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Radical intermediates in peroxide-dependent reactions catalyzed by cytochrome P-450

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Abstract. Highly purified liver microsomal cytochrome P-450 acts as a peroxygenase in catalyzing the reaction,

$$RH + XOOH \rightarrow ROH + XOH,$$

Where RH represents any of a large variety of foreign or physiological substrates and ROH the corresponding product, and XOOH represents any of a series of peroxy compounds such as hydroperoxides or peracids serving as the oxygen donor and XOH the resulting alcohol or acid. Several experimental approaches in this and other laboratories have yielded results compatible with a homolytic mechanism of oxygen-oxygen bond cleavage but not with the heterolytic formation of a common iron-oxo intermediate from the various peroxides.

Recently, we have found a new reaction, catalyzed by the reconstituted system containing the phenobarbital-inducible form of P-450, which catalyzes the reductive cleavage of hydroperoxides:

$$XRR'C\text{-}OOH + NADPH + H^{+} \rightarrow XR'CO + R'H + H_2O + NADP^{+}.$$

Thus, cumyl hydroperoxide yields acetophenone and methane, and 13-hydroperoxyoctadeca-9,11-dienoic acid yields pentane and an as yet unidentified additional product. Since hydroperoxide reduction does not produce the corresponding alcohol, it is concluded that homolytic cleavage of the oxygen-oxygen bond occurs with rearrangement of the resulting alkoxy radical. Studies are in progress to determine how broad a role the new hydroperoxide cleavage reaction plays in the biological peroxidation of lipids.

Keywords. Cytochrome P-450; peroxides; oxygen radicals; lipid peroxidation; hydroxylation reactions; hydroperoxides.

Introduction

As is now widely known, the term cytochrome P-450 is used to refer to a group of heme proteins that are apparently unique in having a sulphur atom ligated to the iron and that form carbon monoxide complexes that have a major absorption band at about 450 nm (White and Coon, 1980). Cytochrome P-450 catalyzes not only aliphatic and aromatic hydroxylation, but also N-oxidation, sulphoxidation, epoxidation, N-, S-, and O-dealkylation, peroxidation, deamination, desulphuration, and dehalogenation, but also reactions such as reduction of azo groups, nitro groups, N-oxides, and epoxides that involve only electron transfer and partially justify the term 'cytochrome' for this enzyme. The substrates include physiologically occurring lipid such as fatty acids, prostaglandins, and steroids, as well as a host of foreign compounds including drugs, petroleum products, anesthetics, insecticides, and carcinogens. In recent years, cytochrome P-450 has been purified to apparent homogeneity from numerous sources, and from the primary structures established by protein and DNA sequencing it is clear that the various forms of P-450 are distinct

gene products (Black and Coon, 1986). Progress has also been made in understanding the regulation of P-450 gene expression; for example, Ravishankar and Padmanaban (1985) have proposed that heme is a general regulator of expression at the level of transcription, whereas a particular drug or its metabolite would impart the specificity needed for the induction of a particular form of the cytochrome. The various forms or isozymes of P-450 participate in metabolically important transformations of lipids and also catalyze the alteration of xenobiotics in ways that usually lead to detoxification but in some instances yield products with greater cytotoxic, mutagenic, or carcinogenic properties.

The purpose of this communication is to provide a brief review of our mechanistic studies on the role of peroxides in P-450-catalyzed reactions. Emphasis is placed on evidence for radical species derived from hydroperoxides in P-450-dependent reactions.

Catalytic cycle for oxygen-dependent hydroxylation reactions catalyzed by cytochrome P-450

As background for our recent investigations on peroxides, the scheme we have proposed for O_2-dependent reactions, based on findings in this and numerous other laboratories, is given in figure 1. The overall scheme is in accord with the known stoichiometry of such reactions (Gorsky *et al.,* 1984) and also takes into account the known regioselectivity and partial loss of configuration during oxidation of prochiral

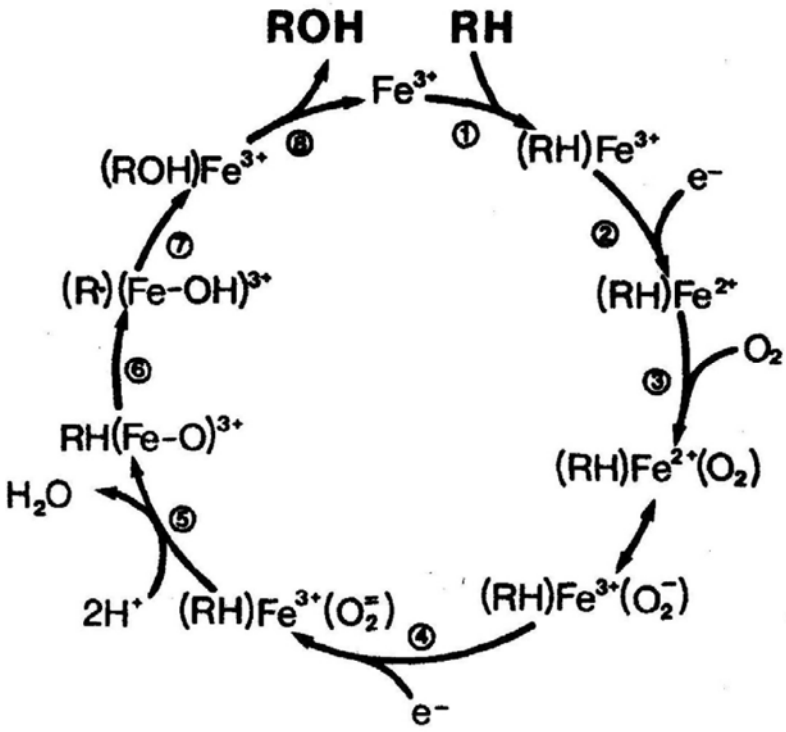


Figure 1. Proposed scheme for mechanism of action of cytochrome P-450 in hydroxylation reactions, taken from White and Coon (1980). RH represents a substrate, ROH the corresponding product, and Fe the heme iron atom in the cytochrome.

centers (Groves *et al.,* 1978). No attempt has been made to show the details of proton uptake.

The steps are as follows: (i) Substrate binding, in which the principal contributor to the binding energy is probably the hydrophobic effect. (ii) First electron transfer, the primary donor being the FMN- and FAD-containing reductase in the case of the microsomal system and an iron-sulphur redoxin in the case of the bacterial and mitochondrial hydroxylation systems. (iii) Dioxygen binding to give a ferrous dioxygen complex with sufficiency stability to be detectable by Mössbauer and stopped flow spectrophotometry; a resonance form, the ferric-superoxide species, serves as the source of the superoxide radical coming from this 'leaky' enzyme system, (iv) Second electron transfer, in which cytochrome b_5 sometimes substitutes effectively for the P-450 reductase (Pompon and Coon, 1984) and the product is at the redox level of hydrogen peroxide, (v) Splitting of the oxygen-oxygen bond to yield water and an iron-oxene species. (vi) Hydrogen abstraction from the substrate (Groves *et al.,* 1978). (vii) Recombination of the substrate carbon radical and proposed hydroxyl radical species to give the product, ROH. (viii) dissociation of the product with recovery of the cytochrome in the resting, ferric state.

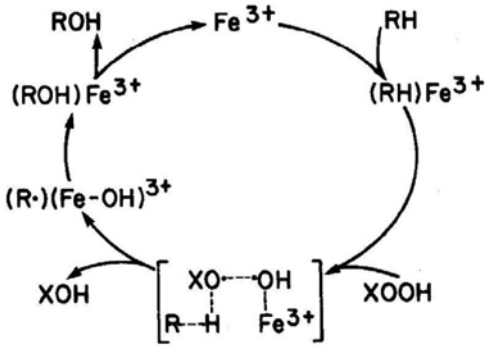
Catalytic cycle for peroxide-dependent hydroxylation reactions catalyzed by cytochromeP-450

In addition to catalyzing various O_2-dependent reactions, P-450 also brings about the oxygenation of organic substrates at the expense of peroxy compounds. This reaction, first shown with drugs and cumyl hydroperoxide in microsomal suspensions (Kadlubar *et al.,* 1973; Rahimtula and O'Brien, 1974), occurs with a number of oxidants other than alkyl hydroperoxides (such as peroxyacids, sodium periodate, iodosobenzene, iodosobenzene diacetate, and N-oxides) and involves cytochrome P-450, as reviewed elsewhere (Black and Coon, 1986b). This laboratory showed that electrophoretically homogeneous P-450 isozyme 2 catalyzes such reactions in the absence of NADPH, NADPH-cytochrome P-450 reductase, and molecular oxygen and that the peroxygenase reaction has the following stoichiometry:

$$RH + XOOH \rightarrow ROH + XOH,$$

where RH and ROH again represent the substrate and product, respectively, and XOOH is the peroxy compound serving as oxygen donor. A difference has been noticed between peroxide-based oxidants and their iodine-based counterparts. Specifically, with peroxides the oxygen atom incorporated into the product alcohol is derived exclusively from the peroxide (Nordblom *et al.,* 1976), whereas the oxygen atom in alcohols formed in the presence of agents such as iodosobenzene appears to be derived from water (Heimbrook and Sligar, 1981; White and McCarthy, 1984) because of a solvent exchange reaction (White, 1986). Thus, the various available oxidants may not all be equally good mechanistic models.

The peroxide shunt is shown in figure 2. Several experimental approaches were used in this laboratory in an attempt to determine whether peroxide oxygen-oxygen bond cleavage occurs by a homolytic or heterolytic mechanism, as summarized elsewhere (Black and Coon, 1987; Coon and Blake, 1982). The reaction of rabbit P-



Figured 2. Peroxide shunt, in which a peroxy compound, XOOH, serves as the oxygen donor to the substrate.

450 isozyme 2 with peroxides differs in several important respects from that of peroxidases. In the case of P-450 only, the intermediates are formed reversibly, and their spectra vary with structural differences in the peroxy compounds (Blake and Coon, 1980). The absolute spectra as well as the difference spectra with the various peroxides differ not only in the magnitude but also in the positions of the maxima and minima. Secondly, several P-450's were shown to bring about the decarboxylation of peroxyphenylacetic acid in a reaction presumably involving the phenylacetoxy radical as an intermediate (White *et al.,* 1980). Finally, studies with a series of *m*- and *p*-substituted derivatives of cumyl hydroperoxide as the oxygen donor and of toluene as the substrate showed that the hydroxylation rate constant is sensitive to alterations in both reactants (Blake and Coon, 1981). The reaction of toluene with cumyl hydroperoxide to form benzyl alcohol and cumyl alcohol was followed by stopped flow spectrophotometry; the two spectral complexes formed were examined, along with the rate of benzyl alcohol formation in the presence of 3-acetylpyridine adenine dinucleotide and alcohol dehydrogenase. In such studies a number of *m*- and *p*-substituted toluenes and cumyl hydroperoxides were employed. Additional work by McCarthy and White (1983) established that peroxy acid decarboxylation is unique to P-450 enzymes. Thus, whereas all P-450's examined have been found catalytically competent in decarboxylation, other heme proteins, including peroxidases, promote only heterolytic cleavage. The 3 dimensional crystal structure of bacterial $P\text{-}450_{cam}$ (Poulos *et al.,* 1985) reveals that this enzyme has none of the general acid-base and charge stabilization machinery normally considered necessary for heterolytic oxygen-oxygen bond cleavage. In summary, these results are compatible with a homolytic mechanism of oxygen-oxygen bond cleavage but not with the heterolytic formation of a common iron-oxo intermediate from the various peroxides, as generally seen with peroxidase-catalyzed reactions.

NADPH oxidation by hydroperoxides: Catalysis by cytochrome P-450 and evidence for homolytic cleavage of the peroxide bond

In a recent study on the reduction of hydroperoxides, undertaken to develop a model for the 4-electron reduction of dioxygen by cytochrome P-450 (Gorsky *et al.,* 1984),

we have found that simple 2-electron transfer to cumyl hydroperoxide to yield the corresponding alcohol does not occur; acetophenone was produced instead (Vaz and Coon, 1985). The other product was then identified as methane by GC-mass spectrometry (Vaz and Coon, 1986), and the stoichiometry was shown to correspond to the following general reaction for the reductive cleavage of peroxides:

$$XRR'C\text{-}OOH + NADPH + H^+ \rightarrow XRCO + R'H + H_2O + NAD\,P^+.$$

The data in table 1 show that the hydroperoxide-dependent NADPH oxidation requires both P-450 and the reductase and that the native enzymes are necessary for activity. In other experiments the addition of benzphetamine had no effect, whereas carbon monoxide inhibited the reaction about 65%. Our current view of the mechanism of the reaction is that alkyl hydroperoxides are reduced stepwise as shown here for cumyl hydroperoxide (Vaz and Coon, 1985):

$$\text{(a) } PhC(CH_3)_2OOH + P\text{-}450^{II} [PhC(CH_3)_2O\cdot] + OH^- + P\text{-}450^{III}$$

$$\text{(b) } [PhC(CH_3)_2O\cdot]\ PhCOCH_3 + [CH_3\cdot]$$

$$\text{(c) } [CH_3\cdot] + H^+ + P\text{-}450^{II} \rightarrow C\,H_4 + P\text{-}450^{III}.$$

Homolytic cleavage of the oxygen-oxygen bond with transfer of one electron from P-450 is pictured as yielding the cumyloxy radical and hydroxide ion; the radical rearranges to give acetophenone and a transient methyl radical, and the latter with uptake of the second electron produces methane.

Table 1. Requirements for cumyl hydroperoxide-dependent NADPH oxidation[a].

System	NADPH oxidation (nmol/min/nmol P-450)
Complete	30·0
P-450 omitted	0·1
Reductase omitted	3·6
P-450 and reductase omitted	0·1
P-450 heat-inactivated	0·8
Reductase heat-inactivated	3·6

[a]Taken from Vaz and Coon (1985). The complete system contained P-450 form 2 (0·05 μM), NADPH-cytochrome P-450 reductase (0·05 μM), dilauroylglyceryl-3-phosphorylcholine (30 μg/ml), and cumyl hydroperoxide (0·4 mM) in 0·05 M potassium phosphate buffer, pH7·4, containing EDTA (10 mM). For inactivation, the enzyme was boiled for 5 min. The incubations were at 25°C.

In addition to cumyl hydroperoxide, α-methylbenzyl hydroperoxide, benzyl hydroperoxide, and *t*-butyl hydroperoxide also yield products in accord with the reductive cleavage already shown. Furthermore, we have added 13-hydroperoxy-octadeca-9,ll-dienoic acid to the reconstituted system containing phenobarbital-inducible P-450 form 2, the reductase, and NADPH, and have identified pentane as a product as well as a compound not yet fully characterized but having the properties of the other presumed cleavage product, an aldehyde acid (Coon and Vaz, 1986). No

attempt will be made to review here the biochemical literature on microsomal lipid peroxidation, other than to indicate that NADPH-cytochrome P-450 reductase is usually regarded as the chief source of oxygen species that initiate the conversion of polyunsaturated fatty acids to hydroperoxides (Aust *et al.,* 1972; Lai *et al.,* 1979). The role of cytochrome P-450 in lipid peroxidation has been the subject of investigation but remains uncertain (Hrycay and O Brien, 1971; Svingen *et al.,* 1979; Ekström and Ingelman-Sundberg, 1984). Although much remains to be learned, our present findings with phenobarbital-inducible rabbit liver microsomal P-450 form 2 and with model hydroperoxides as well as a hydroperoxide derived from linoleic acid establish a new reaction catalyzed by this cytochrome. The reductive cleavage of lipid hydroperoxides by P-450 would account for many of the final products that are believed to arise biologically, including aldehydes and alkyl hydrocarbons (Wendel and Dumelin, 1981).

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