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Hydrocarbon formation in the reductive cleavage of hydroperoxides by cytochrome P-450

(radical intermediates in hydroperoxide cleavage/homolysis of the oxygen–oxygen bond in hydroperoxides/rabbit cytochrome P-450 form 2/lipid peroxidation)

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ABSTRACT **Evidence is presented that cytochrome P-450 catalyzes the reductive cleavage of hydroperoxides. For example, in a reconstituted system containing rabbit liver microsomal P-450 form 2, NADPH-cytochrome P-450 reductase, and NADPH, cumyl hydroperoxide yields acetophenone and methane, but no cumyl alcohol is formed. The stoichiometry of the reaction and similar results with α-methylbenzyl, benzyl, and *t*-butyl hydroperoxides are in accord with the following general equation, in which X represents an alkyl group and R and R′ are either alkyl groups or hydrogen atoms in the starting peroxide: XRR′C-OOH + NADPH + H^+ → XRCO + R′H + H_2O + $NADP^+$. Because 13-hydroperoxy-9,11-octadecadienoic acid yields pentane under these conditions, we propose that the known formation of alkanes and aldehydes in membrane lipid peroxidation involves reductive cleavage by P-450 to give the products predicted by the above equation. The cleavage reaction is thought to involve stepwise one-electron transfer, resulting in homolysis of the peroxide oxygen–oxygen bond and generation of an alkoxy radical, with β-scission of the latter followed by reduction of the secondary radical to the hydrocarbon. In accordance with this scheme, when the cleavage reaction with cumyl hydroperoxide was done in ^2H_2O, deuteromethane was formed.**

The cytochrome P-450 family of enzymes is a versatile group of heme proteins well known for their ability to catalyze the monooxygenation of a large variety of endobiotic and xenobiotic compounds (1–3). Whereas molecular oxygen and a reduced pyridine nucleotide are required for such hydroxylation reactions, these components can be replaced by a peroxy compound as shown with liver microsomal suspensions (4, 5) and subsequently with the reconstituted enzyme system containing purified rabbit liver microsomal cytochrome P-450 [P-450 form (or isozyme) 2] (6). The stoichiometry was determined and found to be in accordance with the following reaction, in which P-450 functions as a peroxygenase, RH and ROH represent the substrate and product, respectively, and XOOH is the peroxy compound serving as oxygen donor (6):

$$RH + XOOH \rightarrow ROH + XOH. \quad [1]$$

Thus, with cumyl hydroperoxide the product is cumyl alcohol. As reviewed elsewhere (1, 7, 8), several lines of evidence indicate that in reaction **1** the peroxide oxygen–oxygen bond cleavage is homolytic and therefore distinct from the heterolytic cleavage typical of reactions catalyzed by peroxidases.

In a recent study on the reduction of hydroperoxides undertaken to develop a model for the four-electron reduction of dioxygen by P-450 (9), we found that simple two-electron transfer to yield the corresponding alcohol does not occur; instead acetophenone is produced (10). The present paper is concerned with the identity of the other product, a 1-carbon compound, and with the mechanism and possible biological significance of hydroperoxide reduction. This P-450-catalyzed reaction may now be written as follows:

$$XRR'C\text{-}OOH + NADPH + H^+ \rightarrow XRCO + R'H + H_2O + NADP^+, \quad [2]$$

where X represents any of a variety of alkyl groups and R and R′ are either hydrogen atoms or alkyl groups, of which only methyl has been studied so far.

The mechanism of hydroperoxide reduction by this class of heme proteins is of biological interest because organic hydroperoxides and peroxides are used extensively in industry as initiators in polymer synthesis (11), and some of these compounds are known to produce malignant tumors (12, 13) or to damage cytochrome P-450 *in vitro* (6). Subsequent to the report that ADP-activated lipid peroxidation, as measured by malonaldehyde formation, is coupled to the NADPH oxidase system of microsomes (14), numerous papers have dealt with this subject. Although NADPH-cytochrome P-450 reductase may serve as the primary source of superoxide (15) and hydroxyl radicals (16) for initiation of lipid peroxidation, P-450 has also been implicated (17, 18). More recently, Lindstrom and Aust (19) have reported the reduction of 13-hydroperoxy-9,11-octadecadienoic acid (13-HOO-$C_{18:2}$) to the corresponding 13-hydroxy compound by a reconstituted rat liver microsomal system containing P-450, and Ekström and Ingelman-Sundberg (20) have concluded that purified rabbit P-450s may contribute to lipid peroxidation in reconstituted membrane vesicles. Various products have been reported to arise from lipid peroxidation, including alkanes, and hydrocarbon exhalation is believed to be a measure of this pathophysiological process *in vivo* (21).

Evidence is presented below for the reductive cleavage of 13-HOO-$C_{18:2}$ acid by purified P-450 in the reconstituted system containing NADPH, with the formation of pentane. This is the expected product according to reaction **2**, and we propose that lipid peroxides may be the physiological substrates for the cleavage reaction we have found using hydroperoxides as model substrates.

MATERIALS AND METHODS

Materials. α,α-Dimethylbenzyl hydroperoxide (cumyl hydroperoxide) and *t*-butyl hydroperoxide were obtained from MCB Chemical (Norwood, OH) and Aldrich, respectively, and benzyl and α-methylbenzyl hydroperoxides were

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Abbreviations: P-450 form (or isozyme) 2, rabbit liver microsomal cytochrome P-450 form 2; 13-HOO-$C_{18:2}$, 13-hydroperoxy-9,11-octadecadienoic acid.

synthesized from the corresponding bromides with use of 90% hydrogen peroxide and silver trifluoroacetate as described earlier (22). α,α-Dimethylbenzyl, α-methylbenzyl, and benzyl hydroperoxides were purified by the method of Armstrong *et al.* (23). Attempts to obtain *t*-butyl hydroperoxide free of *t*-butanol and acetone were unsuccessful, but control experiments showed that these contaminants had no effect on the rates of NADPH oxidation and product formation from hydroperoxides. The peroxide content of all samples was determined by iodometric titration as described by Martin (24) and was found to be >95% of the theoretical value for the benzyl hydroperoxides and approximately 60% for *t*-butyl hydroperoxide. P-450 form 2 and NADPH-cytochrome P-450 reductase were purified to electrophoretic homogeneity from liver microsomes of phenobarbital-induced, male New Zealand White rabbits by methods previously described (25–27). The specific content of the P-450 was 12.5–16 nmol per mg of protein, and the specific activity of the reductase was 40–55 μmol of cytochrome *c* reduced per min per mg of protein at 30°C; the results obtained in the studies to be described were independent of the particular enzyme preparations used.

Linoleic acid obtained from Sigma was used for the preparation of 13-HOO-$C_{18:2}$. The starting acid (100 mg in 50 μl of ethanol) was added to 100 ml of 50 mM sodium borate, pH 9.0, and the suspension was kept at 23°C under an atmosphere of oxygen in a two-necked flask capped with a rubber septum. A 1-ml aliquot of a solution of 20 mg of soybean lipoxidase (Sigma) in 20 ml of borate buffer was injected every 20 min into this mixture. The suspension turned clear, and 30 min after the last addition the reaction was quenched with 2 M phosphoric acid, and the pH was adjusted to 4.0. The mixture was extracted with two 50-ml portions of chloroform, and the extract was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The residual oil was dissolved in 1.0 ml of chloroform and applied to an E. Merck (Darmstadt, F.G.R.) Lobar Lichroprep Si 60 column (particle size, 40–63 μm). The column was developed with ethyl acetate/chloroform (vol/vol) 1:2, 5-ml fractions were collected, and those giving a positive hydroperoxide reaction with potassium iodide were pooled and evaporated to dryness. Iodometric titration of this oil indicated a hydroperoxide content of 95%. An absorption coefficient of 11.0 $mM^{-1}\cdot cm^{-1}$ at 236 nm was determined for the product in 50 mM potassium phosphate buffer, pH 7.4. The yield of 13-HOO-$C_{18:2}$ after purification was 60%.

Reconstitution of Enzyme System and Assay for NADPH Oxidation. Stock solutions of P-450 form 2 (40–95 μM) and the reductase (14–22 μM) were mixed to give a 1:1 molar ratio of the two proteins, which was found to be optimal for NADPH oxidation by all of the hydroperoxides used in this study. An appropriate volume of a freshly sonicated aqueous dispersion of dilauroylglyceryl-3-phosphorylcholine (1.0 mg per ml) was added in an amount so that in the final reaction mixture, 1.0 ml in volume, the concentration of each enzyme would be 0.1 μM and of the phospholipid would be 30 μg per ml. The concentrated system was allowed to stand at 4°C for 1–2 hr before use. Potassium phosphate buffer, pH 7.4, containing EDTA (to give final concentrations of 50 and 10 mM, respectively) was added to a cuvette with a 1.0-cm optical path length designed for anaerobic work and equipped with two sidearms (28), and the cuvette was made anaerobic by purging with oxygen-free, water-saturated nitrogen gas. The reconstituted enzyme system was then added from one sidearm, followed by a solution containing 150 nmol of NADPH from the other sidearm. A constant absorbance at 340 nm during a 15-min equilibration at 25°C provided assurance that the mixture was anaerobic. The reaction was initiated by the injection of a 10-μl sample of an anaerobic aqueous solution of an organic hydroperoxide, and absorbance changes were monitored at the same temperature for 2–3 min. The initial rate of NADPH oxidation was determined over the first minute with use of an absorption coefficient of 6.22 $mM^{-1}\cdot cm^{-1}$.

Product Analysis. When hydroperoxide disappearance and product formation were to be measured as well as NADPH oxidation, the conditions were the same as described above, except that the NADPH concentration was 200 μM, the final concentration of each enzyme was increased to 2.0 μM and that of dilauroylglyceryl-3-phosphorylcholine was increased to 102 μg per ml, and the volume of the reaction mixture was 3.0 ml. The three benzyl hydroperoxides and their corresponding alcohols as well as products such as acetophenone and benzaldehyde were analyzed by HPLC with an IBM LC/9533 liquid chromatography system coupled to a Hewlett–Packard model LC/9540 data integrator. An IBM C_{18} reverse-phase column was used at room temperature with an isocratic solvent system of 30% acetonitrile in water at a flow rate of 1 ml/min. Preliminary experiments indicated that the extraction of benzaldehyde and acetophenone from reaction mixtures by 1.0 ml of petroleum ether was quantitative, whereas that of benzyl alcohol, 1-phenylethanol, cumyl alcohol, cumyl hydroperoxide, α-methylbenzyl hydroperoxide, and benzyl hydroperoxide gave recoveries of 40, 45, 60, 56, 46, and 41%, respectively. The areas of the peaks were found to be linear with respect to known amounts of the standards in the range of 5 to 1000 pmol for benzaldehyde and acetophenone, 2.5 to 75 nmol for benzyl alcohol, 1-phenylethanol, and cumyl alcohol, and 2 to 150 nmol for cumyl, α-methylbenzyl, and benzyl hydroperoxides. Remaining hydroperoxide in reaction mixtures was determined iodometrically and for the benzyl hydroperoxides the amount was confirmed by HPLC. Gas chromatographic analysis of methane in the head space was done on a 2-ft Supelco Carbosieve G glass column at 100°C with a 7 ml/min flow rate of N_2 as carrier gas, and analysis of acetone and pentane in the head space (after the reaction mixture was heated for 20 min at 60°C) was done on a 2-ft 5% Carbowax 20M glass column. For acetone the initial temperature was 80°C for 2 min with an increase of 10°C per min to a final temperature of 210°C, and for pentane the initial temperature was 130°C

Table 1. Requirements for hydroperoxide-dependent NADPH oxidation

System	Rate of NADPH oxidation (nmol/min/nmol of P-450) with various hydroperoxides			
	Cumyl	α-Methylbenzyl	Benzyl	*t*-Butyl
Complete	30	24	5.6	60
No hydroperoxide	<1.0	<1.0	1.3	<1.0
No P-450, no reductase	<1.0	<1.0	<1.0	<1.0
No P-450	<1.0	1.1	1.3	1.2
P-450 heat-inactivated	<1.0	1.1	<1.0	<1.0
No reductase	3.6	2.2	1.6	8.0
Reductase heat-inactivated	2.0	1.8	1.1	7.5
Aerobic	30	6.9	<1.0	50

The complete system was anaerobic with hydroperoxides at the following starting concentrations (mM): cumyl, 0.4; α-methylbenzyl, 0.4; benzyl, 0.4; and *t*-butyl, 1.0. For heat inactivation, the enzyme solution was placed in a boiling water bath for 10 min and then cooled before addition to the system. The data are an average of the results from at least three experiments. Under anaerobic conditions no NADPH oxidation occurred without a hydroperoxide present. The values given for aerobic conditions have been corrected for the rate observed without a hydroperoxide present, 6.1 nmol/min/nmol of P-450. Rates of 1.0 or lower are not significant.

Table 2. Products resulting from hydroperoxide cleavage in an anaerobic system

Hydroperoxide	Starting amount, nmol	NADPH oxidized, nmol	Products detected, nmol						
			Cumyl alcohol	Acetophenone	Benzaldehyde	Acetone	Methane	α-Methylbenzyl alcohol	Benzyl alcohol
Cumyl	100	99	0	91	0	0	70		
α-Methylbenzyl	150	157		116	10	0	<25*	<1.0	
Benzyl	200	174			124				0
t-Butyl	200	211				174	190		

The complete anaerobic system was described elsewhere. When NADPH oxidation had ceased, as judged spectrophotometrically, the reaction mixtures were analyzed. The hydroperoxides were found to be completely consumed. The amounts of products, with the exception of methane and acetone, are corrected for efficiency of extraction from the reaction mixtures. Results are an average of three independent experiments. *Methane was detected, but the amount was too low for accurate determination.

for 7 min with an increase of 30°C per min to a final temperature of 210°C. The flow rate was 20 ml per min in each case. For acetone, pentane, and methane the peak heights were linear with respect to the amount of the authentic standards in the ranges used. GC/MS analysis was done with a Carbosieve S-2 (6 ft × 2 mm i.d.) glass column maintained at 225°C coupled to a Finnigan 40-21GC-MSDS mass spectrometer set at an electron multiplier voltage of −1245 V, emission current of 0.3 mV, electron energy of 70 eV (1 eV = 1.602×10^{-19} J), and ionizer temperature of 250°C with sensitivity at 10^{-9} amp/volt. These analyses were done by James Windak, Department of Chemistry, the University of Michigan.

RESULTS AND DISCUSSION

The requirements for hydroperoxide-dependent NADPH oxidation in the reconstituted enzyme system are shown in Table 1. With all of the alkyl hydroperoxides examined—cumyl, α-methylbenzyl, benzyl, and t-butyl—the rate of NADPH oxidation was greatly decreased upon the omission of P-450 form 2 or NADPH-cytochrome P-450 reductase. Furthermore, the heat-denatured enzymes were inactive. Such assays were routinely carried out under anaerobic conditions to avoid the complexity of side reactions such as NADPH-dependent hydrogen peroxide formation (29). However, under aerobic conditions cumyl hydroperoxide-, α-methylbenzyl hydroperoxide-, and t-butyl hydroperoxide-dependent NADPH oxidation also occurs; the values given are considered to be minimal because they have been corrected for the aerobic rate without a hydroperoxide present, and such a control experiment is difficult to evaluate without information on products formed from the hydroperoxides. Thus, the apparent absence of the benzyl hydroperoxide-dependent reaction under aerobic conditions is considered to be equivocal. In other experiments the omission of EDTA from the buffer was found to have no effect.

Reaction mixtures incubated anaerobically with an excess of NADPH over the starting hydroperoxide were then examined for the products formed, with the results shown in Table 2. With cumyl hydroperoxide, cumyl alcohol was thought to be the likely product, but none could be detected in the petroleum ether extract of the reaction mixture by HPLC as compared with the behavior of the known alcohol as a standard. Instead, acetophenone was identified. At this point it was apparent that cumyl hydroperoxide had undergone a rearrangement, probably involving radical intermediates, with the formation of an additional 1-carbon product. This was identified in the head space of the reaction mixture by GC/MS analysis as methane, and the amount of methane was determined by GC analysis on a Carbosieve G column. The stoichiometry, expressed as the molar ratio of hydroperoxide consumed to that of NADPH oxidized, acetophenone formed, and methane formed, was 1.0:1:0:0.9:0.7. In similar experiments with α-methylbenzyl hydroperoxide, acetophenone was the major product, along with a small amount of benzaldehyde and methane; the level of the methane was too low for accurate determination. The stoichiometry, hydroperoxide: NADPH: acetophenone plus benzaldehyde, was 1.0:1.0:0.8. With benzyl hydroperoxide,

Table 3. Requirements for product formation from model hydroperoxides

System	Hydroperoxide used	Product formed, nmol			
		Acetophenone	Benzaldehyde	Acetone	Methane
Complete	Cumyl	91			80
	α-Methylbenzyl	99	20		<25
	t-Butyl			174	177
No NADPH	Cumyl	23			ND
	α-Methylbenzyl	24	<0.01		ND
	t-Butyl			50	ND
No P-450	Cumyl	<0.01			
	α-Methylbenzyl	6	<0.01		ND
	t-Butyl			0	ND
P-450 heat-inactivated	Cumyl	6			ND
	α-Methylbenzyl	4	<0.01		ND
	t-Butyl			0	ND
No reductase	Cumyl	40			ND
	α-Methylbenzyl	27	<0.01		ND
	t-Butyl			44	ND
Complete, but aerobic	Cumyl	88			65
	α-Methylbenzyl	129	<0.01		
	t-Butyl			153	165

The complete anaerobic system was as in Table 2. For methane formation, ND indicates that none was detected.

benzaldehyde was the only product detected, with an observed stoichiometry for the reaction of 1.0:0.9:0.6. With *t*-butyl hydroperoxide, the stoichiometry, hydroperoxide:NADPH:acetone:methane, was determined as 1.0:1.1:0.9:1.0.

The components required for product formation from three of the hydroperoxide substrates are evident from the results given in Table 3. Under the usual anaerobic conditions, product formation was greatly decreased in the absence of P-450 or when the P-450 had been heat-denatured. The omission of NADPH or the reductase resulted in no detectable methane formation from any of the hydroperoxides, but, in the case of cumyl and α-methylbenzyl hydroperoxides, this omission gave acetophenone in lower but still significant amounts. Under aerobic conditions cumyl and *t*-butyl hydroperoxides yielded the expected products in comparable amounts to those found anaerobically.

The products obtained from model hydroperoxides (Table 2) are indicative of the following mechanism:

$$\text{XRR'C-OOH} + \text{P-450(II)} \rightarrow [\text{XRR'C-O}\cdot] + \text{OH}^- + \text{P-450(III)} \quad [2a]$$

$$[\text{XRR'C-O}\cdot] \rightarrow \text{XRCO} + [\text{R}'\cdot] \quad [2b]$$

$$[\text{R}'\cdot] + \text{H}^+ + \text{P-450(II)} \rightarrow \text{R'H} + \text{P-450(III)} \quad [2c]$$

$$\text{XRR'C-OOH} + \text{H}^+ + 2\text{e} \rightarrow \text{XRCO} + \text{R'H} + \text{OH}^- \quad [2]$$

The hydroperoxy bond undergoes a stepwise one-electron reduction in which the first reductive step yields an alkoxy radical. This radical undergoes the well-known β-scission reaction (30) to yield a ketone or aldehyde and a derived radical, R'·. Uptake of the second electron by the latter radical species results in hydrocarbon formation, and the overall reaction is thus represented by Eq. 2. The lack of alcohol formation indicates that neither heterolysis of the peroxy bond nor reduction of the intermediate alkoxy radical is significant in the reaction described. That hydrocarbon formation occurs by reduction of the derived radical is clearly demonstrated by the incorporation of one deuterium atom into the methane formed from the reaction of cumyl hydroperoxide in 70% deuterium oxide as solvent (Fig. 1). Ethane was not detected in the reaction, indicating that radical dimerization does not occur.

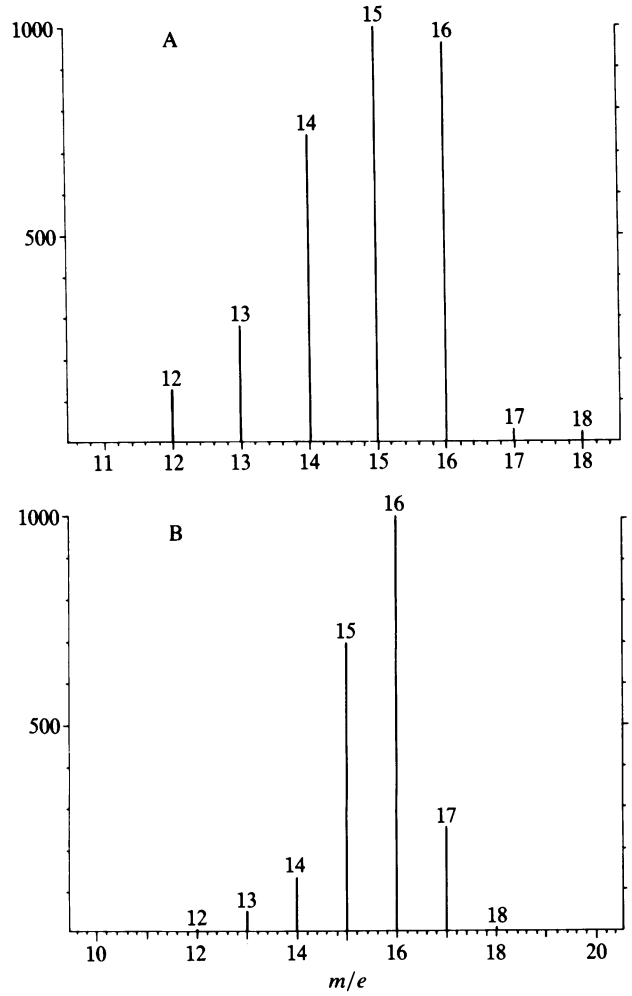

FIG. 1. Electron impact fragmentation pattern for enzymatically generated methane in H_2O (*A*) and in 70% D_2O (*B*), with mass per unit charge (*m/e*) shown as a function of ion intensity. The intensity at *m/e* (or *m/z*) = 17 is a measure of $CH_3{}^2H$.

Table 4. Requirements for pentane formation from 13-HOO-$C_{18:2}$ acid

System	Hydroperoxide consumed, nmol	NADPH oxidized, nmol	Pentane formed, nmol
Complete	31	37	25
No P-450	0	2	0
No reductase	31	5	0
Both P-450 and reductase heat-inactivated	0	0	0

The complete anaerobic system was as described in *Materials and Methods*, but with 1.0 μM P-450 and reductase, 60 μg of phospholipid per ml, 150 μM NADPH, and 15.7 μM 13-HOO-$C_{18:2}$ in a total volume of 2.0 ml. Pentane was determined by gas chromatography, and the identity was confirmed by GC/MS.

A variety of other reductive reactions catalyzed by cytochrome P-450 has been reported, such as the reduction of *N*-oxides (31), epoxides (32), and alkyl halides (33, 34). Mechanistic studies with alkyl halides also indicate a stepwise one-electron reduction of the carbon-halogen bond (34, 35). These results suggest that P-450-catalyzed reductive reactions generally involve stepwise one-electron transfer processes, as would be predicted from the nature of the catalyst.

Although several investigators have proposed that the cytochrome P-450-containing mixed function oxidase system of liver microsomes is somehow involved in lipid peroxidation, the role of the individual enzymes has remained uncertain. In view of the results already given with model hydroperoxides, we considered that lipid hydroperoxides might undergo a similar reductive cleavage and looked for alkane formation from 13-HOO-$C_{18:2}$ with the results given in Table 4. Pentane was identified as the product, and both P-450 and the reductase were found to be required. In the complete system, the stoichiometry of the reaction, peroxide consumed to NADPH oxidized to pentane formed, was 1.0:1.2:0.8. Evidence has been obtained for the other product (E. S. Roberts, A.D.N.V., and M.J.C., unpublished data), apparently an unsaturated aldehyde acid, but the identification is not yet complete.

Some of the cytochrome P-450 form 2 and NADPH-cytochrome reductase preparations used in this study were prepared by Robert L. Clark. This work was supported by Grant DK-10339 from the National Institutes of Health.

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