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Destruction of Cytochrome *P*-450 by Olefins: *N*-Alkylation of Prosthetic Heme

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SUMMARY

ORTIZ DE MONTELLANO, P. R., K. L. KUNZE AND B. A. MICO: Destruction of cytochrome *P*-450 by olefins: *N*-Alkylation of prosthetic heme. *Mol. Pharmacol.* **18:** 602–605 (1980).

Destruction of phenobarbital-inducible cytochrome *P*-450 during metabolism of 4-ethyl-1-hexene and ethylene results in accumulation of abnormal hepatic porphyrins. Field desorption mass spectrometric analysis of these "green" porphyrins has shown that they are 1:1:1 stoichiometric adducts of protoporphyrin IX (isolated as the dimethyl ester), the olefin in question, and an oxygen atom. The electronic absorption spectra of the adducts, as both free bases and zinc complexes, are virtually superimposable with the corresponding spectra of the dimethyl ester (DME) of synthetic *N*-methylprotoporphyrin IX. The zinc complexes of both *N*-methylprotoporphyrin IX (DME) and the 4-ethyl-1-hexene adduct exhibit mass spectrometric molecular ions attributable to the porphyrin plus zinc plus a chloride ion, although evidence for thermal generation of protoporphyrin IX (DME) from both of these porphyrins in the mass spectrometer is provided. These results establish that *N*-alkylation of prosthetic heme during attempted metabolism of olefinic bonds is the cause of cytochrome *P*-450 destruction. They also suggest that *N*-(2-hydroxyethyl)protoporphyrin IX (dimethyl ester) is the probable structure of the ethylene adduct.

INTRODUCTION

Hepatic cytochrome *P*-450 monooxygenases, particularly the isozymes inducible by phenobarbital pretreatment, are selectively destroyed during attempted metabolism of certain unsaturated substrates. The first observed, and subsequently most investigated, of these destructive interactions is that of cytochrome *P*-450 with 2-allyl-2-isopropylacetamide (AIA)[1] (1–3). Destruction of the enzyme by this substrate requires enzyme turnover, depends on the presence in the substrate of an unsaturated carbon–carbon bond, and results in conversion of the enzyme prosthetic heme group into an unidentified "green" porphyrin (2–4). We have shown by radiolabeling (5) and mass spectrometric (6) experiments that this abnormal green porphyrin is a 1:1 covalent adduct of AIA with protoporphyrin IX, the organic framework of heme, and have consequently established that alkylation of the prosthetic group is the cause of enzyme loss.

Other substrates with carbon–carbon double bonds, among them secobarbital (3, 7), novonal[2] (8), fluroxene[2] (9), and vinyl chloride (10), are now known also to destroy cytochrome *P*-450. Differences exist in the mechanism of action of these substrates, however, since only the first three result in the accumulation of hepatic porphyrins spectroscopically analogous to those obtained with AIA. We have recently established, in a systematic effort to define the minimum structural requirements for cytochrome *P*-450 destruction, that simple terminal olefins like 4-ethyl-1-hexene and ethylene efficiently destroy the enzyme by a mechanism which results in abnormal porphyrin formation (11). A mechanistic parallel was suggested for the action of AIA and of simple olefins by the virtual identity in electronic absorption properties of the resulting green porphyrins, although firm evidence for this mechanistic analogy was not provided (11). We now report mass spectrometric characterization of the abnormal hepatic porphyrins which accompany *in vivo* destruction of cytochrome *P*-450 by 4-ethyl-1-hexene and ethylene. A comparison is also made between the electronic absorption and mass spectrometric properties of these biologically obtained porphyrins with those of *N*-methylprotoporphyrin IX (DME).[1] This *N*-methylated structure, to our knowledge the first *N*-alkyl derivative

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[1] Abbreviations used: AIA or allylisopropylacetamide, 2-allyl-2-isopropylacetamide; novonal, 2,2-diethyl-4-pentenamide; fluroxene, 2,2,2-trifluoroethyl vinyl ether; DME, dimethyl ester.

[2] Typical hepatic pigments have been isolated in our laboratory after administration of these agents to phenobarbital-pretreated rats.

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of protoporphyrin IX to be described, has recently been synthesized in this laboratory.[3]

MATERIALS AND METHODS

Ethylene gas (CP grade) was purchased from Matheson Gas Products, Newark, Calif.; 4-ethyl-1-hexene was obtained from Chemical Samples Co., Columbus, Ohio, and was distilled before use; all other reagents and solvents were of the highest grade commercially available. The dimethyl ester of *N*-methylprotoporphyrin IX was prepared by alkylation of the dimethyl ester of protoporphyrin IX with methyl fluorosulfonate. This procedure, to be reported separately,[3] yields the four possible isomers expected from alkylation of the four distinguishable nitrogen atoms in protoporphyrin IX. Since the electronic absorption and mass spectra of the four isomers are virtually identical, the spectra of an unresolved mixture of isomers are used for the comparisons in this communication.

Hepatic pigments were isolated and purified exactly as previously described (11) from the livers of phenobarbital-pretreated Sprague–Dawley male rats to which 4-ethyl-1-hexene (400 μl/kg intraperitoneally) or ethylene gas (by inhalation) had been administered. In this procedure, the zinc complexes of the porphyrins (as dimethyl esters due to the workup) are first isolated and the free bases are then obtained by demetalation in acid (11). The zinc complexes of the porphyrin pigments were purified by high-pressure liquid chromatography using a previously described procedure (12). Although the zinc complexes of the ethylene and 4-ethyl-1-hexene pigments chromatographed as single peaks, the possibility of unresolved isomers cannot at this time be excluded.

Absorption spectra of the porphyrins were recorded in dilute chloroform solution on a Varian–Cary 118 spectrophotometer. Field desorption mass spectra were obtained on a Kratos/AEI MS-902 instrument as previously reported (6), except that, due to ongoing efforts at the Biomedical Mass Spectrometry Resource to optimize the properties of the emitters utilized for sample introduction, the method used to prepare emitters was somewhat altered. Although the natures of the desorption process and of chemical reactions occurring on the surface of emitters are poorly understood, substantial evidence exists that these processes are sensitive to changes in the still empirical methods used to prepare emitters (13).

RESULTS AND DISCUSSION

The field desorption mass spectrum of the abnormal porphyrin obtained from rats treated with 4-ethyl-1-hexene (Fig. 1A) exhibits an unprotonated molecular ion at *m/e* 718. This corresponds to a structure whose molecular weight can be rationalized by summation of the molecular weights of the dimethyl ester of protoporphyrin IX (MW 590), 4-ethyl-1-hexene (MW 112), and an oxygen atom (AW 16). The structural validity of this summation, consistent in any case with that of the abnormal porphyrins accompanying destruction of cytochrome *P*-450 by acetylenic agents (12), is substantiated by the mass spectrum of the pigment isolated from ethylene-treated rats. Again, the unprotonated molecular ion peak at *m/e* 634 (spectrum not shown) corresponds to a structure due to stoichiometric combination of protoporphyrin IX (as the dimethyl ester), ethylene, and an oxygen atom. Clearly, the abnormal porphyrins generated during olefin-mediated destruction of cytochrome *P*-450 reflect covalent attachment of the olefinic substrate to the protoporphyrin IX skeleton of prosthetic heme by a mechanism which also results in incorporation of an oxygen atom (or some other 16-mass unit structural element). Given that cytochrome *P*-450 monooxygenases catalyze their own destruction, and that molecular oxygen is required for destruction to occur, the presence of an oxygen atom in the substrate–porphyrin adduct is presumptive evidence for oxidative activation of the substrate prior to adduct formation. An exactly analogous mechanism has also been found to rationalize the destruction of cytochrome *P*-450 by acetylenes (12). Two particularly salient points emerge from these results. First, the data confirm that the catalytic addition of olefins to prosthetic heme depends on the presence of no functional group other than the unsaturated carbon bond and, thus, as recently suggested (11), that the altered carbonyl group in the allylisopropylacetamide adduct results from a lactonization reaction occurring subsequent to, rather than consequent with, the alkylative event. More importantly, the conclusion that similar mechanisms govern covalent binding of

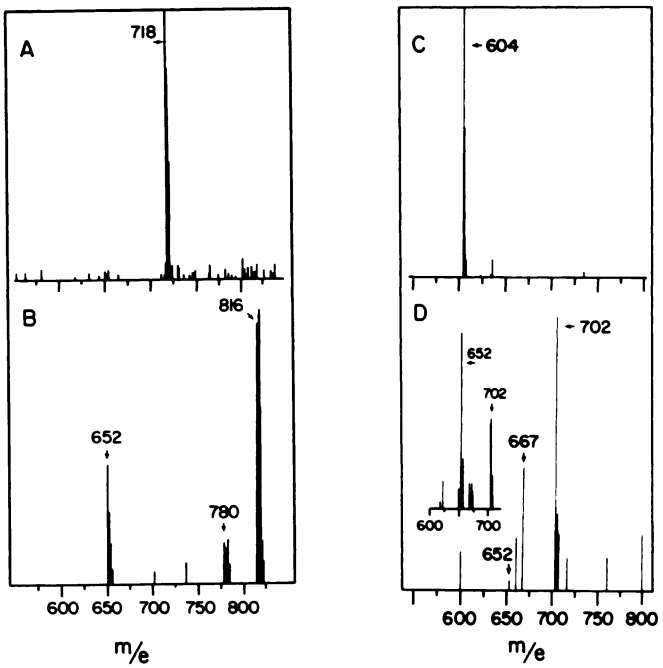

FIG. 1. *Field desorption mass spectra of the abnormal porphyrin obtained with 4-ethyl-1-hexene as (A) the free base and (B) the divalent zinc complex and of the dimethyl ester of N-methylprotoporphyrin IX as (C) the free base and (D) the zinc complex*

The spectrum of the zinc-complexed 4-ethyl-1-hexene adduct reproduced in B was taken at a time when both the *m/e* 816 and the *m/e* 652 peaks were present, earlier spectra in the series favoring the *m/e* 816 peak and later spectra the *m/e* 652 peak. In D, a spectrum taken early in the mass spectrometric run is presented with a later spectrum reproduced as an inset. The labels correspond to the *m/e* value of the first peak in the given cluster.

[3] P. R. Ortiz de Montellano and K. L. Kunze, manuscript in preparation.

olefins and acetylenes to prosthetic heme, and therefore that porphyrin adducts differing in oxidation state by two electrons are produced, makes the virtually identical electronic absorption spectra of both types of adducts (5, 6, 11, 12) of structural diagnostic value. Given the difference in oxidation state of the attached group, it is difficult to rationalize the observed bathochromic shifts (relative to protoporphyrin IX) by introduction in both cases of the same conjugating functionality, as required if the protoporphyrin IX vinyl groups are the site of alkylation. Direct evidence for alkylation not at a vinyl group but at nitrogen, a chromophore perturbation insulated from differences in the shape or oxidation state of the substituent and consequently not in conflict with the spectral data, is presented below.

Bathochromic shifts in porphyrin absorption bands can be engineered by extension of conjugation (for example, at a vinyl group) (3, 14), by steric deformation of the porphyrin ring due to introduction of a *meso* substituent (15), or by ring deformation and bond fixation due to alkylation at nitrogen. Unfortunately, although protoporphyrin IX derivatives with extended vinyl conjugation are known, to our knowledge no *meso*- or *N*-alkylated protoporphyrin IX analogues have been reported. Nevertheless, a recent analysis of the magnitude of the shifts caused by *meso*- and *N*-alkylation of simple porphyrins has been found most consistent with the presence of *N*-alkylation in the biological porphyrin adducts (17). In order to obtain directly related chromophore models for such comparisons, a task made particularly desirable by the relatively small differences in shifts caused by *meso*- and *N*-alkylation and by the possible effect on such shifts of sterically determined alterations in vinyl group conjugation, we have recently completed synthesis of all four possible isomers of *N*-methylprotoporphyrin IX (DME).[3] The absorption spectra of an equal mixture of these isomers and of the porphyrin adduct obtained with 4-ethyl-1-hexene, in both the free-base and the zinc-complexed form, are compared in Fig. 2. As shown, the spectra of these two porphyrin systems are practically superimposable. This remarkable spectral identity provides firm support for the contention (17) that the abnormal porphyrins are *N*-alkylated protoporphyrin IX derivatives. Further support is provided by the field desorption mass spectra of the zinc complexes of these porphyrins. Two features of the spectra are particularly relevant, the more so because the identical behavior is exhibited by both the 4-ethyl-1-hexene adduct and *N*-methylprotoporphyrin IX (DME), a well-characterized model system. The first striking aspect of the spectra of each of these two zinc complexes is that the observed molecular ion is 35 mass units ***higher*** than expected, indicating the association of a chloride[4] ion with the desorbed zinc–porphyrin complex (Figs. 1B and D). This observation is consistent with the structure of *N*-methylprotoporphyrin IX (DME), since the divalent zinc complex would be monocationic and would thus be

[4] Field desorption emitters are a ready source of chloride ions. The peak clusters found in these spectra are due to the significant natural abundance of four zinc and two chloride isotopes, ^{64}Zn and ^{35}Cl being the major isotopes. Isotope ratios are not quantitatively reflected in the observed clusters, however, due to ion current instabilities inherent in the field desorption method.

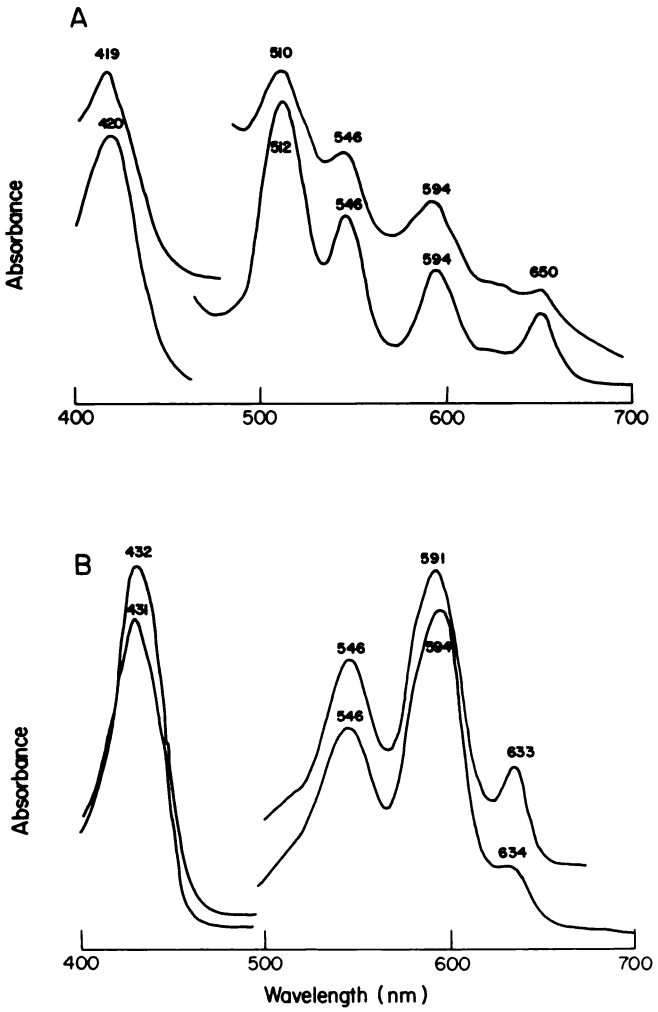

FIG. 2. *Comparison of the electronic absorption spectra of the 4-ethyl-1-hexene adduct with those of authentic N-methylprotoporphyrin IX (dimethyl ester), as both (A) free bases and (B) divalent zinc complexes*

The upper curve in each panel (note that the baselines have been displaced) is due to the 4-ethyl-1-hexene adduct, and the lower to *N*-methylprotoporphyrin IX. The Soret bands were recorded at a 10-fold higher attenuation than the rest of the spectrum. The corresponding spectra for the ethylene adduct are essentially the same (6) as those shown above.

a neutral species only in the presence of a counterion. The fact that a chloride *also* is associated with the zinc complex of the 4-ethyl-1-hexene adduct thus argues strongly for its formulation as an *N*-alkylated structure. The absence of such a counterion in our previously reported spectrum of the allylisopropylacetamide adduct led us, in fact, to suggest that *N*-alkylation was not involved (6). Peaks due to zinc–porphyrin complexes without associated counterions, however, are also found in the present spectra (at *m/e* 780/781 in Fig. 1B and at *m/e* 667 in Fig. 1D). The reason for the presence of both chloride-associated and counterion-free peaks in the current spectra, but not in the earlier work (6), cannot be precisely defined but is probably due to differences in the surface properties of the emitters utilized in the mass spectrometric determinations (see Ref. 13). The importance of emitter surface chemistry is emphasized in the present studies by the observation that, during the course of mass spectrometric runs with *either* *N*-methylproto-

porphyrin IX (DME) (Fig. 1D) or the 4-ethyl-1-hexene adduct (Fig. 1B), a peak at *m/e* 652 appeared and gradually increased. The peak at *m/e* 652, at least in the case of *N*-methylprotoporphyrin IX (DME), can be assigned to the zinc complex of the dimethyl ester of protoporphyrin IX itself. The appearance of this peak undoubtedly reflects thermal *N*-dealkylation on the emitter surface, a reaction for which ample chemical precedent exists (16). The fact that the 4-ethyl-1-hexene adduct mimics the action of an authentic *N*-alkyl compound and also reverts thermally to protoporphyrin IX confirms that the alkyl group is attached to the porphyrin at a ring nitrogen. Contamination of the samples by protoporphyrin IX (DME), resulting in artifactual observation of the *m/e* 652 peak, is highly unlikely since (a) the radically different chromatographic properties of the dimethyl ester of protoporphyrin IX make its detection and removal a simple task, and (b) a peak due to the dimethyl ester of protoporphyrin IX (at *m/e* 590) was *not* observed in the spectra of the uncomplexed bases (Figs. 1A and C). As in solution chemistry, the zinc ion is apparently required for facile *N*-dealkylation to occur (16).

In summary, convincing evidence has been obtained that olefins alkylate the prosthetic heme of cytochrome *P*-450 to give, after demetalation, carboxyl group esterification, and isolation, *N*-alkylated protoporphyrin IX derivatives. In view of the stoichiometry of the isolated adducts, the involvement of only the carbon–carbon unsaturated bond in the destructive event (11), and the spectroscopic evidence for retention of an intact protoporphyrin IX ring system in the adducts, a very limited number of structures differing only in the precise nature of the *N*-alkyl group can be envisioned for each adduct. In the case of ethylene, the most symmetrical of olefins, it is difficult to formulate a reasonable structure other than *N*-(2-hydroxyethyl)protoporphyrin IX (DME). Definitive evidence on the detailed structure of these adducts is currently being sought and will be reported in due course.

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