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# Cytochrome P-450 Inactivation: Structure of the Prosthetic Heme Adduct with Propyne†

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ABSTRACT: Hepatic microsomal cytochrome P-450 from phenobarbital-pretreated rats is destroyed by propyne in a reduced nicotinamide adenine dinucleotide dependent process which also results in vivo in the accumulation of an abnormal green porphyrin. The green porphyrin has been identified by its electronic absorption, mass spectrometric, and nuclear magnetic resonance properties as the isomer of *N*-(2-oxopropyl)protoporphyrin IX in which the alkylated nitrogen is that of pyrrole ring A. Alkylation of the other nitrogens in the parent heme is quantitatively unimportant, although evidence for traces of the resulting adducts has been obtained. The green porphyrin exhibits a circular dichroism spectrum and is therefore the result of a chirally selective or specific interaction. The structure of the green porphyrin implicates a cytochrome P-450 destructive mechanism in which a species formed by catalytic oxidation of the acetylenic moiety reacts with the nitrogens of prosthetic heme. The possible nature of the reactive intermediate is discussed.

Hepatic microsomal cytochrome P-450 is destroyed during catalytic processing of olefins (De Matteis, 1971, 1978; Levin et al., 1972; Ortiz de Montellano & Mico, 1980), acetylenes (White & Müller-Eberhard, 1977; Ortiz de Montellano & Kunze, 1980a), and allenes (Ortiz de Montellano & Kunze, 1980b). Suicidal $\pi$-bond metabolism by the cytochrome P-450 enzyme appears to underlie the destructive process, although its suicidal nature has only been explicitly documented in the case of 2-isopropyl-4-pentenamide (Ortiz de Montellano & Mico, 1981). The destruction of the enzyme by monosubstituted olefins and acetylenes is paralleled by an approximately equimolar decrease in microsomal heme content (De Matteis, 1971; Bradshaw et al., 1978; White, 1978) and by the concurrent appearance of abnormal hepatic green porphyrins (De Matteis, 1978; White & Müller-Eberhard, 1977; Ortiz de Montellano & Kunze, 1980a; Ortiz de Montellano & Mico, 1980). Analogous green porphyrins, however, have not been observed to accompany destruction of the enzyme by disubstituted olefins and acetylenes or by allenes (Ortiz de Montellano & Kunze, 1980a,b; Ortiz de Montellano & Mico, 1980). We have isolated the green porphyrins obtained with a number of unsaturated agents and have shown that their mass spectrometric molecular ions correspond to the stoichiometric sum of the molecular weights of protoporphyrin IX (as the dimethyl ester due to the isolation procedure) plus the administered agent plus an oxygen atom (Ortiz de Montellano et al., 1979, 1980; Ortiz de Montellano & Kunze, 1980a). These results have led us to postulate that terminal olefins (Ortiz de Montellano et al., 1979, 1981a,b) and acetylenes (Ortiz de Montellano & Kunze, 1980a) are oxidized by cytochrome P-450 to transient intermediates that irreversibly alkylate the prosthetic heme of the enzyme. The validity of this proposal has remained uncertain, however, and the details of the interaction have remained experimentally inaccessible, due to the absence of precise structural information on the isolated green porphyrins. We have recently, as the result of a major effort to obtain such structural information, unambiguously identified the ethylene-derived green porphyrin as one of the four possible regioisomers of *N*-(2-hydroxyethyl)protoporphyrin IX (Ortiz de Montellano et al., 1980, 1981a,b). Similar efforts to determine the structure of the porphyrin isolated from acetylene-treated rats have been

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0006-2960/81/0420-7266$01.25/0 

frustrated due to its unusual instability, although sufficient data have been obtained to tentatively identify it as a mixture of the isomers of *N*-(2-oxoethyl)protoporphyrin IX (Kunze et al., 1981). Unequivocal identification of the structure of an adduct between prosthetic heme and an acetylene nevertheless remains an essential key to the mechanism of the destructive interaction of cytochrome P-450 with this class of substrates. The structure of the prosthetic heme adduct with propyne and some of the mechanistic implications of the observed regio- and stereoselectivity of the alkylation process are presented here.

## Experimental Procedures

*Isolation of the Porphyrin Adduct.* Male Sprague-Dawley rats, injected intraperitoneally once a day for 4 days with an 80 mg/kg dose of sodium phenobarbital in water (80 mg/mL), were placed for 4 h in a chamber through which a 1:1 mixture of air/propyne (Farchan Division, Story Chemical Corp.) was passed. The livers of the rats were then perfused and homogenized, and the homogenate was allowed to stand in 5% (v/v) $H_2SO_4$/methanol overnight at 0 °C, as previously described (Ortiz de Montellano & Mico, 1980). The methylene chloride extract of the acidic methanol mixture, obtained as before (Ortiz de Montellano & Mico, 1980), was concentrated and chromatographed on 2000-$\mu$m silica gel G preparative thin-layer plates (Analtech) after addition of a few drops of 0.5% (w/v) zinc acetate in methanol. The plates were developed with 2:1 (v/v) chloroform/acetone. The green (red-fluorescing) band on the plates was extracted with acetone, and the extract was rechromatographed on a 500-$\mu$m silica gel plate developed with 3:1 (v/v) chloroform/acetone. The recovered red-fluorescing fraction was then subjected to high-pressure liquid chromatography (HPLC) on a Partisil 10-PAC magnum 9 column eluted with a 15-min 0–100% gradient of methanol into 1:1 (v/v) hexane/tetrahydrofuran (variable wavelength detector set at 590 nm). The single porphyrin fraction thus obtained was demetalated by treatment with 5% (v/v) $H_2SO_4$/methanol (Ortiz de Montellano & Kunze, 1980a). The metal-free porphyrin fraction was further purified by high-pressure liquid chromatography on the Partisil 10-PAC magnum 9 column eluted isocratically with a ternary solvent mixture consisting of 5% methanol and 95% 1:1 (v/v) hexane/tetrahydrofuran (detector set at 510 nm). The purified porphyrin was reconverted to its zinc complex when required by addition of zinc acetate. The acetate counterion was exchanged for a chloride by repeated washing of a methylene chloride solution of the complex with NaCl solution.

*In Vitro Destruction of Cytochrome P-450.* The loss of spectroscopically observable cytochrome P-450 due to incubation of hepatic microsomes from phenobarbital-pretreated rats with propyne was measured by a slight modification of the procedure used in the analogous study of ethylene (Ortiz de Montellano & Mico, 1980). The propyne was not bubbled through the incubation mixture, as with ethylene, but was passed over its surface as a 10% (v/v) mixture in air at a rate of 100 mL/min for 5 min before the incubation was started by addition of NADPH.[1] The final incubation mixture contained, in addition to substrate, the following: microsomal protein (1 mg/mL), KCl (150 mM), EDTA (1.5 mM), and NADPH (1.0 mM) in 0.1 N sodium/potassium phosphate buffer (pH 7.4).

*Spectroscopic Elucidation of the Structure of the Green Porphyrin.* Electronic absorption spectra were obtained in methylene chloride on a Varian-Cary Model 118 instrument. Field desorption mass spectra were recorded on a modified AEI MS-902 instrument at the Berkeley Biomedical and Environmental Mass Spectrometry Resource (Berkeley, CA) with previously reported conditions (Ortiz de Montellano & Kunze, 1980a). NMR spectra were obtained in deuteriochloroform solution on a Nicolet NT-360 FT NMR spectrometer at the University of California (Davis) NMR facility. The conditions employed for measurement of relaxation times and of nuclear Overhauser effects have been described (Kunze & Ortiz de Montellano, 1981). The 99.96% deuterated chloroform used in the NMR studies was stored over $K_2CO_3$ to remove all traces of acids. Chemical shifts in the NMR spectrum are assigned with respect to the chloroform peak at 7.21 ppm. Circular dichroism spectra were obtained on a Jouan Dichrograph II instrument with a cell of 2-cm light path. Assuming an extinction coefficient at 432 nm of approximately 125 000 for the zinc complex of the green porphyrin, based on the extinction coefficient of the *N*-methylprotoporphyrin IX complex (Ortiz de Montellano et al., 1981c), we used a 100 $\mu$M concentration of the porphyrin in methylene chloride for circular dichroism measurements.

## Results

Incubation of hepatic microsomes from phenobarbital-pretreated rats under an atmosphere of propyne and air resulted in time-dependent loss of spectroscopically measurable cytochrome P-450. Approximately 48 ± 2% of the enzyme was lost after 15 min of incubation and 56 ± 2% after 30 min. The loss of enzyme was insignificant (less than 2%) in the absence of NADPH or in the presence of NADPH but in the absence of propyne. These results rule out destruction of the enzyme by an adventitious process such as lipid peroxidation and also suggest catalytic participation of the enzyme in its own destruction. Propyne, as expected, thus exhibits the cytochrome P-450 destructive activity characteristic of most (Ortiz de Montellano & Kunze, 1980a; White, 1978, 1981) but not all (Ortiz de Montellano et al., 1981b; White, 1981) terminal acetylenes.

A green hepatic porphyrin was isolated from the livers of phenobarbital-pretreated rats exposed for 4 h to a 1:1 mixture of propyne/air. The porphyrin was isolated by a procedure that results in methylation of free carboxyl groups and that removes metal ions from weak complexes such as those formed with N-alkylated porphyrins (Ortiz de Montellano et al., 1981a; Ortiz de Montellano & Kunze, 1980a). The green porphyrin was purified by a series of steps involving zinc complexation–demetalation coupled with both thin-layer and high-pressure liquid chromatography. A single band or peak was observed for the green porphyrin throughout this purification sequence. Analysis of the final purified sample by HPLC (Figure 1), however, using conditions that have been found to resolve isomeric *N*-alkylprotoporphyrin IX structures (Ortiz de Montellano et al., 1981a,c), revealed the presence of trace components which may be isomers of the overwhelmingly dominant porphyrin.

The electronic absorption spectra (Figure 2) of the purified porphyrin and of its zinc complex are essentially identical with the corresponding spectra of *N*-alkylprotoporphyrin IX derivatives (Ortiz de Montellano et al., 1980, 1981a–c). The spectrum of the zinc complex is particularly informative because the Soret band has the longer wavelength shoulder which we have suggested is characteristic of *N*-alkylprotoporphyrin IX structures in which the *N*-alkyl group is borne by a vi-

[1] Abbreviations used: NADPH, reduced nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

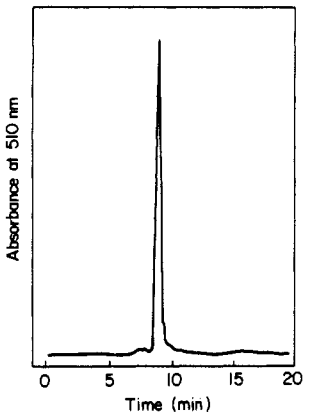


FIGURE 1: Analysis of the purified green porphyrin by high-pressure liquid chromatography on a Partisil 10-PAC magnum 9 column. The elution solvent is a ternary mixture consisting of 5% methanol and 95% 1:1 hexane/tetrahydrofuran.

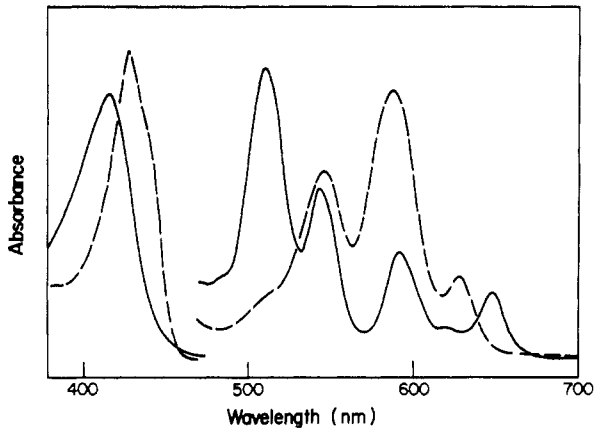


FIGURE 2: Electronic absorption spectra of the purified green porphyrin (solid line) and of its zinc complex (dotted line) in methylene chloride. The Soret band is reproduced at a 10-fold higher attenuation than the rest of each spectrum.

nyl-substituted pyrrole ring (Ortiz de Montellano et al., 1981c; Kunze & Ortiz de Montellano, 1981). The field desorption mass spectrum of the purified green porphyrin (not shown) consists essentially of only two peaks, a molecular ion at $m/e$ 646 and a monoprotonated molecular ion at $m/e$ 647. The molecular constitution suggested by this molecular ion, equal in magnitude to the sum of the molecular weights of the dimethyl ester of protoporphyrin IX ($M_r$ = 590) plus propyne ($M_r$ = 40) plus an oxygen atom ($M_r$ = 16), is consistent with that inferred for other acetylene-derived green porphyrins (Ortiz de Montellano & Kunze, 1980a). The actual incorporation of protoporphyrin IX into the structure is confirmed by NMR analysis of the zinc-complexed green porphyrin (Figure 3). Signals assignable to all the protons of the original protoporphyrin IX are found in the NMR spectrum: four meso proton singlets (10.1–10.4 ppm), two internal vinyl proton multiplets (at approximately 7.9 and 8.2 ppm), a four-proton terminal vinyl multiplet (6.1–6.3 ppm), four side-chain protons adjacent to the ring (approximately at 4.3 ppm), six methyl group singlets (3.5–3.8 ppm), and a multiplet for the four side-chain protons adjacent to the carbonyl groups (3.3 ppm). In addition to these signals and to extraneous peaks at 7.21 ppm (chloroform) and 0.8–1.8 ppm (impurities), two high-field signals are present in the spectrum, one at −0.26 ppm (three protons) and one at −4.43 ppm (two protons). The exceptional position of these signals requires that the associated protons be placed over the porphyrin ring current. In view of the retention of the protoporphyrin IX functionality in the green

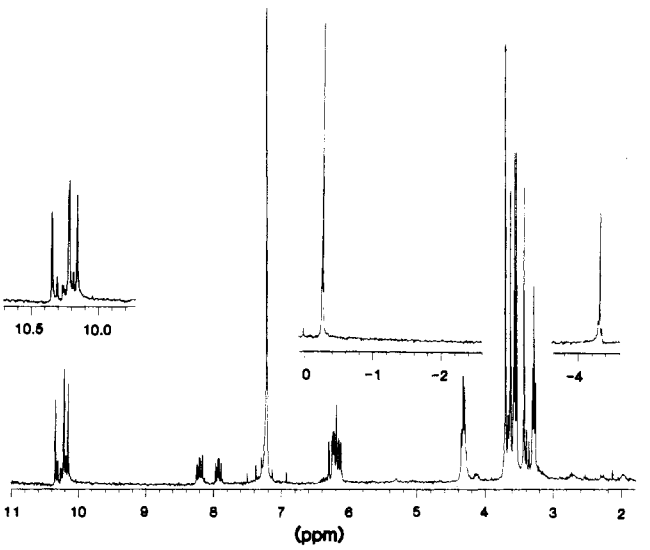


FIGURE 3: NMR spectrum of the zinc complex of the green porphyrin in deuteriochloroform. The region of the spectrum between 0 and 2 ppm is not shown. The only signals in the deleted region are due to impurities and water (0.8–1.8 ppm). The base line of the upfield region of the spectrum is displaced upward and to the left. The meso proton signals are shown at higher resolution in the inset.

porphyrin, of the electronic absorption spectra characteristic of an *N*-alkylprotoporphyrin IX derivative, and of the molecular weight difference between the green porphyrin and protoporphyrin IX, the five upfield protons must be associated with an *N*-alkyl residue with the molecular composition $C_3H_5O$. The absence of scalar coupling between the methylene and methyl group protons in the *N*-alkyl group, and the fact that the methylene is upfield of the methyl and is thus closer to the center of the ring current, is only consistent with identification of the residue as a 2-oxopropyl ($CH_3COCH_2$–) moiety. The absence of scalar coupling was demonstrated by the observation that irradiation of the methyl protons had no effect on the methylene proton signal (not shown). In sum, spectroscopic analysis of the green porphyrin uniquely identifies it as *N*-(2-oxopropyl)protoporphyrin IX dimethyl ester.

Four regioisomers of *N*-(2-oxopropyl)protoporphyrin IX are possible due to the nonequivalence of the four nitrogens in protoporphyrin IX. The chromatographic analysis (Figure 1) and the NMR spectrum (Figure 3) clearly establish that in quantitative terms essentially only one isomer is formed, although one or more of the other three isomers may be formed in trace amounts. Minor signals possibly due to such isomers are present in the NMR spectrum (Figure 3) and are particularly noticeable in the regions where the meso and *N*-alkyl protons are found. It has not been possible, however, to obtain definitive evidence for the formation of minor isomers due to their low concentration. The *N*-alkyl group in the dominant (or exclusive) isomer of the green porphyrin is located on one of the two vinyl-substituted pyrrole rings of the protoporphyrin IX framework. Firm evidence for this conclusion is provided by the already mentioned shoulder on the Soret band of the zinc complex and by the presence in the NMR spectrum of two well-resolved signals for the two internal vinyl protons but of only one unresolved signal for each of the two types of methylene protons in the propionic acid side chains (Figure 3). This signal pattern, which reflects differentiation in the NMR of the peripheral substituents on the vinyl-substituted but not on the propionic acid substituted rings, has been shown to be diagnostic for N-alkylation of either pyrrole ring A or B (Ortiz de Montellano et al., 1981c; Kunze & Ortiz de Montellano, 1981). It is much more difficult, however, to

Table I: Chemical Shifts, Relaxation Times, and Nuclear Overhauser Effects

| group identity | chemical shift (ppm) | $T_1$ (s)[a] | meso position exhibiting NOE[b] |
|---|---|---|---|
| α meso | 10.341 | 1.04 | |
| β meso | 10.220 | 1.02 | |
| δ meso | 10.213 | 0.95 | |
| γ meso | 10.156 | 0.70 | |
| 4-vinyl, internal proton | 8.20 | 0.76 | β |
| 2-vinyl, internal proton | 7.94 | 0.76 | α |
| methylenes vicinal to ring | 4.31 | 0.43 | γ |
| methoxy | 3.701 | 1.24 | none |
| methoxy | 3.697 | 1.05 | none |
| 3-methyl | 3.626 | 0.68 | α |
| 8-methyl | 3.562 | 0.67 | δ |
| 5-methyl | 3.536 | 0.68 | β |
| 1-methyl | 3.426 | 0.69 | δ |

[a] $T_1$, spin-lattice relaxation time. [b] NOE, nuclear Overhauser effect.

determine whether ring A or ring B is N-alkylated. This task has been accomplished by a variant of the procedure we recently developed for identification of the four *N*-methylprotoporphyrin IX isomers (Kunze & Ortiz de Montellano, 1981), the variation in procedure being required by the fact that only one of the four isomers is available in the present case. In essence, relaxation times and nuclear Overhauser effects have been used to identify the specific proton(s) associated with each signal in the NMR spectrum. As a result, it has been possible to determine to which pyrrole ring the upfield of the two internal vinyl protons is attached and consequently to identify the alkylated ring since the upfield shift reflects tilting of the ring caused by N-alkylation (Kunze & Ortiz de Montellano, 1981).

The spin–lattice relaxation time ($T_1$ value), the positions which exhibit a nuclear Overhauser signal enhancement on irradiation of the given proton, and the eventually determined identity of each signal are given in Table I. The γ-meso proton and the two methoxy and methyl groups are directly identified by the relaxation time data, the methoxy groups relaxing more slowly than the ring methyls due to their reduced interaction with other nuclei and the γ-meso proton more rapidly than the other meso protons due to interaction with the flanking propionic acid side chains (Kunze & Ortiz de Montellano, 1981; Sanders et al., 1978). These assignments are confirmed by the absence of a nuclear Overhauser enhancement of any meso proton on irradiation of the methoxy groups and by the observation of a nuclear Overhauser enhancement of the γ-meso proton on irradiation of the side-chain methylene protons at 4.31 ppm. The δ-meso proton is also easily identified because it is uniquely vicinal to two different methyl groups and exhibits nuclear Overhauser enhancement on irradiation of both groups. The two methyls, in turn, must be those at positions 1 and 8, although which is which cannot be determined without further information. Each of the two remaining methyl group signals is specifically associated with one of the two still unassigned meso protons by the observation of a nuclear Overhauser enhancement of the signals due to the latter on irradiation of the former, although again further information is required for specific identification of the two *meso*-methyl pairs. The additional information required for resolution of this problem, in the case of the four *N*-methylprotoporphyrin IX isomers, was obtained by synthesis of the isomers with different but known incorporation of deuterium label into the four meso positions (Kunze & Ortiz de Montellano, 1981). The absence of a synthetic route to the present porphyrin has required the use of a direct instrumental approach based on the report by Sanders et al. (1978) that long-range coupling exists between each internal vinyl proton and the vicinal methyl group. This observation was confirmed in our earlier study of *N*-methylprotoporphyrin IX (Kunze & Ortiz de Montellano, 1981). Irradiation in turn of the four methyl groups resulted in well-defined sharpening of the internal vinyl proton signals in only two instances, the upfield of the two internal vinyl protons responding to irradiation of one of the two methyls already assigned on the basis of the nuclear Overhauser data to position 1 or 8. The methyl in question must therefore be that at position 1 and the responsive vinyl proton that associated with the vinyl group on ring A. The methyl that is coupled to the other vinyl group proton must be that at position 3. The assignment of all the NMR signals (Table I) follows directly from the relationships established by the nuclear Overhauser experiments and from the vinyl proton decoupling results. The assignments are confirmed by the observation that irradiation of each of the two internal vinyl protons results in nuclear Overhauser enhancement of the signal attributed to the appropriate vicinal meso proton (Table I). The internally consistent assignment of all the signals in the NMR spectrum unambiguously establishes that the methyl group and the internal vinyl proton at highest field are both on pyrrole ring A. We have previously observed that N-alkylation causes an upfield shift of the protons associated with the alkylated ring relative to those of the similarly substituted but not alkylated protoporphyrin IX pyrrole ring (Kunze & Ortiz de Montellano, 1981). The 2-oxopropyl moiety in the present green porphyrin therefore is attached to the nitrogen of pyrrole ring A (Figure 4).

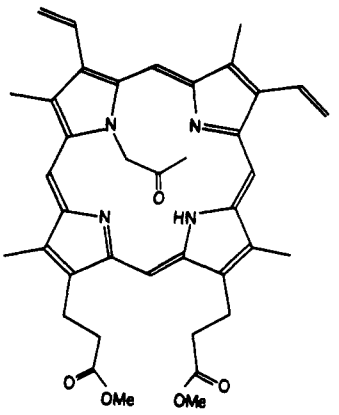


FIGURE 4: Structure of the isolated green porphyrin.

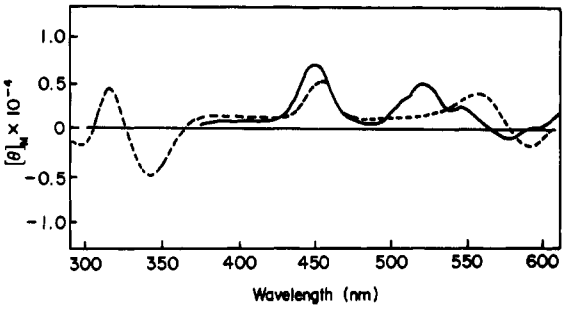


FIGURE 5: Circular dichroism spectra of the green porphyrin (solid line) and of its zinc complex (dotted line) in methylene chloride. The high absorbance of the sample precluded determination of the shorter wavelength region of the spectrum of the free base form of the porphyrin. Ellipticity has been calculated on the assumption that the extinction coefficient of the zinc-complexed porphyrin at 432 nm is 125 000.

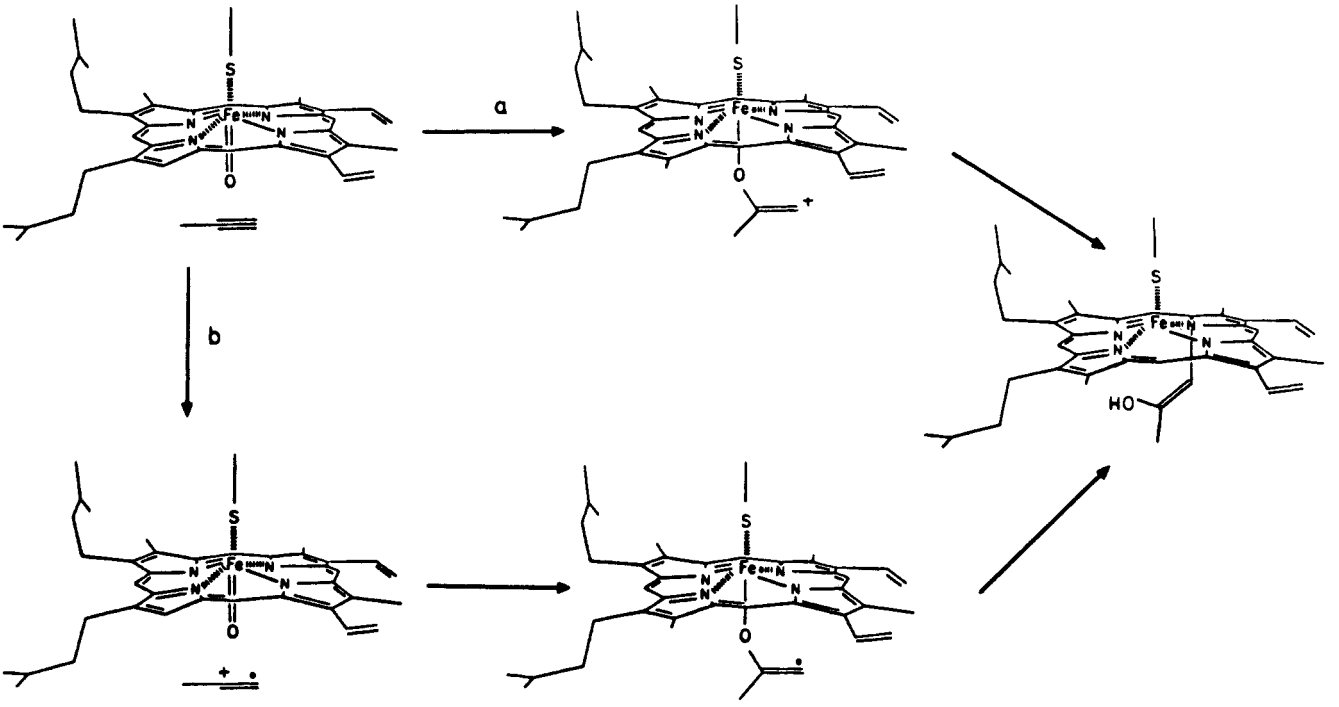


FIGURE 6: Alternative mechanisms for alkylation of the prosthetic heme in cytochrome P-450 by oxidatively activated propyne. The prosthetic heme carboxyl groups are not explicitly shown. The enzymatically activated oxygen is assumed to be an oxene–iron complex, although a specific distribution of the electrons in the complex is not to be inferred.

The purified green porphyrin and its zinc complex exhibit circular dichroism spectra (Figure 5). The positions of the peaks in the circular dichroism spectra, and the shift in peak position caused by complexation with zinc, clearly identify the green porphyrin as the chiral species. This result establishes that the green porphyrin is formed in a chirally selective or specific process, that is, that heme alkylation occurs preferentially from one side of the heme molecule, although neither the degree of chiral specificity nor the absolute stereochemistry of the alkylated porphyrin can now be specified. The biological formation of optically active *N*-methylprotoporphyrin IX has been described (Ortiz de Montellano et al., 1981c).

## Discussion

Hepatic microsomal cytochrome P-450 from phenobarbital-pretreated rats is efficiently inactivated by propyne, a not unexpected result in view of the previously demonstrated ability of acetylenes to destroy cytochrome P-450 (Ortiz de Montellano & Kunze, 1980a; White, 1978). The destructive activity of propyne is only expressed in the presence of NADPH, is not due to substrate-independent processes such as lipid peroxidation, and is accompanied in vivo by the formation of an abnormal green porphyrin. The green porphyrin, isolated as the metal-free dimethyl ester, is uniquely identified by its electronic absorption properties (Figure 2), its molecular weight ($M_r$ = 646), and the NMR spectrum of its zinc complex (Figure 3) as essentially a single isomer (Figure 1) of *N*-(2-oxopropyl)protoporphyrin IX (Figure 4). The key facts supporting this structural assignment are (a) that the electronic absorption spectra of the porphyrin in its metal-free and zinc-complexed forms are virtually identical with the corresponding spectra of other *N*-alkylprotoporphyrin IX derivatives (Ortiz de Montellano et al., 1980; Kunze & Ortiz de Montellano, 1981; Ortiz de Montellano et al., 1981a), (b) that the mass spectrometrically determined molecular weight is equal to the sum of the molecular weights of the dimethyl ester of protoporphyrin IX plus propyne plus an oxygen atom, and (c) that the NMR spectrum, in addition to signals for all the protons of a protoporphyrin IX substructure, exhibits two diagnostic uncoupled high-field signals due to a methylene and a methyl centered in the porphyrin ring current. The nitrogen in pyrrole ring A of protoporphyrin IX has been shown to bear the *N*-alkyl group by the finding, after specific identification of all the signals in the NMR spectrum (Table I), that the protons of the substituents on ring A are displaced to higher field relative to those of the analogous substituents on ring B.

The earlier finding that addition of the substrate plus an oxygen atom to heme rationalizes the molecular weights of porphyrins isolated from the catalytic interaction of cytochrome P-450 with acetylenes (Ortiz de Montellano & Kunze, 1980a) led us to propose an autocatalytic mechanism in which oxygen transfer to the acetylene group gives rise to a highly unstable species that then reacts with the prosthetic heme of the enzyme. The validity of this mechanism and the details of the interaction, however, remained uncertain due to our ignorance of the actual molecular structure of the prosthetic heme adducts. Our first efforts to define the structure of one of these adducts, using acetylene as the substrate, have only met with qualified success due to the exceptional instability of the resulting green porphyrin (Kunze et al., 1981). The present demonstration that the porphyrin framework of the propyne–heme adduct is *N*-(2-oxopropyl)protoporphyrin IX, however, provides solid support for the proposed mechanism. The structure of the porphyrin categorically excludes mechanisms involving oxidation of the porphyrin skeleton of heme and explicitly demonstrates that the oxygen atom is incorporated into the adduct as part of the substrate-derived *N*-alkyl group. Incorporation of oxygen into the substrate with concomitant N-alkylation of prosthetic heme, implied by this result, is most consistent with initial oxidation of the triple bond to an oxirene or to an acylic transient precursor of the oxirene. Independent evidence for cytochrome P-450 catalyzed oxidation of acetylenes to such intermediates is provided by the finding that oxidation of biphenylacetylene to biphenylacetic acid is accompanied by a 1,2 shift of the acetylenic proton (Ortiz de Montellano & Kunze, 1980c). Neither the structure

of the propyne adduct nor the metabolites obtained by acetylene oxidation, however, can be used to differentiate between the involvement in the alkylative process of an oxirene or of an acylic oxirene precursor. The high instability of oxirenes (Strausz et al., 1976; Tanaka & Yoshimine, 1980), which has precluded their direct observation [see Tanaka & Yoshimine (1980) and Ciabattoni et al. (1970)], provides little encouragement for the formulation of oxirenes as distinct reaction intermediates. Indirect evidence furthermore suggests that the reactive species, whatever its nature, is short-lived and unable to diffuse away from the heme. The intervention of a short-lived, nondiffusible species is supported by the observation that essentially only one of the four possible protoporphyrin IX nitrogens is alkylated by propyne. The nitrogen of pyrrole ring A is not inherently the only reactive one because propene and ethylene react exclusively with a different heme nitrogen atom of (presumably) the same phenobarbital-inducible cytochrome P-450 isozyme (unpublished data). Highly regiospecific heme alkylation is most consistent with short-lived, oxygen-anchored species such as those in Figure 6, even though it is not definitive evidence. Further support for the absence of a diffusible intermediate is provided by the observation that norethisterone-inactivated cytochrome P-450 can be reconstituted by replacement of its prosthetic heme (Correia et al., 1981). A diffusible intermediate such as an oxirene would be expected to also alkylate the protein and thus to more permanently damage the enzyme, although the strength of this evidence is tempered by the absence of data on the fraction of inactivated enzyme that can be rescued by exogenous heme. Nevertheless, despite the bias introduced by the indirect evidence, the electronic structure and nature of the intermediate formed by oxygen transfer to the acetylenic $\pi$ bond remain open to question. Two attractive alternatives to the intermediacy of an oxirene are given in Figure 6, a two-electron oxidation to give an iron-coordinated enol cation (path a) and a single-electron process to give the corresponding radical (path b). The reactive species in path a can also be written as a ketocarbene, although localization of negative charge on the oxygen as shown is favored by coordination with the iron. The single-electron process of path b can be envisioned to involve a direct one-electron reaction of the oxygen with the $\pi$ bond but could also involve initial transfer of the $\pi$-bond electron to give a transient radical cation which subsequently collapses to the prealkylation complex shown in Figure 6.

Oxidative alkylation of prosthetic heme by propyne can in principle yield two structurally distinct adducts, the actually observed ketone due to reaction of the heme nitrogen with the unsubstituted acetylenic carbon and an aldehyde due to analogous reaction with the substituted acetylene carbon. Isolation of only the ketone product indicates that the aldehyde derivative either is not formed or is too unstable to be isolated by our usual procedures. Our success in isolating the adduct with acetylene, which is N-substituted with a 2-oxoethyl moiety (Kunze et al., 1981), argues that the aldehyde derivative expected with propyne would have been detected if formed in significant amounts. Failure to do so thus suggests that the aldehyde product is not formed, a result which implies that prosthetic heme alkylation is limited by steric and possibly electronic effects to reaction with the unsubstituted end of acetylenic substrates. This conclusion is consistent with the observation that disubstituted acetylenes destroy cytochrome P-450 but do not give isolable prosthetic heme adducts (Ortiz de Montellano & Kunze, 1980a). The destructive action of disubstituted acetylenes may therefore reflect protein rather than heme alkylation.

The high regioselectivity of heme alkylation by propyne, determined by the orientation of the substrate when bound in the active site, clearly establishes that the alkylation process is governed by the topology of the active site. This conclusion is strengthened by the finding that the heme adducts yield optically active porphyrins (Figure 5) and must therefore have been formed within the chiral confines of the active site. The use of the regiospecificity and chiral specificity of heme alkylation to explore the active sites of cytochrome P-450 isozymes, suggested by these results, is under investigation.

Acknowledgments

The assistance of Dr. Jerry Dallas of the University of California (Davis) NMR facility is gratefully acknowledged.

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