rapidly degraded by excess H_2O_2 in aqueous solution. The products of the reaction were methylated with $(CH_3)_3OBF_4$ as described under Experimental Procedures and separated by reverse-phase HPLC (Figure 3). Peaks a and b correspond to hematinic acid methyl ester and MVM (Figure 4), respectively, as determined by comigration with standards. The four large peaks c–f eluting later were identified as PDPs by the Stokvis reaction and were designated PDP1, PDP2, PDP3, and PDP4, respectively. Isolation of each methylated PDP by preparative reverse-phase HPLC permitted characterization and structural determination. UV spectra showed respective λ_{max} values of 279, 270, 296, and 294 nm for PDP1, PDP2, PDP3, and PDP4. Mass spectra (FAB) corresponded to methoxy PDP methyl esters, in contrast to the hydroxy Et_4-PDP, which was not subject to methylation. However, only elimination products (loss of CH_3OH) of each methylated PDP were observed with CH_4 CI mass spectrometry, analogous to the situation with Et_4-PDP. Presumably, hydroxy PDPs are

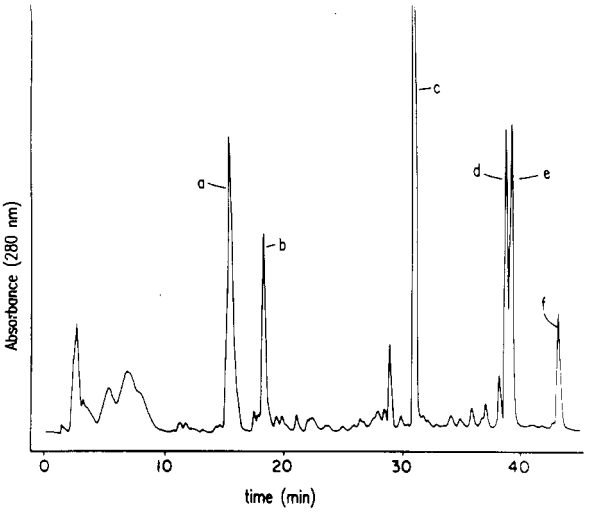

FIGURE 3: Separation of products produced from reaction of H_2O_2 with FePPIX by reverse-phase HPLC. Conditions are given under Experimental Procedures. (a) Hematinic acid, (b) MVM, (c) PDP1, (d) PDP2, (e) PDP3, and (f) PDP4.

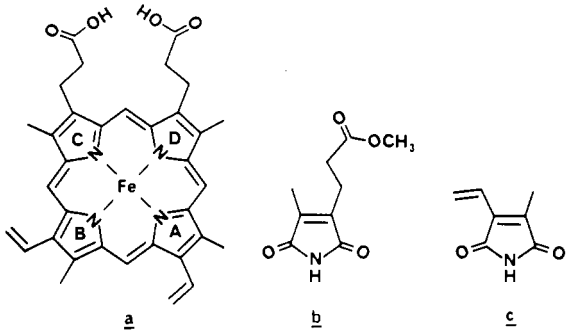

FIGURE 4: Structures of FePPIX (a), hematinic acid (b), and MVM (c).

the actual degradation products. PDP1 showed an M^{+1} ion at m/z 407 by FAB mass spectrometry and an M^+ ion at m/z 375 by CH_4 CI mass spectrometry, corresponding to a PDP of the C and D rings of FePPIX. Identical M^+ ions were found for both PDP2 and PDP3 at m/z 347 and 315 by FAB and CH_4 CI mass spectrometry, respectively. Thus, either could consist of rings A and D or of rings B and C from FePPIX. Exact assignments were not possible without NMR spectra (vide infra). Finally, PDP4 showed an M^+ ion at m/z 287 by FAB mass spectrometry and an apparent M^+ ion at m/z 255 by CH_4 CI mass spectrometry, consistent only with a PDP from FePPIX rings A and B.

Structural Determination of FePPIX-Derived PDPs by ^1H NMR. The structures of the methylated FePPIX-derived PDPs were elucidated by ^1H NMR with strategies similar to those used for Et_4-PDP. Again, experiments involving NOEs were valuable, and in addition, long-range decoupling experiments were useful for assigning methyl and vinyl side chains. Long-range coupling constants arising over four or more bonds and which are too small for splitting to be observed can sometimes be detected by measurement of peak widths with and without the decoupler turned on. Methyl–vinyl pairs in PDP2 and PDP4 were identified in this manner. The structures of the propentdyopents are shown in Figure 5, and the ^1H NMR spectra are in Figures 6–9. The large multiplet at 2.05 ppm is due to isotopic impurities in the solvent, $[^2H_6]$-acetone. The peaks at 2.83 and 2.87 ppm are attributed to H_2O and an unidentified impurity, respectively, and the small singlet at 3.30 ppm is residual CH_3OH from sample preparations.

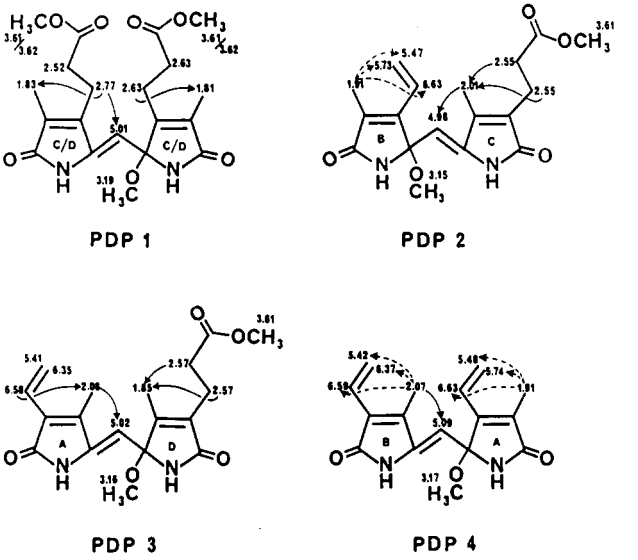

FIGURE 5: Structures of PDPs produced from FePPIX. The letters correspond to the rings of FePPIX from which the PDPs were derived (Figure 4), and the numbers represent the ^1H NMR chemical shifts (ppm). Solid arrows point to NOEs and originate at the position of irradiation. Broken arrows show long-range coupling; the arrows originate at the position irradiated and terminate at the decoupled positions.

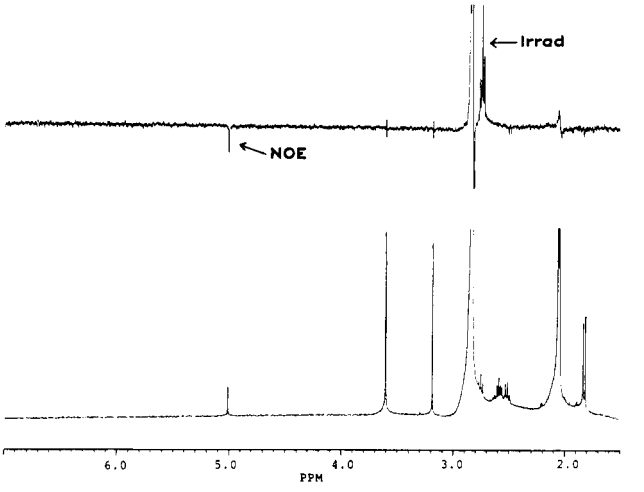

FIGURE 6: NMR spectra of PDP1. The ^1H NMR spectrum of PDP1 is shown at the bottom. The NOE difference spectrum (above) is the result of subtraction of the irradiated spectra from the control spectra; thus, the NOE appears negative although it is actually an enhancement. The assignments of the peaks and NOE are discussed in the text. "Irrad" denotes the site of irradiation.

PDP1. Figure 6 shows the spectrum of PDP1. The mass spectra suggested a methylated PDP derived from the C and D rings of FePPIX. Consonant with this view, no vinyl side chains were visible in the NMR spectrum. The peak at 5.01 ppm represents the hydrogen on the methene bridge between pyrrole rings. The singlet at 3.19 ppm corresponds to the methoxy attached to the pyrrole ring, and the signals at 3.61 and 3.62 ppm are each methyl esters. The complex multiplet between 2.55 and 2.70 ppm represents the propionyl on the ring with the methoxy. The extensive splitting is attributed to the chiral center at the methoxylated position on the ring. Irradiations at 2.61 and 2.65 ppm both show NOEs to the singlet at 1.82 ppm, which corresponds to the methyl side chain on the same ring. The propionyl side chain on the other pyrrole ring consists of two triplets at 2.77 and 2.52 ppm. When the methylene at 2.77 ppm was irradiated, strong NOEs were observed with the methene bridge H at 5.01 and at 1.85 ppm

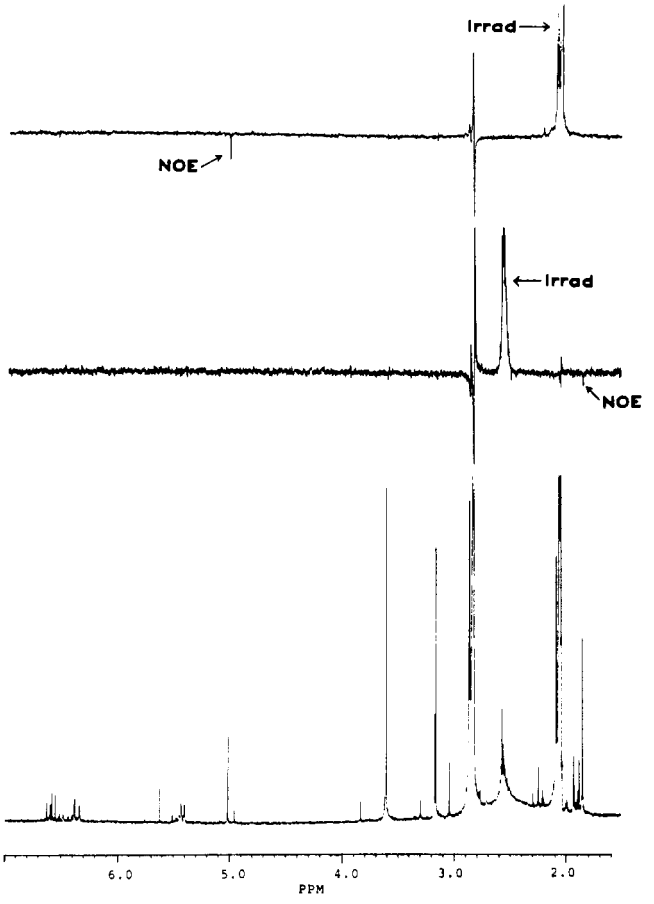

FIGURE 7: NMR spectra of PDP3. ^1H NMR spectrum of PDP3 (bottom) with NOE difference spectra (above) obtained as described in Figure 6. Peak assignments are discussed in the text.

assigned to the methyl side chain on the same ring. Amide protons were not visible and were believed to have rapidly exchanged with H_2O/D_2O in the [$^2H_6$]acetone.

PDP3. The ^1H NMR spectrum of PDP3 is shown in Figure 7. The singlets at 1.85 and 2.08 ppm were assigned to methyls and the multiplet at 2.57 ppm was assigned to the propionyl methylenes. The singlets at 5.02 and 3.61 ppm represent the hydrogens on the methene bridge carbon and the methyl ester, respectively. The peaks at 5.41, 6.35, and 6.58 ppm correspond to vinyl side chains. Irradiation of the methyl at 2.08 ppm showed NOEs to both the 6.58 ppm proton of the vinyl side chain and the 5.02 ppm proton on the methene bridge carbon. This pattern indicates that the methyl is spatially located between the vinyl side chain and the bridge. A PDP containing A and D rings of heme is consistent with this structure. Also, a double bond must connect the A ring to the bridge. Therefore, the methoxy (3.16 ppm) is attached to the D ring. An NOE was also observed between the 1.85 ppm methyl and the propionyl methylenes, indicating that they are adjacent. The structure of FePPIX dictates the positions of the propionyl and the adjacent methyl; however, the NOE experiments were required to determine the positions of the methyl and vinyl side chains on the other ring.

PDP4. The mass spectrum showed an M^+ ion at m/z 287, suggesting a methylated PDP comprised of the A and B ring from heme. Figure 8 shows the ^1H NMR spectrum of PDP4. Consistent with the A and B rings of heme, a propionyl side chain and its methyl ester are not found in the spectrum. Similar to other PDPs, the singlet at 5.09 ppm represents the methene bridge hydrogen, singlets at 1.91 and 2.07 ppm are methyl side chains, and the 3.17 ppm singlet is the methoxy

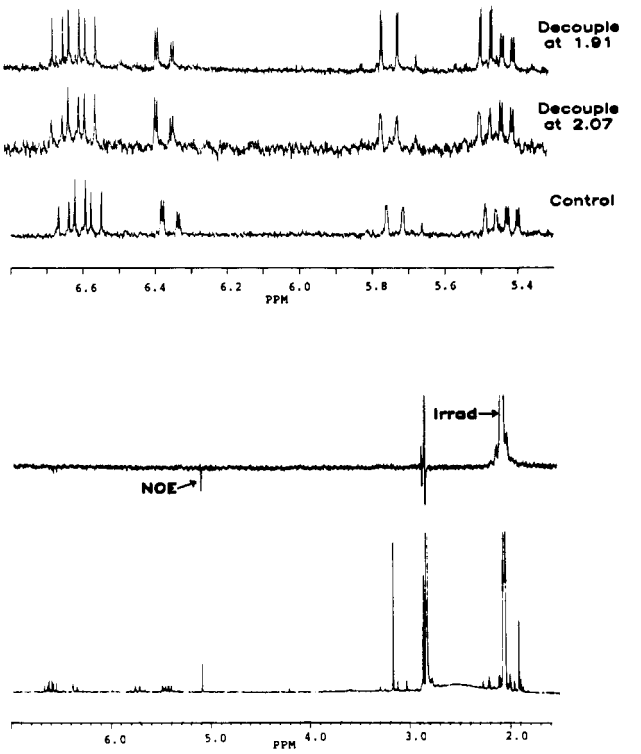

FIGURE 8: NMR spectra of PDP4. The ^1H NMR spectrum of Me-PDP4 is shown in the bottom panel with an NOE difference spectrum (middle panel) that is described in the text. The upper panel shows long-range decoupling experiments for the two vinyl side-chains of Me-PDP4. The control spectrum shows the vinyl region that has not been irradiated. The decoupled spectra resulted from irradiations at the 2.07 ppm methyl side chain and the 1.91 ppm methyl side-chain, respectively.

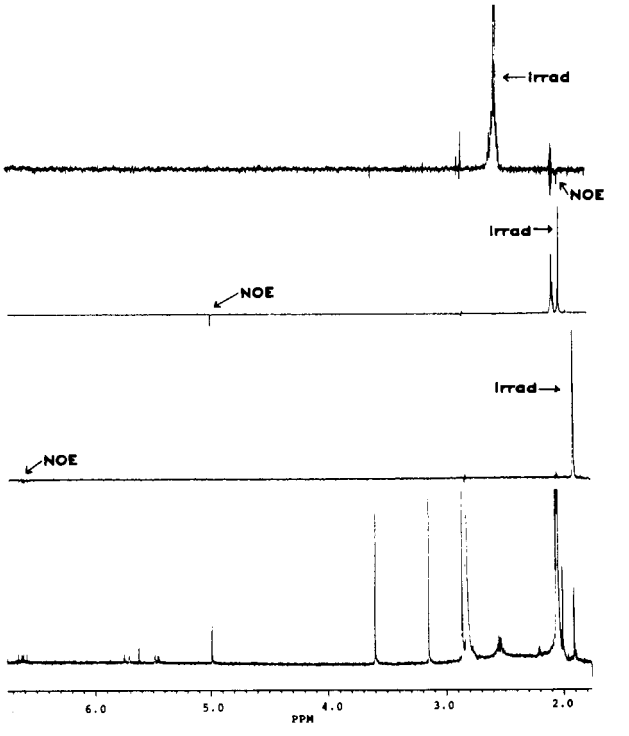

FIGURE 9: NMR spectra of PDP2. The ^1H NMR spectrum of PDP2 (bottom) and NOE difference spectra were obtained as described in Figure 6. Peak assignments are explained in the text.

group. Peaks between 5.42 and 6.63 ppm are consistent with two vinyl side chains. Decoupling showed that the peaks at 5.48, 5.74, and 6.63 ppm belong to one vinyl, and those at 5.42, 6.37, and 6.59 ppm belong to the other. In long-range de-

Table I: Heme Degradation Products Formed from FePPIX, Purified P-450, and Microsomal P-450

system	incubation time (min)	total heme destroyed (%)	P-450 destroyed (%)	heme degradation products (expressed on a molar basis relative to hematinic acid) hematinic acid	MVM	PDP1	PDP2	PDP3	PDP4
FePPIX, reductase, NADPH[a,c]	60	36		100	59	4	3	4	2
$P\text{-}450_{PB\text{-}B}$, reductase, NADPH[a,d]	30	53	55	100	83	<0.2	1	5	3
microsomes, NADPH, N_3^-[b]	30	59	42	100	9	<0.2	3	1	<0.2
microsomes, 5 mM iodosylbenzene[b,f]	30	>90	>90	100	7	<0.2	<0.2	<0.2	<0.2
microsomes, 0.5 mM cumene hydroperoxide[b,f]	15	80	90	100	13	<0.2	<0.2	<0.2	<0.2
microsomes, NADPH, Fe(III)–ADP[b,g]	30	33	60	100	17	<0.2	4	<0.2	<0.2

[a] The products were separated by HPLC as described under Experimental Procedures, and quantities of products from FePPIX and purified P-450 were estimated by using area under the peak and ϵ_{280}. [b] The products were separated by HPLC as described under Experimental Procedures, and quantities of products from microsomes were estimated by measuring radioactivity of each product. Microsomes were prepared from phenobarbital-induced rats pretreated with 5-amino[4-^{14}C]levulinic acid to label microsomal heme. [c] FePPIX (20 μM) was incubated at 37 °C with 0.42 μM P-450 reductase, 0.1 mM $NADP^+$, 10 mM glucose 6-phosphate, 1.0 IU of glucose-6-phosphate dehydrogenase mL^{-1}, 30 μM di-12-GPC, 0.25 mM NaDOC, and 50 mM potassium phosphate buffer (pH 7.7). [d] The reaction mixtures contained 5 μM P-450, 0.63 μM 45 μg/mL P-450 reductase, 0.2 mM $NADP^+$, 5 mM glucose 6-phosphate, 1.0 IU of glucose-6-phosphate dehydrogenase mL^{-1}, 30 μM di-12-GPC, 0.25 mM NaDOC, and 50 mM potassium phosphate buffer (pH 7.7). Incubations were carried out at 37 °C. [e] Incubations (37 °C) contained 2 mg of liver microsomes mL^{-1} (prepared from rats treated with 5-amino[4-^{14}C]levulinic acid), 0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, 1.0 IU of glucose-6-phosphate dehydrogenase mL^{-1}, 1 mM NaN_3, and 0.1 M potassium phosphate buffer (pH 7.7). [f] Liver microsomes (2 mg mL^{-1}) containing ^{14}C-labeled heme (footnote *e*) were incubated at 37 °C with 50 mM potassium phosphate buffer, 1 mM NaN_3, and either 5 mM iodosylbenzene or 0.5 mM cumene hydroperoxide. [g] Reaction mixtures contained 2 mg of microsomes containing ^{14}C-labeled heme mL^{-1} (footnote *e*), 1.7 mM ADP, 0.1 mM $FeCl_3$, 1 mM NADPH, and 50 mM Tris-HCl (pH 7.5 at 37 °C).

coupling experiments, irradiating at the 1.91 ppm methyl decoupled the 5.48, 5.74, and 6.63 ppm vinyls, while irradiating at the 2.07 ppm methyl decoupled the 5.42, 6.37, and 6.59 ppm vinyls. NOE experiments aided in determining the positions of the latter pair of methyl and vinyl side chains. NOEs were observed between the 2.07 ppm methyl and the bridge hydrogen but not between the bridge hydrogen and the 1.91 ppm methyl or the 6.63 ppm vinyl side chain. Thus, the 2.07 ppm methyl is closest to the bridge, and its ring (B) is attached to the bridge by a double bond. This portion of the PDP4 is identical with ring A of PDP3. In addition, the chemical shifts of the methyl and vinyl side chains of ring A of PDP3 are identical with those of ring B of PDP4. The configuration of the other ring of PDP4 could not be determined by NOE experiments. Considering the structure of FePPIX and assuming no rearrangements during preparation, the other ring must be ring A of FePPIX, and the vinyl must be proximal to the bridge and the methyl adjacent to the carbonyl. Interestingly, the chemical shifts of these two side chains on ring A are identical with the methyl and vinyl side chains, respectively, of PDP2 (vide infra).

PDP2. The ^{1}H NMR spectrum of PDP2 is shown in Figure 9. Immediately obvious are the singlet assigned to the methene bridge hydrogen at 4.98 ppm, one methyl ester at 3.61 ppm, the methoxy at 3.15 ppm, and the multiplet at 2.55 ppm corresponding to the methylenes of the propionyl side chain. The methyl side chains appear at 1.91 and 2.01 ppm. In addition, peaks indicating a vinyl side chain are present downfield at 5.47, 5.73, and 6.63 ppm. It is important to note that the chemical shifts of this vinyl are different from those of PDP3. Long-range decoupling revealed that the 1.91 ppm methyl and the vinyl are on the same ring. When the propionyl methylenes at 2.55 ppm were irradiated, an NOE was observed with the methyl side chain at 2.01 ppm. NOEs were also found between the 2.01 ppm methyl and methene bridge hydrogen. This indicates a double bond between the bridge carbon and the ring with the propionyl side chain. Thus, the methoxy is attached to the ring with the vinyl and 1.91 ppm methyl groups. The chemical shifts of the vinyl and methyl hydrogens are identical with those on the methoxylated pyrrole ring of PDP4 (ring A), suggesting identical structures. Therefore, PDP2 must have a vinyl proximal to the bridge carbon and be derived from B and C rings of heme.

FePPIX Degradation Products Formed in Various Enzymatic Systems. Table I shows the products from destruction of both free and P-450-bound FePPIX under several conditions. NADPH-P-450 reductase destroys FePPIX in the presence of NADPH (Guengerich, 1978; Yoshinaga et al., 1982) and P-450 FePPIX (Guengerich & Strickland, 1977; Guengerich, 1978). Both systems produce primarily hematinic acid and MVM. PDPs are also formed, in agreement with previous results from this laboratory (Guengerich, 1978) and the subsequent work of Yoshinaga et al. (1982), but at much lower levels than the maleimides. Microsomes incubated with NADPH and NaN_3 also show significant loss of FePPIX from P-450 (Guengerich, 1978). Without NaN_3, FePPIX destruction is greatly reduced, presumably due to breakdown of H_2O_2 by the catalase that contaminates microsomal preparations. In systems using microsomes from phenobarbital-induced rats containing ^{14}C-labeled FePPIX, hematinic acid was the predominant product with smaller quantities of MVM, PDP2, and PDP3 also formed. PDP1 and PDP4 were not detected.

Iodosylbenzene and cumene hydroperoxide are both oxygen donors capable of replacing NADPH-P-450 reductase, NADPH, and O_2 in many oxidation reactions catalyzed by P-450; however, they are believed to act by different mechanisms (Mansuy et al., 1982). Both compounds are very potent in destroying the FePPIX of P-450. Cumene hydroperoxide (0.5 mM) incubated with microsomes (2 mg of microsomal protein mL^{-1}) from phenobarbital-induced rats for 15 min at 37 °C resulted in loss of 95% of the P-450 and 79% of the total microsomal FePPIX. Iodosylbenzene was even more potent, destroying greater than 90% of the P-450 in microsomes in less than 5 min. The FePPIX degradation products formed were similar for both compounds. Hematinic acid was the major product, and MVM was found in small quantities. PDPs were not detected.

P-450 is also destroyed during lipid peroxidation (Nishibayashi et al., 1968; Levin et al., 1973; DeMatteis et al., 1977). The mechanism of this process is not knwon; however, lipid hydroperoxides generated during lipid peroxidation may be involved. Destruction of the FePPIX of P-450 by cumene hydroperoxide supports this hypothesis, since cumene hydroperoxide may be considered a model organic hydroperoxide. Microsomes containing ^{14}C-labeled FePPIX that were free of

BHT and EDTA (preservatives normally used to inhibit lipid peroxidation) were prepared from phenobarbital-treated rats and stored under argon. Lipid peroxidation was produced in these microsomes with NADPH and an Fe(III)–ADP complex (Svingen et al., 1978), and NaN_3 was omitted from these incubations to abolish the heme destruction directly due to H_2O_2. The relative extent of lipid peroxidation was monitored by measuring malondialdehyde by the thiobarbituric acid assay (Svingen et al., 1978). After 45 min at 37 °C, 90 nmol of malondialdehyde (mg of microsomal protein)$^{-1}$ was produced; 60% of the P-450 and 33% of the total microsomal FePPIX were destroyed. Hematinic acid and MVM, as well as a small amount of PDP2, were identified as FePPIX degradation products in this system.

Involvement of the Chelated Metal in the Reaction of OEP with H_2O_2. Free OEP and OEP chelates of Fe(III), Co(II), Mn(III), Mg(II), Cr(III) (porphyrin dimer), Cu(II), and Zn(II) were examined for reaction with H_2O_2. Each porphyrin was dissolved at a concentration of 0.1 mg mL^{-1} in methyl ethyl ketone and treated with 1.5% H_2O_2 [final concentration (w/v)]. Reactions were monitored by repeatedly scanning the visible spectra. Fe(III) and Co(II) OEPs reacted rapidly (pseudo-first-order degradation rates of 0.22 min^{-1} and 0.072 min^{-1}, respectively). Co(II) OEP showed an immediate shift in the visible spectrum upon addition of H_2O_2, followed by destruction of the new species (data not shown). Fe(III)OEP showed no such change before degradation. Mn(III) OEP also reacted with H_2O_2, but at a much slower rate (0.006 min^{-1}). The products formed from the Co(II) and Mn(III) OEPs were diethylmaleimide and Et_4-PDP, the same as were formed from Fe(III) OEP. Mg(II), Cr(III), Cu(II), and Zn(II) OEPs showed no reaction with H_2O_2 under these conditions. Metal-free OEP also did not react. These results suggest that a redox-active metal chelated in the porphyrin is required for porphyrin degradation by H_2O_2.

Degradation of Octaethylbilitriene and Biliverdin. Biliverdin and bilirubin have not been detected as products of FePPIX destroyed by P-450 reductase or of degraded microsomal P-450 heme (Guengerich, 1978; Yoshinaga et al., 1982). Conceivably, a biliverdin (or bilitriene) could be a short-lived intermediate formed following cleavage of one of the porphyrin bridge carbons as occurs with the ascorbate-coupled oxidation of FePPIX. The susceptibility of bilitrienes to attack by H_2O_2 was first examined with octaethylbilitriene dissolved in methyl ethyl ketone, as in the case of the OEP studies. Reactions were monitored by repeatedly scanning the visible spectra of the reaction mixtures. Octaethylbilitriene (15 μM) treated with 1.5% (w/v) H_2O_2 showed a very slow breakdown (0.0065 min^{-1}, estimated first-order rate constant). Controls without H_2O_2 proved that this was not due to photodegradation in the spectrophotometer. When 1 mol equiv of $FeCl_3$ was mixed with the bilitriene, 1.5% (w/v) H_2O_2 caused more rapid destruction of the tetrapyrrole (0.028 min^{-1}). Increasing the $FeCl_3$ concentration to 100 μM and reacting with 15 μM octaethylbilitriene and 1.5% (w/v) H_2O_2 increased the rate of destruction to 0.093 min^{-1}. Thus, iron significantly increases the rate of reaction of H_2O_2 with octaethylbilitriene, as in the case of OEP.

Purified biliverdin IXα incubated with 1.1 equiv of $FeCl_3$, NADPH-P-450 reductase, and an NADPH-generating system was destroyed at a rate of 0.004 min^{-1} (first-order rate constant determined spectrally). A 10-fold excess of $FeCl_3$ did not increase the rate of biliverdin degradation appreciably. FePPIX treated under identical conditions, except without any $FeCl_3$ added, was destroyed at a rate of 0.093 min^{-1}. These results suggest that biliverdin is not an intermediate in this mechanism of heme breakdown. The slow rate at which biliverdin is destroyed would allow it to accumulate during heme degradation; however, biliverdin has not been detected.

Table II: Degradation of Et_4-PDP by Various FePPIX-Destroying Systems

incubation conditions	Et_4-PDP destroyed (%)
50 μM Et_4-PDP, 5 mM H_2O_2[a]	4
50 μM Et_4-PDP, 5 mM H_2O_2, 0.5 mM $FeSO_4$[a]	12
50 μM Et_4-PDP, microsomes, NADPH, 1 mM NaN_3[a]	3
50 μM Et_4-PDP, microsomes, NADPH, 1 mM NaN_3, 1.7 mM ADP, 0.1 mM $FeCl_3$[b]	8
50 μM Et_4-PDP, microsomes, NADPH, 1 mM NaN_3, 1 μM hemin[a]	4

[a] Incubations were carried out in 0.1 M potassium phosphate buffer (pH 7.7) for 45 min at 37 °C. The products were extracted into CH_2Cl_2 and analyzed by HPLC (see Experimental Procedures). [b] Incubations performed as in footnote *a* except in 50 mM Tris-HCl buffer (pH 7.4).

The products produced from reaction of biliverdin IXα with NADPH-P-450 reductase and an NADPH-generating system were analyzed. Only biliverdin IXα was tested and the other biliverdins (IXβ, IXγ, and IXδ) were assumed to give similar product distributions. Hematinic acid, methylvinylmaleimide, PDP2, and PDP3 were identified, but their relative quantities were much different from the products from FePPIX. Biliverdin IXα formed hematinic acid, methylvinylmaleimide, and total PDPs in equimolar quantities. FePPIX, however, yielded primarily hematinic acid and methylvinylmaleimide with total PDPs accounting for only a small percentage of the total products. These results further indicate that degraded FePPIX does not pass through a biliverdin intermediate.

Breakdown of PDPs to Maleimides. Maleimides appear to be the major degradation products derived from FePPIX, purified P-450, and microsomal P-450 in each of the systems we examined. PDPs were also detected but at very low levels. Conceivably, PDPs could be the products initially generated from heme that are further degraded to maleimides. To test this hypothesis, Et_4-PDP was incubated under a variety of conditions that would be expected to destroy it to form diethylmaleimide. The results are shown in Table II. Because Fe(II) PPIX is much more susceptible to degradation by H_2O_2 than Fe(III) PPIX (Guengerich, 1978), Et_4-PDP was incubated with a 10-fold excess of $FeSO_4$ and a 100-fold excess of H_2O_2. Only 12% of the Et_4-PDP was destroyed after 45 min at 37 °C; however, there was no detectable diethylmaleimide produced. Et_4-PDP was also incubated with rat liver microsomes and the Fe(III)–ADP complex or a catalytic concentration of hemin. NaN_3 was included to inhibit endogenous catalase. Neither form of complexed iron catalyzed extensive degradation of Et_4-PDP, and diethylmaleimide was not generated. Thus, PDPs do not appear to be intermediates in the formation of maleimides during heme breakdown.

Discussion

PDPs have been shown to be produced from FePPIX degraded by H_2O_2 (Fischer & Mueller, 1937; Guengerich, 1978; Yoshinaga et al., 1982). Theoretically, the porphyrin ring can be split only two ways to yield four different dipyrroles; however, each of these dipyrroles could conceivably form two PDPs differing in the position of hydration. We have found only four PDPs produced from the reaction of FePPIX with H_2O_2 and determined their structures by mass spectrometry and ^1H NMR. The four structures account for all four dipyrroles expected from FePPIX. Interesting, only one hy-

drated PDP was detected for each different dipyrrole. This situation is in contrast to the PDPs identified by Bonnett & Stewart (1975) from photooxidation of bilirubin, where both hydrated configurations were produced for one dipyrrole.

The PDPs, however, were not the primary products produced from the reaction of FePPIX with H_2O_2. The maleimides, hematinic acid and MVM, were formed in much larger quantities. Fe(III) OEP degraded by H_2O_2 in acetone/water showed a similar distribution of products with diethylmaleimide being the most abundant product and smaller quantities of Et_4-PDP being formed. The microsomal and purified enzyme systems tested also fit this same pattern. Microsomal FePPIX destroyed by cumene hydroperoxide or iodosylbenzene showed formation of hematinic acid and MVM; however, PDPs were not detected. P-450 heme degraded by NADPH-P-450 reductase and NADPH, microsomes incubated with NADPH and N_3^-, and microsomes undergoing lipid peroxidation all formed large quantities of hematinic acid and MVM and very low levels of some of the PDPs. Only FePPIX destroyed by NADPH-P-450 reductase and NADPH yielded all four PDPs, but still in small quantities relative to the maleimides. Because PDP2 and PDP3 are the only dipyrroles formed in the microsomal systems, the α and γ bridges of P-450-bound FePPIX are most susceptible to attack. PDPs 1 and 4 would require cleavage at the β- and δ-bridge positions. The membrane-bound P-450 apoprotein may shield β- and δ-positions from attack and leave the α- and γ-positions unprotected.

Although the maleimides and PDPs were the major degradation products that were recovered, they only account for up to 15% of the heme destroyed in these enzyme systems. In microsomes containing ^{14}C-labeled heme, 30–60% of the heme-derived radioactivity became covalently bound to microsomal protein during heme destruction. We (Guengerich, 1978) previously showed that when purified $P\text{-}450_{PB\text{-}B}$ containing ^{3}H-labeled heme was incubated with NADPH-P-450 reductase, NADPH, and excess bovine serum albumin, the ^{3}H label became covalently bound to the P-450 apoprotein, and no ^{3}H was bound to the albumin. These results suggest that a reactive species is formed which quickly binds to the P-450 before it can migrate away from the apoprotein. The nature of the reactive species has not been determined; however, MVM could account for at least some of the bound products. MVM was always found at lower levels than hematinic acid. The reactivity of MVM toward protein nucleophiles (or with H_2O_2) could account for this observation. MVM reacts with glutathione nonenzymatically, while hematinic acid does not (data not shown).

The studies with the reaction of H_2O_2 with metal–OEP complexes suggest that the metal plays a role in the porphyrin breakdown mechanism. Only OEP complexed with Fe(III), Co(II), and Mn(III) was degraded by H_2O_2. These metals all have relatively high one-electron reduction potentials, while the other metals tested [Mg(II), Cr(III), Cu(II), and Zn(II)] do not and do not support OEP breakdown. Similarly, Bonnett & Dimsdale (1972) have shown that Fe(III)–, Co(II)–, and Mn(III)–OEP complexes form a *meso*–oxyoctaethylporphyrin product when treated with H_2O_2 in pyridine under anaerobic conditions. Exposure of the oxyoctaethylporphyrin metal complex to oxygen and H_2O, however, yields octaethylbilitriene and other pigments. Thus, this mechanism appears to be distinct from the mechanism of FePPIX degradation that we are studying.

Biliverdins have not been detected from FePPIX or P-450 heme destroyed by NADPH-P-450 reductase and NADPH

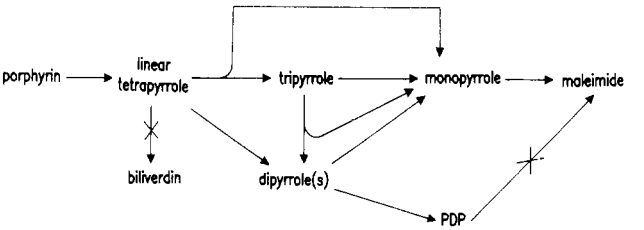

FIGURE 10: Proposed scheme for the breakdown of FePPIX upon reaction with H_2O_2. The rationale is discussed in the text.

(Guengerich, 1978; Yoshinaga et al., 1982). Further, they do not appear to be intermediates in the breakdown of FePPIX to PDPs and maleimides. FePPIX is degraded by NADPH-P-450 reductase and NADPH at a much faster rate than is biliverdin IXα in the presence of excess Fe(III). Thus, biliverdins, if formed, would be expected to accumulate with time during FePPIX breakdown. In addition, biliverdin IXα destroyed by NADPH-P-450 reductase and NADPH in the presence of Fe(III) produced hematinic acid, MVM, and total PDPs (2 and 3) in equimolar quantities. FePPIX yields low levels of PDPs relative to the maleimides, suggesting a different reaction mechanism.

Maleimides and PDPs appear to be end products of FePPIX breakdown. Under a variety of conditions that produce maleimides from FePPIX, PDPs are not destroyed and do not form maleimides as judged from experiments with Et_4-PDP. A pathway is conceivable, however, whereby a dipyrrolic species could form either PDPs or maleimides. Figure 10 shows a scheme for FePPIX breakdown that incorporates these mechanistic considerations. The porphyrin may first be opened to a linear tetrapyrrole other than biliverdin. This could either be split into two dipyrroles or a tripyrrole and a monopyrrole (maleimide). The tripyrrole may then be cleaved into a dipyrrole and a monopyrrole. The dipyrroles are either converted to PDPs or monopyrroles.

Porphyrin bridge carbons are lost during formation of PDPs and maleimides, but the nature of this species has not been elucidated. Production of CO and CO_2 from degraded P-450 heme was assayed by the method of Ahr et al. (1980) with microsomes from rats treated with 5-amino[5-^{14}C]levulinic acid. This heme precursor results in labeling of hepatic and renal heme at the methene bridge positions, as well as in the pyrrole rings (Bissell & Hammaker, 1976). When the FePPIX in these microsomes was destroyed by cumene hydroperoxide, NADPH and NaN_3, or lipid peroxidation, the levels of CO and CO_2 account for <10% of the FePPIX lost. In control experiments, FePPIX was extracted from the ^{14}C-labeled microsomes and incubated with rat spleen microsomes, which contain high levels of heme oxygenase. The recovered ^{14}CO accounted for 97% of the FePPIX destroyed. These results are in agreement with those of DeMatteis et al. (1977), which showed that labeled CO production accounted for only 6–7% of microsomal FePPIX destroyed by lipid peroxidation. HCHO was also considered as a possible product. FePPIX was incubated with NADPH-P-450 reductase and NADPH (conditions described in Table I, footnote *d*). HCHO was assayed after derivitization with 2,4-dinitrophenylhydrazine (Feiser & Feiser, 1967) and quantitated by HPLC [Altex octyl reverse-phase column; 30% H_2O, 70% MeOH (v/v)]. Again, HCHO accounted for <10% of the FePPIX degraded. Cantoni et al. (1981) have suggested that HCO_2H is produced when FePPIX is reacted with H_2O_2. More recently, Docherty et al. (1984) showed that HCO_2H was the major one-carbon product formed from methemalbumin degraded by NADPH-P-450 reductase and NADPH. Our efforts to measure

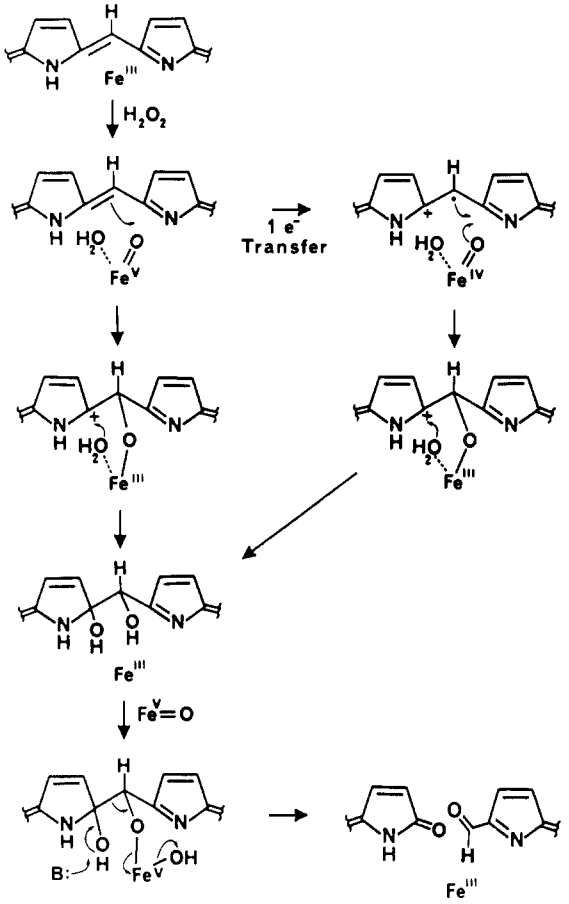

FIGURE 11: Proposed mechanism for the cleavage of the FePPIX ring system that does not yield biliverdin. This mechanism is proposed to be repeated at other bridge positions to generate mono- and dipyrrolic species. "B:" denotes a general base.

HCO_2H from the reaction of FePPIX with P-450 reductase and NADPH were unsuccessful due to high levels of HCO_2H contaminating the cofactors and the insensitivity of the assays; however, HCO_2H does appear to be a likely species.

A mechanism consistent with the scheme in Figure 10 and iron-catalyzed oxidations involving H_2O_2 is presented in Figure 11 for FePPIX breakdown. The iron, presumably complexed in the porphyrin, reacts with H_2O_2 to form a formal perferryl oxygen species (Groves et al., 1981). This complex reacts with the olefinic porphyrin bridge to form a glycol. The resulting Fe(III) can reactivate another H_2O_2, which oxidatively cleaves the glycol to yield an amide and an α-formyl pyrrolic species. The reaction could also be envisioned to proceed through a one-electron radical mechanism involving the perferryl species instead of the two-electron process discussed above. Another radical mechanism is shown in Figure 11. It proceeds through a one-electron transfer from the porphyrin π system to the perferryl to yield a π-cation radical. Such a species has been proposed to be formed in catalase during reaction with H_2O_2 (Dolphin et al., 1971), as well as during inactivation of horseradish peroxidase by cyclopropanone hydrate (Wiseman et al., 1982).

The reaction can be repeated at different bridge carbons to eventually break the porphyrins into monopyrroles and dipyrroles. Most of these structures should contain an α-formyl group. To generate maleimides and PDPs, this α-formyl group may be oxidatively cleaved as shown in Figure 12. The hydrated pyrrole could be attacked by the perferryl species, again generated from reaction of Fe(III) with H_2O_2, to form an α-ketone on the pyrrole ring and HCO_2H.

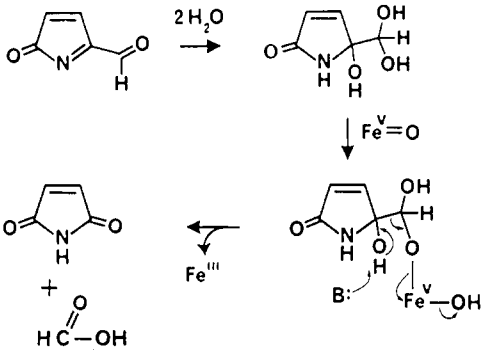

FIGURE 12: Proposed mechanism for the oxidative cleavage of a formyl group from a 2-formylpyrrolonone to generate a maleimide. "B:" denotes a general base. A similar mechanism is proposed for cleavage of the formyl group from a dipyrrolic species to yield a PDP. The mechanism in Figure 11 accounts for formation of the 2-formyl compounds.

These studies describe the degradation of P-450 heme in vitro; however, the in vivo relevance of this mechanism is currently under investigation. Such a mechanism could account for the catabolism of P-450 heme by a pathway that does not form CO, the existence of which has been shown in liver cells (Bissell & Guzelian, 1980).

ACKNOWLEDGMENTS

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Registry No. MVM, 21494-57-5; PDP1, 96328-83-5; PDP2, 96328-84-6; PDP3, 96328-85-7; PDP4, 96328-86-8; Et_4-PDP, 20884-36-0; Fe(III) OEP, 28755-93-3; FePPIX, 14875-96-8; Co(II) OEP, 17632-19-8; Mn(III) OEP, 28265-17-0; NADPH, 53-57-6; P-450, 9039-06-9; Fe(III) ADP, 51595-41-6; reductase, 9039-06-9; N_3^-, 14343-69-2; Fe, 7439-89-6; H_2O_2, 7722-84-1; hematinic acid, 487-65-0; 2,3-diethylmaleimide, 34085-07-9; iodosylbenzene, 536-80-1; cumene hydroperoxide, 80-15-9; octaethylbilitriene, 35050-46-5; biliverdin IXa, 114-25-0.

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Stereochemistry of Lysine Formation by *meso*-Diaminopimelate Decarboxylase from Wheat Germ: Use of ^1H–^{13}C NMR Shift Correlation To Detect Stereospecific Deuterium Labeling†

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ABSTRACT: The stereochemical course of the wheat germ *meso*-diaminopimelate (DAP) decarboxylase reaction is compared to that of the decarboxylase isolated from *Bacillus sphaericus*, which has been reported to proceed with an unusual inversion of configuration [Asada, Y., Tanizawa, K., Sawada, S., Suzuki, T., Misono, H., & Soda, K. (1981) *Biochemistry 20*, 6881–6886]. Reaction of each enzyme with either unlabeled diaminopimelic acid in D_2O or [2,6-$^2H_2$]diaminopimelic acid in H_2O gave stereospecifically deuterium-labeled lysine samples that were derivatized with (−)-camphanoyl chloride and diazomethane. Analysis by two-dimensional ^1H–^{13}C heteronuclear NMR shift correlation spectroscopy with ^2H decoupling confirmed the stereochemistry of the *B. sphaericus* enzyme reaction and showed that the eukaryotic wheat germ *meso*-DAP decarboxylase also operates with inversion of configuration. This suggests similar mechanisms for the prokaryotic and eukaryotic enzymes and contrasts the retention mode observed with other pyridoxal phosphate dependent α-decarboxylases.

The enzyme *meso*-diaminopimelate (DAP)[1] decarboxylase (EC 4.1.1.20) is pyridoxal 5′-phosphate (PLP) dependent and catalyzes the decarboxylation of the R_D center of *meso*-diaminopimelic acid to give L-lysine (Figure 1) (Patte, 1983; White, 1983). This enzyme has been isolated from both plants (Vogel & Hirvonen, 1971; Mazelis & Creveling, 1978; Sodek, 1978; Kato, 1979; Rosenthal, 1982) and bacteria (White, 1971; Rosner, 1975; Asada et al., 1981a; Lakshman et al., 1981) and is the only PLP-dependent α-decarboxylase known to act on a D-amino acid. The enzyme isolated from *Bacillus sphaericus* has been reported by Soda and co-workers (Asada et al., 1981b) to operate with inversion of configuration, in direct contrast to other PLP-dependent α-decarboxylases for which the stereochemistry of reaction has been determined. In all other cases examined, the reaction proceeds with retention of configuration—that is, the incoming proton occupies the same stereochemical position as did the departing carboxyl group (Dunathan, 1971; Snell, 1982; Floss & Vederas, 1982; Akhtar et al., 1984; Palcic & Floss, 1985; Nakazawa et al., 1981; Orr

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; DAP, diaminopimelate; DTE, dithioerythritol; EtOH, ethanol; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetate; Me_4Si, tetramethylsilane.

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