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The Cytochrome P-450 Active Site

REGIOSPECIFICITY OF PROSTHETIC HEME ALKYLATION BY OLEFINS AND ACETYLENES*

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Hepatic microsomal cytochrome P-450 from phenobarbital-pretreated rats is inactivated during the metabolism of linear olefins (ethylene, propene, and octene) and acetylenes (acetylene, propyne, and octyne). As expected from previous work, the inactivation is due to *N*-alkylation of the prosthetic heme group by the substrate. The *N*-alkyl group in each adduct is formally obtained by addition of a porphyrin nitrogen to the terminal carbon and of an oxygen atom (as a hydroxyl function) to the internal carbon of the π-bond. The oxygen is shown here by ^{18}O studies to be catalytically introduced by the enzyme. The olefins exclusively alkylate the nitrogen of pyrrole ring D, but the acetylenes alkylate that of pyrrole ring A. Acetylene is an exception in that it reacts with more than one nitrogen. Circular dichroism studies of the ethylene adduct and of the ring D regioisomer of *N*-ethylprotoporphyrin IX obtained by alkylation of the prosthetic heme of hemoglobin have been used to determine which face of cytochrome P-450 heme is alkylated by the unsaturated substrates. These results implicate an active site that is sterically encumbered in the region over pyrrole ring B and has a lipophilic binding site that accommodates chains of at least six carbon atoms over pyrrole ring C.

The mechanism by which cytochrome P-450 transfers activated oxygen to its substrates and the features of the active site that govern substrate binding have proven to be experimentally elusive. The numerous studies of substrate selectivity available in the literature provide important information on the substrate preference of various isozymes but are not amenable to coherent interpretation in terms of the active site topology of any one isozyme. A salient exception is the recent exploration by Jerina and his collaborators, using the stereochemistry of epoxidation of a large number of polycyclic aromatic hydrocarbons as the experimental probe, of the topology of cytochrome P-450$_c$ from rat liver (1, 2). Imai has independently investigated the topology of the analogous isozyme from rabbit liver (P-448$_1$) by measuring the effect of one ligand on the binding of a second (3). Indirect and sometimes cumbersome methods such as these, in the absence of crystallographic structures for the cytochrome P-450 isozymes, are the only means now available for the acquisition of topological information.

The use of heme[1] alkylation to probe the topology and mechanism of cytochrome P-450 is suggested by our finding that the prosthetic heme of the phenobarbital-inducible rat enzyme is alkylated during catalytic turnover of terminal olefins and acetylenes (4–7). Heme alkylation by olefins and acetylenes involves addition of an oxygen atom to one carbon of the π-bond and of a heme pyrrole nitrogen to the other. The *N*-alkyl group in the resulting adducts, which are isolated as the iron-free *N*-alkylprotoporphyrin IX derivatives, is a 2-hydroxyethyl function in the case of ethylene (4) and a 2-oxopropyl moiety in the case of propyne (5). The advantages of prosthetic heme alkylation as a probe of the enzyme are that (*a*) the heme, as an integral component of the active site, by definition does not perturb the normal catalytic sequence, (*b*) the heme provides an absolute set of coordinates within the active site to which topological data can be related, and (*c*) the heme, because of its intimate involvement with the catalytic process, provides "real time" information on the fleeting events occurring during catalysis. The use of prosthetic heme in this context requires that heme alkylation occur during, rather than subsequent to, the catalytic event. The alkylation reaction that accompanies the metabolism of terminal olefins and acetylenes, according to various lines of evidence, fulfills this condition (see introduction to the accompanying article) (8). Prosthetic heme alkylation has recently provided evidence in support of a nonconcerted, probably free radical, mechanism for olefin epoxidation (6, 8). The use of heme alkylation to investigate the orientation of substrates in the active site, suggested by our finding that propyne reacts almost exclusively with the nitrogen of pyrrole ring A (5) but ethylene with that of either pyrrole ring C or D, is reported here (4). We have determined (*a*) the origin of the oxygen atom incorporated into the porphyrin adducts, (*b*) the absolute stereochemistry of the adduct obtained with ethylene, and (*c*) the regiochemistry of heme alkylation by three olefins and three acetylenes. The results confirm that the enzyme catalytically initiates alkylation of its own prosthetic heme, establish which face of heme reacts with the substrates, and define key elements of the active site topology.

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[1] The trivial name and abbreviation used are: heme, iron protoporphyrin IX regardless of the iron oxidation state; DDEP, 3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine.

MATERIALS AND METHODS[2]

RESULTS

Octene, octyne, and propene decrease the concentration of cytochrome P-450 when incubated with hepatic microsomes from phenobarbital-pretreated rats. The time-dependent decrease in spectroscopically measured cytochrome P-450 is essentially complete within 30 min. The total cytochrome P-450 content was reduced at this time by 22 ± 2, 32 ± 6 and 32 ± 1%, respectively. Enzyme loss is prevented by omission of either NADPH or the substrate from the incubation mixture.

Treatment of phenobarbital-induced rats with octene (500 mg/kg), octyne (500 mg/kg), and propene (40% in air) resulted in the accumulation of abnormal liver porphyrins. The porphyrins were converted to their metal-free dimethyl esters by treatment with acidic methanol and were extracted and purified by thin layer and high pressure liquid chromatography according to previously developed protocols (4, 5). Each porphyrin migrated as a single band or peak during the multiple purification steps. The electronic absorption spectra of the porphyrins, both in the metal-free and zinc-complexed state, were characteristic of *N*-alkylprotoporphyrin derivatives (λ_{max} free base: 418, 512, 546, 592, and 652 nm; zinc complex: 432, 545, 592, and 630 nm). A long wavelength shoulder on the Soret band was only observed with the zinc complex of the porphyrin obtained with octyne.

The molecular ion (MH^+) in the field desorption mass spectrum of each of the metal-free porphyrins (propene adduct, *m/e* 649; octene adduct, *m/e* 719; octyne adduct, *m/e* 717) corresponds, as found before with ethylene and propyne (4, 5), to the sum of the molecular weights of the dimethyl ester of protoporphyrin IX plus the destructive agent plus an oxygen atom. The source of the oxygen atom, however, has remained unknown. The ethylene adduct has therefore been generated *in vitro* under an atmosphere highly enriched in $^{18}O_2$. The resulting adduct was purified by the standard procedure and was compared by field desorption mass spectrometry with the porphyrin previously obtained from ethylene-treated rats (4). The mass spectra of the two samples were obtained on the same day and with the same emitter to minimize instrumental variations (Table I, Miniprint). The porphyrin obtained *in vivo* has the expected molecular ion at *m/e* 634 and monoprotonated molecular ion at *m/e* 635, but the porphyrin obtained in the incubation with $^{18}O_2$ exhibits a large monoprotonated molecular ion at *m/e* 637 with the attendant isotope peak at 638 and only a minor peak at *m/e* 635. The ratio of protonated to unprotonated molecular ions for *N*-alkylporphyrins has been found to be highly variable (12). The two mass unit difference observed here nevertheless clearly establishes that approximately 85% of the porphyrin is labeled with $^{18}O_2$. The 15% of unlabeled oxygen incorporated into the adduct is not unexpected because the gentle purging required to maintain enzyme activity is not sufficient to remove all the dissolved oxygen from the incubation mixture.

Alkylation of prosthetic heme by ethylene, acetylene, and propyne involves formal addition of oxygen to one end of the π-bond and of a heme pyrrole nitrogen to the other (4–6). This reaction pattern could give rise in the present instance to a total of eight distinct porphyrins (not counting stereoisomers) if each of the four nonidentical nitrogens reacts with the two ends of the asymmetric π-bond. The 360 MHz NMR spectra of the zinc-complexed octene, octyne, and propene adducts (Fig. 1, Miniprint), however, clearly establish that essentially only one isomer is actually formed in each instance. The formation at best of traces (approximately 5%) of other isomers is indicated by the presence of essentially only one set of signals for each proton. The observed adducts result from addition of a nitrogen to the terminal carbon of the π-bond. The spectrum of the octyne adduct, for example, exhibits the 2-proton singlet at −4.37 ppm expected for terminal *N*-alkylation rather than the one-proton multiplet expected for the internal alkylation product. Terminal *N*-alkylation is likewise confirmed for the octene and propene adducts by the presence of two-proton multiplets in the appropriate region of their NMR spectra (Fig. 1).

The *N*-alkyl proton signals in each spectrum have been identified by spin decoupling experiments. Irradiation of the multiplet at −5 ppm in the $ABMX_3$ spin system of the *N*-(2-hydroxypropyl) moiety in the propene adduct (Fig. 1) has no effect on the doublet at −1.3, but causes subtle changes in the 0.9 ppm region. Irradiation of this region reduces the methyl doublet at −1.3 ppm to a singlet and the 8-line methylene proton pattern at −5 ppm to 4 lines. The methylene protons therefore are nonequivalent and geminally coupled (J_{gem} = 15 Hz). The different vicinal coupling constants of the two methylene protons with the methine proton (J_{vic} = 2 and 7 Hz) indicate that rotation about the carbon-carbon bond is slow on the NMR time scale at room temperature. Similar results are obtained with the octene adduct (see Ref. 8). The remaining alkyl group proton signals for the octene and octyne pigments are identified in Table II (see Miniprint). The presence of two chiral centers in the olefin adducts make each set of methylene protons, including those of the propionate side chains, diastereotopic. The C-3 and C-4 pairs of methylene protons of the *N*-alkyl group in the octene adduct are sufficiently dissimilar to be resolved in the NMR spectrum.

The porphyrins isolated here from rats treated with propene, octene, and octyne, like those previously obtained with ethylene (4) and propyne (5), result from highly regioselective alkylation of a single pyrrole nitrogen. A technique has been developed to assign such isomers (11) and has been used to identify the nitrogen alkylated by propyne (5). The method hinges on the fact that an *N*-alkyl group on a porphyrin pyrrole ring causes the signals associated with the peripheral substituents of that ring to appear at higher field in the NMR spectrum relative to their position when on a nonalkylated ring. This reflects the different relationship of the substituents relative to the porphyrin ring current brought about by the associated tilting of the alkylated ring. The two internal vinyl protons of the octyne adduct, each a 4-line signal due to spin coupling with the terminal vinyl protons, differ in chemical shift by 0.3 ppm, while the same protons in the octene, propene, and ethylene (4) adducts differ by only 0.06 ppm (Fig. 1). Conversely, the four methylene protons adjacent to the porphyrin ring (4.0–4.4 ppm) are chemical shift equivalent in the spectrum of the octyne adduct, as are the four methylene protons adjacent to the propionate carboxyl groups (3.0 to 3.3 ppm), but these protons are widely separated in the spectra of the three olefin adducts. The octyne adduct therefore bears the *N*-alkyl group on either ring A or B (the vinyl-substituted rings), whereas the olefin adducts are alkylated on either ring C or D (the rings with propionic acid side chains).

In order to differentiate between rings A and B or C and D it is necessary to specifically assign the six methyl and four *meso* proton signals in each spectrum (11). This is done by

[2] Portions of this paper (including "Materials and Methods," Tables I and II, and Figs. 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-2726, cite authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

defining a unique set of structural connectivities based on the fact that (*a*) nuclear Overhauser enhancement of *meso* proton signals is observed when adjacent methyl, internal vinyl, or benzylic methylene protons are irradiated, and (*b*) the γ *meso* proton has a shorter T_1 than the other three *meso* protons, whereas the two methoxy group protons relax more slowly than the ring methyls. The identification of the nitrogen alkylated by ethylene, the most difficult of the examples in this study, is outlined below to illustrate the logic involved. The γ *meso* proton at 10.322 ppm (Fig. 2, Miniprint) in the NMR spectrum of the ethylene adduct (the full spectrum has been published) (4) is identified by its short T_1 (Table II, Miniprint) and by the enhancement of its intensity upon irradiation of the benzylic methylene protons at 4.3 ppm. One ester methyl (3.705 ppm) is identified by its long T_1 (Table II) and the other (3.555 ppm), which overlaps with a ring methyl signal, by integration. Further assignments are hampered by the fact that two of the remaining *meso* proton signals are superimposed (Fig. 2). Irradiation of three separate methyls enhances the signal due to the overlapping *meso* protons. One of the two protons in question must therefore be at the δ *meso* position. Sequential spin decoupling of each set of methyl group protons to determine which ones are spin-coupled (J = 0.8Hz) to internal vinyl protons identifies the 1 and 3 methyls. A distinct sharpening of the 4-line signal due to an internal vinyl proton is observed when these two methyls are decoupled (*middle two spectra*, Fig. 3). Because a nuclear Overhauser enhancement of the overlapping *meso* proton signal was observed when the now identified 1- and 3-methyl protons were irradiated, the overlapping protons must be those at the α and δ *meso* positions. The remaining *meso* proton (10.236 ppm), by difference, is that at the β position. This is confirmed by enhancement of its signal when the internal vinyl protons are irradiated. Since the β *meso* proton is flanked by the 5-methyl group, the dipolar coupling observed in the nuclear Overhauser experiment between the two assigns the latter (3.555 ppm). The 8-methyl group (3.388 ppm) can then be identified because it is the only methyl group not yet assigned. Irradiation at 8.17 ppm (differential irradiation of the two internal vinyl protons was not possible) more strongly enhanced the β than the α *meso* proton signal. Irradiation at 8.22 ppm, on the other hand, more strongly enhanced the α signal. The vinyl proton dipolar coupled to the β *meso* proton is therefore the one centered at 8.17 ppm (that on the 4-vinyl). The 3-methyl group on the same ring as the 4-vinyl is then located by spin decoupling. The final complete *meso* and methyl assignments are given in Table II. The porphyrins obtained with octyne, octene, and propene were similarly analyzed. The results of these experiments are also given in Table II. It was not necessary to examine the coupling of the internal vinyl and *meso* protons in the propene and octene adducts, nor the existence of spin coupling between the internal vinyl and methyl protons in the octyne adduct, to make the assignments.

A single methyl group signal is shifted upfield (Table II) relative to its expected position in each of the adducts. The range of chemical shifts for the 3- and 5-methyl signals are essentially invariant but the 1-methyl signal of the octyne adduct is approximately 0.2 ppm upfield from its position in the other three porphyrins. The octyne adduct therefore is alkylated on ring A (Fig. 4). The 8-methyl signals of the three olefin adducts likewise occur at significantly higher field (0.15 ppm) than the 8-methyl signal of the octyne adduct (or of the three isomers of *N*-methylprotoporphyrin IX not alkylated on ring D) (11). The three olefin adducts therefore are alkylated on ring D (Fig. 4).

The circular dichroism spectrum of the zinc-complexed

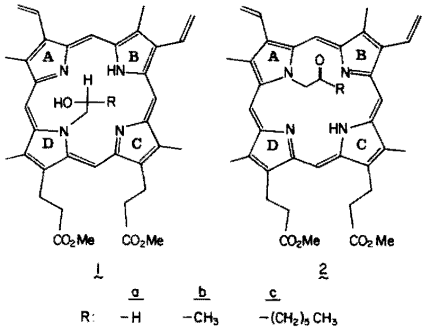

FIG. 4. **Porphyrins derived from prosthetic heme adducts.** The porphyrins derived from the prosthetic heme adducts with ethylene (**1a**), propene (**1b**), octene (**1c**), acetylene (**2a**), propyne (**2b**) (5), and octyne (**2c**). Except for acetylene, which reacts with more than one nitrogen, the nitrogen alkylated is that shown in the structures.

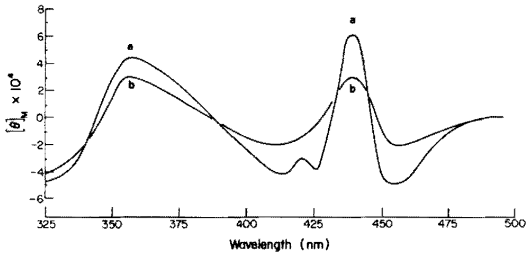

FIG. 5. **Circular dichroism spectra.** Circular dichroism spectra of (*a*) the zinc-complexed dimethyl ester of *N*-(2-hydroxyethyl)-protoporphyrin IX from ethylene-treated rats and (*b*) the ring D isomer of dimethyl esterified chlorozinc *N*-ethylprotoporphyrin IX from DDEP-treated rats. The spectra were recorded in CH_2Cl_2.

dimethyl ester of the ethylene adduct exhibits bands of the same sign and at the same positions as does the spectrum of the ring D isomer of chlorozinc *N*-ethylprotoporphyrin IX (dimethyl ester) (Fig. 5) obtained by alkylation of the prosthetic heme of hemoglobin with ethylhydrazine (14).[3]

DISCUSSION

Oxidation of terminal olefins by the reactive iron-coordinated species of atomic oxygen produced by catalytic turnover of cytochrome P-450 results in epoxide formation and heme *N*-alkylation. Heme alkylation, like olefin epoxidation, requires catalytic turnover of the enzyme in the presence of NADPH and oxygen (7), is inhibited by carbon monoxide and SKF-525A (7), and, as shown here (Table I), results in incorporation of 1 atom of molecular oxygen into the *N*-alkyl group of the heme adduct. The parallels between epoxidation and heme alkylation argue that both reactions spring from interaction of the olefin with a single (or very closely related) activated oxygen species. This mechanistic analogy underlies the use of heme alkylation as a probe of the mechanism by which cytochrome P-450 oxidizes π-bonds. Of particular topological relevance is the fact that catalytic incorporation of molecular oxygen into the olefin-derived *N*-alkyl group constrains the spatial relationship of the olefin relative to the prosthetic heme group during the reaction because the internal carbon of the unsaturated bond must be juxtaposed with

[3] Ortiz de Montellano, P. R., Kunze, K. L., and Beilan, H. S. (1983) *J. Biol. Chem.* **258,** 45–47

the activated oxygen that initiates the reaction.

The structures of the propene, octene, and octyne prosthetic heme adducts are formally obtained by addition of an oxygen (as a hydroxyl group) to the inside carbon of the π-bond and of a nitrogen from the protoporphyrin IX framework of heme to the terminal carbon (Fig. 4). These structures, in conjunction with those of the ethylene (4), acetylene (6), and propyne (5) adducts, clearly establish that reaction of the heme nitrogen with the terminal (unsubstituted) carbon of the π-bond is overwhelmingly favored. High regiospecificity is also observed with respect to the nitrogen of the heme that is alkylated. The three olefins (ethylene, propene, and octene) react almost exclusively with the nitrogen of pyrrole ring D but the two terminal acetylenes (propyne and octyne) react with that of pyrrole ring A (Fig. 4). Only acetylene, among the linear unsaturated hydrocarbons so far tested, is not highly regiospecific and alkylates at least two of the nitrogens (6). The high specificity of the alkylation reaction points to a well defined active site topology.

If one end of the π-bond (the substituted end if a substituent is present) is fixed approximately over the activated oxygen and the oxygen reacts with the π-bond but remains bound to the iron until heme alkylation occurs, additional active site stereoelectronic constraints must exist that prevent reaction of the ethylene with a nitrogen other than that of pyrrole ring D. This assumes, of course, that alkylation specificity is not due to an inherent electronic difference in the reactivity of the four nitrogens. Three observations indicate that even if such an intrinsic difference in nitrogen reactivity exists it is not a major determinant of regiochemical specificity: (*a*) *N*-alkylation in a chemical model proceeds without regiospecificity (15), (*b*) *N*-alkylation of cytochrome P-450 heme by 4-alkyldihydropyridines (16) and ethchlorvynol (13) occurs with relatively low specificity, and (*c*) propyne and octyne, in contrast to ethylene, propene and octene, react almost exclusively with the nitrogen of pyrrole ring A. The stereoelectronic constraints on substrates in the active site must allow acetylene a greater degree of freedom than ethylene in order to explain the lower regiospecificity of acetylene. This can be achieved if a protein residue located over pyrrole ring B blocks the approach of the laterally extended hydrogens of the relatively bulky ethylene terminus to the nitrogen of ring A but does not interfere with that of the cylindrical, sterically compact, acetylene functionality (Fig. 6).

Steric congestion over pyrrole ring B provides a ready explanation for the reaction of acetylene with multiple nitrogens (presumably including those of pyrrole rings A and D), whereas propyne and octyne only react with that of ring A. If

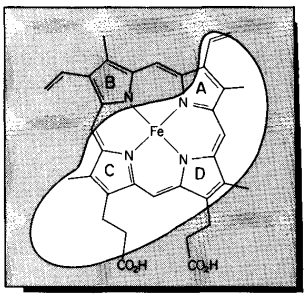

FIG. 6. **Model proposed for the active site of the phenobarbital-inducible cytochrome P-450 isozyme inactivated by unbranched olefins and acetylenes.** Oxygen activation and heme alkylation occur on the reader's side of the heme in the perspective given in the model. Definition of the absolute stereochemistry of the heme in the active site is intended.

the internal acetylenic carbon is anchored by reaction with the iron-bound oxygen, orientation of the terminal carbon toward the nitrogen of ring D forces the hydrocarbon tail of the substrate into the region over pyrrole ring B. If, on the other hand, the terminal carbon is placed over the nitrogen of ring A, the hydrocarbon chain extends over the region defined by ring C. The observed acetylene reaction regiochemistry thus requires *a lipophilic binding site able to accommodate chains of at least six carbons* over pyrrole ring C. Independent evidence for such a binding region is provided by the fact that, if the internal carbon of the π-bond of an olefin is similarly fixed over the iron-bound oxygen and the terminal carbon over the nitrogen of ring D, the hybridization state of the internal carbon (sp^2 going to sp^3) places the hydrocarbon chain in the region over pyrrole ring C.

In order to translate the regiochemical data into a specific active site geometry, it is necessary to know on which side of the prosthetic heme group oxygen activation and prosthetic heme alkylation occur. Heme is a prochiral molecule that gives rise to enantiomeric configurations when the two coordination sites on the iron are differentially occupied. We have recently used the circular dichroism spectra of the ring C isomers of *N*-ethylprotoporphyrin IX obtained from reaction of cytochrome P-450 with DDEP (16) and of hemoglobin with ethylhydrazine (14) to establish that the prosthetic hemes in the two proteins have the same orientation relative to the fifth iron ligand.[3] Analogous comparison of the ethylene adduct (alkylated on ring D) with the corresponding isomer of *N*-ethylprotoporphyrin IX (Fig. 5) shows that the olefin reacts with the same face of heme as DDEP. A model of the active site that incorporates the absolute geometry of the prosthetic heme, the presence of a steric constraint over pyrrole ring B, and the presence of a lipophilic channel over pyrrole ring C, is given in Fig. 6.

The regiochemistry of prosthetic heme alkylation by one branched acetylene, 1-chloro-3-ethyl-1-penten-4-yn-3-ol (ethchlorvynol), is known (13). This sedative hypnotic reacts with at least three of the pyrrole nitrogens, including those of rings A and B. If the same cytochrome P-450 isozyme is inactivated by ethchlorvynol as by unbranched acetylenes, a fact that remains to be established, the active site structure must permit a much lower alkylation regiospecificity when "globular" rather than unbranched substrates are involved. The regiospecificity observed with propyne and octyne is difficult to reconcile, in fact, with metabolism of ethchlorvynol by the same hemoprotein unless the active site has some conformational flexibility. The constraints on reactions in one conformation are unlikely to be the same as those in another. A conformationally flexible active site would not be surprising for a catalytic system as complicated and promiscuous as cytochrome P-450 in view of the fact that even myoglobin, a dedicated monofunctional hemoprotein, apparently undergoes active site conformational breathing (17).

The active site model proposed here predicts that heme alkylation should result from oxidation of the *re* face of the double bond in 1-octene, the exposed face when the internal carbon is fixed over the iron and the terminal carbon is over pyrrole ring D. The stereochemical studies reported in the accompanying paper confirm this prediction and furthermore establish that the opposite (*si*) face of the olefin can be oxidized but does not result in heme alkylation, a result consistent with the proposed active site structure (8).

The model formulated for the active site leaves unexplained the fact that ethylene does not detectably alkylate the nitrogen of pyrrole ring C. The absence of ring C alkylation by olefins larger than ethylene is readily explained because this would involve intrusion of their substituents into the sterically

encumbered pyrrole ring A/B region, but the failure of ethylene to react cannot be explained in the same manner. The model proposed here also does not explicitly incorporate regiochemical imperatives defined by the still unknown detailed mechanism of the oxidation reaction. The regiochemical specificity, for example, may be affected if alkylation by acetylenes involves an iron-carbene intermediate or if that mediated by olefins proceeds through an intermediate in which the iron is simultaneously bound to the oxygen and to the terminal carbon of the original π-bond (7). It is to be hoped that future refinements of the active site model, which is consistent with all of the information now available, will confirm its validity and improve its predictive value.

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The Cytochrome P-450 Active Site. Regiospecificity of Prosthetic Heme Alkylation by Olefins and Acetylenes

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MATERIALS AND METHODS

The following chemicals were used without further purification except for octene, which was distilled prior to use: octene (Aldrich Chem. Co.); octyne (Farchan Division, Story Chem. Co.); propene and ethylene (Matheson Chem. Co.); $^{18}O_2$ (Stohler Isotopes); HPLC grade methanol and hexane (Burdick and Jackson); NADPH, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (Sigma Chem. Co.). Tetrahydrofuran was distilled from sodium under an atmosphere of nitrogen immediately prior to use.

Cytochrome P-450 Destruction In Vitro. Detailed protocols have been reported for the isolation of hepatic microsomes and for the spectroscopic measurement of time and NADPH dependent loss of cytochrome P-450 (9). Microsomes from sodium phenobarbital pretreated (80 mg/Kg) 250 g male Sprague-Dawley rats were used in these experiments. Incubation mixtures contained microsomal protein (1 mg/ml), KCl(150 mM), EDTA (1.5 mM), and NADPH (1 mM) in 0.1N Na/K phosphate buffer (pH 7.4). Octyne (3.3 mM) and octene (10 mM) were added without solvent to the incubation mixtures 10 min prior to initiation of the reaction by addition of NADPH. Propene was passed over the incubation mixture in a closed system at a concentration of 5% in air at a flow rate of 100 ml/min. A 5 min equilibration period was allowed before NADPH was added. The enzyme losses reported (as a percent of the original concentrations) are the average of three independent runs.

Isolation of Prosthetic Heme Adducts Formed In Vivo. Octyne and octene (500 mg/Kg) were administered to phenobarbital induced rats by i.p. injection. Propene was administered by placing rats in a chamber through which a continuous flow (1000 ml/min) of a 40% mixture of propene in air was maintained. The animals were sacrificed after 4 hours of exposure to the hydrocarbons and their livers, after perfusion in situ with ice-cold saline, were homogenized. The homogenate, added to a 5% solution of H_2SO_4 in methanol (100 ml/liver, 0°C), was left overnight in the dark. The resulting dimethyl-esterified porphyrins were worked-up and were purified as the zinc complexes by thin layer chromatography on 2 mm silica plates (Analtech; $CHCl_3$/acetone 3:1) (4, 5). The red-fluorescing pigments, eluted from the silica with acetone, were rechromatographed on 1 mm silica plates (Analtech; $CHCl_3$:acetone 3:1). The zinc-complexed pigments were then purified by HPLC on a Whatman 10 μm Partisil PAC column (4.6 x 250 mm) using a 30 min linear gradient of 0–100% methanol into a 1:1 mixture of hexane and tetrahydrofuran. The pigments were demetallated (5) and rechromatographed under the same conditions. The metal-free pigments thus obtained were converted to the corresponding chloro-zinc complexes (5) for NMR studies. The generation and purification of the ethylene adduct has been reported (4).

Formation and Isolation of the ^{18}O-labeled Ethylene Adduct. The incubation mixture contained hepatic microsomes (3 nM cytochrome P-450) obtained from 10 phenobarbital-induced rats, glucose-6-phosphate (300 mg), glucose-6-phosphate dehydrogenase (100 units), NADP (80 mg), and $MgCl_2 \cdot 6H_2O$ (~0 mg), all in 200 ml of 0.1N Na/K phosphate buffer (pH 7.4) containing EDTA (1.5 mM) and KCl (150 mM). The incubation was carried out in two connected 500 ml vacuum flasks equipped with valved connections to a 250 ml sealed flask containing $^{18}O_2$ and to a balloon containing 1000 ml ethylene, as well as with lines to a water aspirator and a nitrogen tank. The system was purged of oxygen by 5 alternating vacuum-nitrogen gas cycles while maintaining the microsomes at 0°C. The labeled O_2 was introduced to the evacuated system by breaking a glass seal on the flask. The valve to the ethylene-containing balloon was then opened and the reaction allowed to proceed for 45 min at 37°C. The mixture was poured into 1800 ml of 5% H_2SO_4/methanol and left overnight at 0°C in the dark. Previously published procedures were used to isolate and purify the adduct (4, 10).

Spectroscopic Studies. Electronic absorption spectra were obtained on a Varian-Cary 11° spectrophotometer in methylene chloride. Dithionite-reduced, carbon monoxide-complexed microsomal cytochrome P450 concentrations were measured on an Aminco DW-2a instrument. Field desorption mass spectra were obtained on a modified AEI MS-9 instrument at the Berkeley Biomedical and Environmental Mass Spectrometry Resource (Berkeley, CA) (4, 5). NMR spectra were obtained in deuterochloroform on a Nicolet NT-360 FT NMR instrument at the University of California (Davis) NMR facility. The deuterochloroform (99.96% atom D, Aldrich), washed with ND_4OD (Aldrich) and then with D_2O to remove acidic impurities and phosgene, was stored over K_2CO_3. Conditions for the nuclear Overhauser and T_1 experiments have been reported (5, 11). Circular dichroism spectra were recorded in methylene chloride on a Jasco J-500A at 23 °C (2 cm cells).

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