CYTOCHROME P450 2

Peroxidative reactions of diversozymes[1]

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In the ensuing review, Coon and colleagues address the peroxidative reactions catalyzed by the more than 300 cytochromes P450 known and suggest the name Diversozymes to designate the members of this giant family. Their treatment of this subject includes consideration of peroxides as oxygen donors, as substrates for reductive β-scission, and as peroxyhemiacetal intermediates in the cleavage of aldehydes to formate and alkenes. One of the products of reductive β-cleavage, *trans*-4-hydroxynonenal, inactivates specific P450s in a putative, negative regulatory process. In these peroxidative reactions, an iron-peroxy species, instead of an iron-oxene species proposed for most hydroxylation reactions, is implicated in the deformylation reactions of many aldehydes. Dr. Coon's view of this very important family of proteins is particularly noteworthy due to his own prolific and seminal contributions to the field of cytochrome P450-mediated electron transport.

—Bettie Sue S. Masters

ABSTRACT **Cytochrome P450, the most versatile biological catalyst known, was originally named as a pigment having a carbon monoxide difference spectrum at about 450 nm and no known function. Recent progress in many laboratories has revealed that the P450 superfamily has immense diversity in its functions, with hundreds of isoforms in many species catalyzing many types of chemical reactions. We believe it is safe to predict that each mammalian species may be found to have up to a hundred P450 isoforms that respond in toto to a thousand or more inducers and that, along with P450s from other sources, metabolize a million or more potential substrates. Accordingly, the name DIVERSOZYMES is proposed for this remarkable family of hemoproteins. This paper reviews the peroxidative reactions of Diversozymes, including peroxides as oxygen donors in hydroxylation reactions, as substrates for reductive β-scission, and as peroxyhemiacetal intermediates in the cleavage of aldehydes to formate and alkenes. Lipid hydroperoxides undergo reductive β-cleavage to give hydrocarbons and aldehydic acids. One of these products, *trans-4-hydroxynonenal*, inactivates P450, particularly alcohol-inducible 2E1, in what may be a negative regulatory process. Although a P450 iron-oxene species is believed to be the oxygen donor in most hydroxylation reactions, an iron-peroxy species is apparently involved in the deformylation of many aldehydes with desaturation of the remaining structure, as in aromatization reactions.—Coon, M. J., Vaz, A. D. N., Bestervelt, L. L. Peroxidative reaction of diversozymes. *FASEB J.* 10, 428–434 (1996)**

Key Words: P450 cytochromes · reductive cleavage of lipid hydroperoxides · inactivation of P450 by trans-4-hydroxynonenal · peroxidative deformylation of aldehydes

IN AN EARLIER REVIEW ON "Cytochrome P450: progress and predictions," Coon et al. (1) described the remarkable versatility of this family of enzymes. Among biological catalysts, P450 is unmatched in its multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. For example, the substrates include physiologically occurring compounds such as fatty acids, steroids, eicosanoids, lipid hydroperoxides, retinoids, arginine, acetone, and acetol. Some of these are highly hydrophilic, despite the earlier idea that the substrates for this enzyme system would all be hydrophobic in nature. Also unexpected is the very large list of xenobiotic substrates, including drugs (with many new ones being synthesized each year by the pharmaceutical industry), procarcinogens, antioxidants, solvents, anesthetics, dyes, pesticides, petroleum products, alcohols, and, as examples of plant products that are foreign to animals, flavorants and odorants (2).Considering the rapid progress made in recent years in the characterization of a large number of isoforms, it may seem surprising that cytochrome P450, a name first used for a red pigment having a reduced CO-difference spectrum with a major band at an unusually long wavelength (about 450 nm) and with no known biological role, has not been replaced by a terminology based on function. Even the term cytochrome is unsuitable, because in most instances the pigment acts as an oxygenase rather than simply as an electron carrier.

[1]Dedicated to Ronald W. Estabrook in recognition of his pioneering contributions to the cytochrome P450 field, his service to science, and his friendship.

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TABLE 1. *Current and predicted diversity of P450 cytochromes and their properties*

Category	Current number known	Predicted total number
Types of chemical reactions catalzed by P450	40	60
P450 substrates	10^3	$>10^6$
P450 inducers	200	$>10^3$
P450s or *CYP*s characterized	300	$>10^3$
P450s or *CYP*s per mammalian species	30	60 to 450

At the opening session of the 10th International Symposium on Microsomes and Drug Oxidations, held in Toronto in July of 1994, the senior author of this paper compared the current status of the P450 field with that at the time of the 1st Symposium held in Bethesda, Maryland, in 1969 and speculated on future developments. The current and predicted numbers for several categories are shown in **Table 1**. Rapid recent progress has revealed the immense diversity of P450s and their functions, with about 300 enzymes or the corresponding genes from various species already characterized and, by a cautious estimate, at least a thousand individual substrates now recognized. The eventual total numbers to be revealed by future research may reach or even exceed 60 types of chemical reactions and a thousand P450 inducers. The total number of substrates is much harder to estimate. However, considering that a vast array of substituents do not eliminate the activity of a long list of organic chemicals that are metabolically altered by P450, a million or more is not an unrealistic guess. This unusual case of diversity in P450 catalysts would justify their being called "Diversozymes"; no one at the Toronto Symposium had a more suitable name to propose.

This paper is concerned with peroxy compounds as oxygen donors, substrates, intermediates, and products of P450-catalyzed reactions, all of which fall under the general heading of peroxidative reactions and extend our knowledge of the diversity of these catalysts. Strictly speaking, the term peroxidases should be reserved for enzymes that catalyze substrate oxidation with reduction of a peroxy compound:

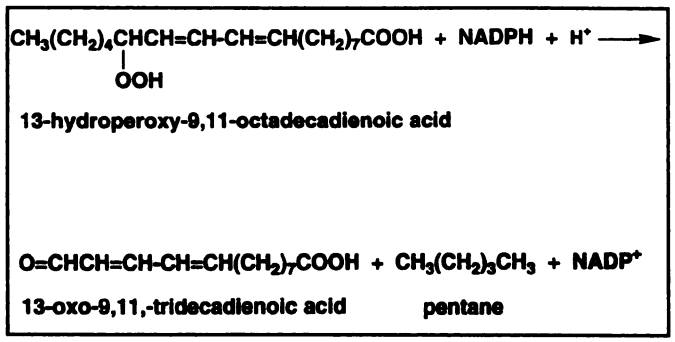

Figure 1. Reductive cleavage of a lipid hydroperoxide by microsomal P450, with the 13-hydroperoxide derived from linoleic acid as an example.

$$(a)\ RH_2 + XOOH \rightarrow R + XOH + H_2O,$$

where RH_2 undergoes oxidation while the hydroperoxide O-O bond is reductively cleaved to give the corresponding alcohol (XOH) and water. As proposed earlier (3), the term peroxygenase should be used for P450s and other enzymes capable of inserting an oxygen atom from a peroxy compound into a substrate (Reaction b):

$$(b)\ RH + XOOH \rightarrow POH + XOH.$$

Reaction b occurs in many cases with P450 as the catalyst and with various oxygen donors including cumyl hydroperoxide, benzyl hydroxperoxide, perbenzoic acid, or even hydrogen peroxide, along with a variety of substrates (4). Thus, the need for an external electron donor is eliminated, in contrast to the typical hydroxylation reactions carried out under aerobic conditions with NADPH as the electron donor:

$$(c)\ RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The more novel "peroxidatic" reactions involving P450 to be considered in this review are the reductive cleavage of organic hydroperoxides, the role of 4-hydroxynonenal (one of the cleavage products) as a P450 inhibitor, and peroxyhemiacetals as proposed intermediates in the scission of a variety of aldehydic compounds to yield formate and alkenes.

REDUCTIVE SCISSION OF LIPID HYDROPEROXIDES

In a study of the reduction of hydroperoxides, undertaken to develop a model for the reductive activation of molecular oxygen by P450, we found that two-electron transfer to yield the corresponding alcohol does not ordinarily occur. Instead, in the case of cumyl hydroperoxide, for example, acetophenone is produced and the missing carbon atom is converted to methane, as shown by gas chromatographic/mass spectrometric analysis. The reaction is as follows, where X represents any of a variety of alkyl groups and R and R′ are either hydrogen atoms or alkyl groups (5):

$$(d)\ XRR'C\text{-}OOH + NADPH + H+ \rightarrow XRCO + R \rightarrow H + H_2O + NADP^+$$

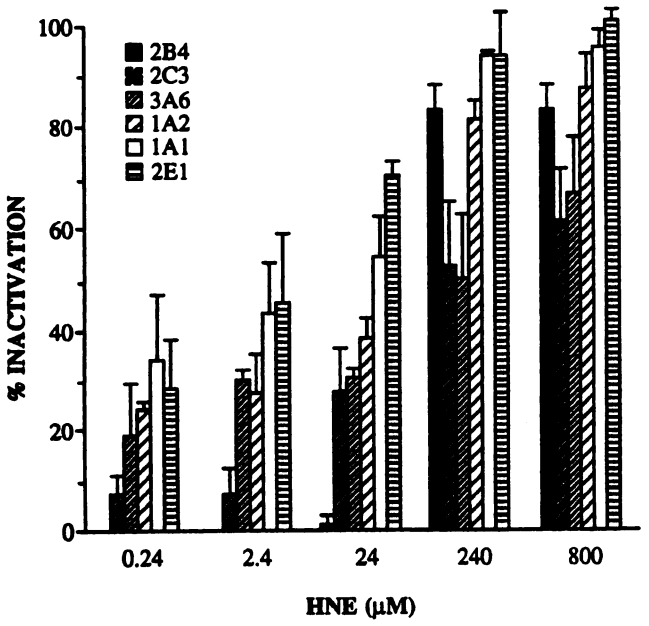

Figure 2. Concentration-dependent HNE inactivation of P450 isozymes. Each purified cytochrome (0.1 nmol of 2E1 or 0.2 nmol of the other forms) was incubated for 15 min with the inhibitor over a range of concentrations, and the remaining catalytic activity was then determined. With P450 2B4, no inactivation occurred with 0.24 or 2.4 μM HNE.

Molecular oxygen plays no role in this conversion, unlike most reactions catalyzed by P450. Reaction d is of considerable biological interest because organic peroxides and hydroperoxides are used widely in industry as initiators of polymer synthesis, and some of these compounds have been shown to produce malignant tumors and to damage P450 in vitro. 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 with the concomitant formation of water. In accordance with this scheme, when the cleavage reaction with cumyl hydroperoxide was carried out in ^2H_2O, deuteromethane was formed. Of particular interest, the occurrence of the reductive cleavage of xenobiotic hydroperoxides suggested that lipid hydroperoxides, which are formed in all biological membranes, might undergo similar transformations. Subsequent to the report of Hochstein and Ernster (6) in 1963 that ADP-activated lipid peroxidation, as shown by malonaldehyde formation, is coupled to the NADPH oxidase system of microsomes, many papers have dealt with this subject. For example, Ekström and Ingelman-Sundberg (7) concluded that rabbit P450s may contribute to lipid peroxidation in reconstituted membrane vesicles. Various alkanes were reported by others to arise from lipid peroxidation, leading to the belief that the exhalation of hydrocarbons in vivo is a measure of this pathological process.

We therefore examined the reductive scission of lipid hydroperoxides by P450 in a reconstituted enzyme system (8). The reaction with 13-hydroperoxy-9,11-octadecadienoic acid, derived from linoleic acid, is shown in **Fig. 1**. The cleavage products were identified as an aldehyde acid, 13-oxo-9,11-tridecadienoic acid, which was isolated and analyzed by NMR, and pentane, which was obtained in the head space of the reaction mixture and analyzed by GC/MS. Of the purified P450 isozymes examined, the alcohol-inducible form, 2E1, is the most active in the reductive β-scission of the 13-hydroperoxide derived from linoleic acid and the 15-hydroperoxide derived from arachidonic acid, followed by 2B4, 3A6, 1A2, and 2C3. The isoforms with lower activity give less of the cleavage products (oxo compound and hydrocarbon), but catalyze direct reduction of hydroperoxides to the corresponding hydroxy compounds. We have suggested that the alcohol-inducible P450, in addition to its known deleterious effects in chemical toxicity and chemical carcinogenesis, may enhance the reductive cleavage of lipid hydroperoxides with a resultant loss in membrane integrity (8). The possible importance of P450 2E1-dependent lipid peroxidation *in vivo* after ethanol abuse has also been pointed out by Ekström and Ingelman-Sundberg (9).

INACTIVATION OF MICROSOMAL P450 ISOZYMES BY *trans*-4-HYDROXY-2-NONENAL, A MAJOR PRODUCT OF MEMBRANE LIPID PEROXIDATION

The formation of reactive aldehydes, including alkenals, 2-alkenals, and 4-hydroxyalkenals, has been proposed to account for the cytotoxicity of lipid peroxidation products (10). *trans*-4-Hydroxy-2-nonenal (HNE),[3] a major product that is particularly damaging, exhibits toxicity to cells (11), lyses erythrocytes (12), and inhibits DNA and protein synthesis (13, 14). HNE reacts with protein sulfhydryl groups to form thioethers (13, 15), with primary amino groups, and with histidine imidazole groups (16). Szweda et al. (17) have described the inactivation of purified bacterial glucose-6-phosphate dehydrogenase by HNE accompanied by reaction with a lysine residue to give a stable secondary amine derivative, and more recently the formation of cross-linked protein less susceptible to proteolysis by the multicatalytic protease has been observed (18). In addition, Uchida and Stadtman (19) have found that HNE inactivates purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and at concentrations lower than 0.5 mM reacts primarily with cysteine and lysine residues.

Because the first target of the toxic products of lipid peroxidation would be expected to be membranous enzymes, such as P450, we have undertaken a study of the sensitivity of hepatic microsomal P450 cytochromes to HNE. As recently reported (20), the purified isozymes

[3]Abbreviations: HNE, *trans*-4-hydroxy-2-nonenal; ODEC, 3-oxodecalin-4-ene-10-carboxaldehyde.

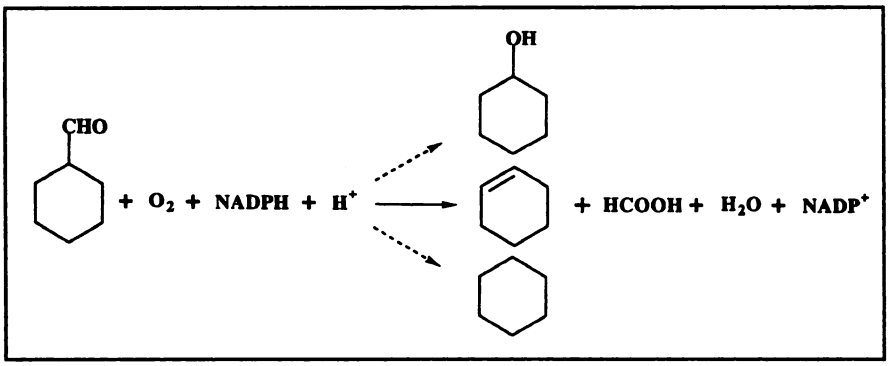

Figure 3. P450-catalyzed deformylation of cyclohexanecarboxaldehyde with cyclohexene as the major product and cyclohexanol and cyclohexane as minor products.

differ in their sensitivity to HNE, with all of the cytochromes having some decreased activity and P450 2E1 being among the most strongly inactivated. Thus, this isoform that is particularly active in generating oxygen radicals, which are capable of initiating lipid peroxidation, and in catalyzing the reductive cleavage of lipid hydroperoxides appears to be negatively regulated in this process by HNE. The observed inhibition of the various purified P450s and of P450 in microsomal membranes is of sufficient magnitude that it could cause significant changes in the metabolism of foreign compounds such as drugs and chemical carcinogens. To our knowledge, the only previous work directly related to our investigation is the report of Benedetti et al. (21) that HNE at a very high concentration (0.45 mM) causes a loss of about 50% of the P450 CO spectrum in liver microsomes.

HNE was incubated with individual P450 cytochromes in the absence of the reductase and NADPH, and the extent of inactivation was then determined by assay of the rate of oxidation of 1-phenylethanol to acetophenone in the presence of the reductase and NADPH (20). From such experiments it was clear that HNE reacts directly with the cytochromes rather than requiring prior metabolic activation. The effects are dependent both on the length of the exposure and the concentration of the inhibitor. HNE at a level as low as 0.24 μM, only slightly more than twice the P450 2E1 concentration, gave significant inactivation, and higher levels had a progressively greater effect. As shown in **Fig. 2**, six purified isozymes of rabbit liver microsomal P450 were examined over a wide concentration range. At 24 μM HNE, for example, 2E1 in particular and also 1A1 are the most extensively inhibited, followed by 1A2, 3A6, and 2C3, with 2B4 almost unaffected.

In related studies, NADPH-cytochrome P450 reductase was found to be only weakly inactivated by HNE. The P450 heme is apparently not altered, and the sulfur ligand is not displaced. Substrate protects against HNE, and the inactivation is reversed upon dialysis; however, the mechanism of inhibition is not yet known. The reaction presumably involves one or more of the protein residues known to be involved in the reaction of soluble enzymes with HNE. Note, however, that purified full-length microsomal P450s exist as aggregates, with P450 2E1 and P450 2B4 being predominantly decameric and hexameric, respectively (22), and their inactivation may be a complex process.

As to the possible biological significance of the inhibition seen with purified P450s in the reconstituted enzyme system, similar results were seen with P450s 2B4 and 2E1 in microsomal membranes from animals induced by the administration of phenobarbital and acetone, respectively. Such findings strongly suggest that HNE produced by lipid peroxidation in the endoplasmic reticulum would similarly inactivate components of the P450 system in vivo. The physiological concentration of HNE in rabbit hepatic microsomes is not known. However, Esterbauer et al. (23) have reviewed data available from their own and other laboratories on the level of this compound in various tissues from several species; the most pertinent value is that for rat liver microsomes, which is 0.03 nmol/mg of protein, and up to 0.2 to 4.5 for animals with enhanced lipid peroxidation activity. A similar upper range was found by Kamimura et al. (24) in microsomes from adult rats on a high-fat diet, particularly after ethanol administration. Although these values may be only a rough guide to the levels produced in various species in vivo, they are within the range of those that led to enzyme inactivation in our studies.

In view of the central role of the P450 system in the metabolism of drugs and chemical carcinogens, alterations in these activities by the products of lipid peroxidation would be of much interest. Only limited information is available on the question of whether membrane peroxidation is enhanced as animals age and might contribute to decreased drug metabolism. Sagai and Ichinose (25) showed that lipid peroxidation increases with age, as determined by higher levels of ethane, ethylene, butane, and pentane in respired gases of rats. Kato and Takanaka (26) reported in 1968 that drug metabolism is decreased in liver microsomes of rats as they age, and Schmucker and Wang (27) observed similar changes in microsomes of senescent rats. Caution is needed, however, in extrapolating results from laboratory rodents to other species, as indicated by the finding that the P450 mixed function oxidase system, NADPH-cytochrome P450 reductase activity, and benzo[*a*]pyrene metabolism do not decrease in older nonhuman primates (28). By immunochemical determinations, the specific content of P450s 2B4 and 1A2 was shown to be maximal in rabbit liver microsomes at

50 to 100 days and then to decrease (29), whereas the levels of several human P450s were found to be unchanged with age (30). However, HNE-modified enzymes would probably also be detected by the antibodies used. In a recent review, Birnbaum (31) has noted that many hepatic drug metabolism activities are decreased in male rats with senescence but that age-related changes may be dependent on the particular substrate, species, strain, sex, and tissue under study. Furthermore, overall P450 activities are regulated in many ways, as by competing substrates and inhibitors, enzyme inducers, and varying rates of biological turnover.

ROLE FOR OXYGEN-DERIVED PEROXIDE IN THE DEFORMYLATION OF ALDEHYDES WITH OLEFIN FORMATION

The oxidative demethylation reaction that accompanies steroid aromatization has been the subject of much mechanistic interest, and several research groups have proposed a role for oxygen-derived peroxide (32–37). We recently examined a number of xenobiotic aldehydes as simple models for the step in which an aldehyde carbon is removed from the steroid, and have established that deformylation with olefin formation is a reaction widely catalyzed by microsomal P450 cytochromes. For example, cyclohexanecarboxaldehyde produces formic acid and cyclohexene in the presence of P450, the reductase, and NADPH under aerobic conditions (38). The reaction is thus a useful model for the final step in the demethylation reaction catalyzed by the steroidogenic P450s aromatase and lanosterol demethylase, where formic acid and an olefinic product are also formed. Whereas H_2O_2 and organic peroxy compounds can be substituted for O_2 and NADPH in many P450-catalyzed reactions, in the case of cyclohexanecarboxaldehyde deformylation only H_2O_2 is active. We therefore concluded that "iron oxene" is not the active oxidant in the reaction and proposed a peroxyhemiacetal-like adduct as a transient enzyme-bound intermediate. Presumably the intermediate rearranges by a concerted or a sequential β-scission to yield formic acid and the olefin. As shown in **Fig. 3**, in addition to cyclohexene as the major product, about one-tenth as much cyclohexane is formed and a small amount of cyclohexanol is predicted. Several P450 enzymes, including 2B4, are active in this reaction, and other cyclic and acyclic aldehydes undergo a similar transformation, as described below.

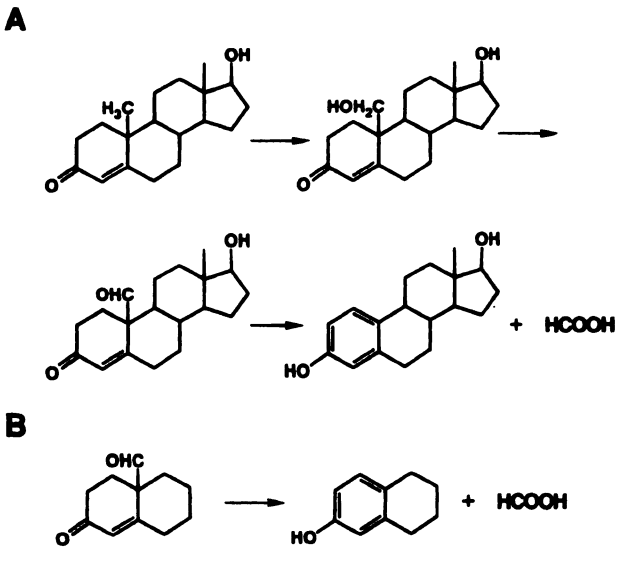

Figure 4. *A)* Conversion of testosterone to estradiol, catalyzed by steroid aromatase; *B)* conversion of ODEC to hydroxytetrahydronaphthalene, catalyzed by P450 2B4.

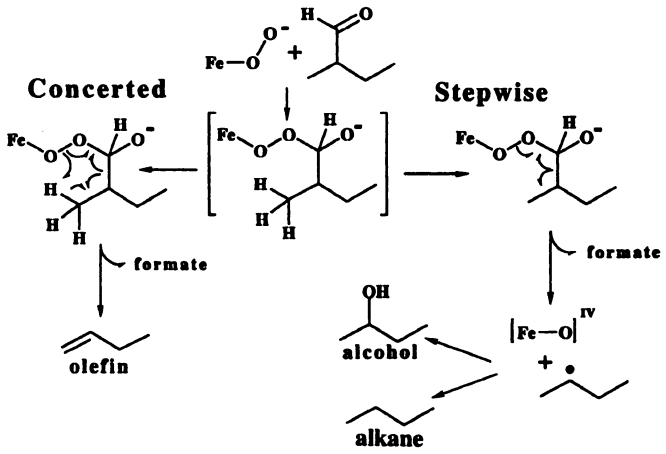

Figure 5. Proposed mechanism for the P450-catalyzed deformylation of 2-methylbutyradehyde, with olefin, alcohol, and alkane formation.

To examine the structural features that are necessary for aldehyde deformylation with olefin formation, we studied a series of xenobiotic compounds (39). Isobutyraldehyde and trimethylacetaldehyde, but not propionaldehyde, are converted to the predicted olefinic products, suggesting a requirement for branching at the α carbon. In addition, the reaction was examined with a group of 5-carbon aldehydes that are about equally hydrophobic but differ structurally, and the rates were determined with five purified enzymes of P450. No detectable 1-butene was formed with straight-chain valeraldehyde, whereas β-branched isovaleraldehyde was converted to the olefin, but at a very low rate. In contrast, 2-methylbutyraldehyde and trimethylacetaldehyde were readily deformylated. Such experiments show that branching, particularly at the α carbon, is an important structural feature for the reaction. This conclusion applies to all of the isozymes studied. Because aldehydes occur as natural flavoring constituents in a variety of foods, it was of interest to examine one of these. Citronellal, a β-branched aldehyde found in many essential oils and widely used as an odorant and flavorant, was found to undergo the oxidative deformylation reaction to give 2,6-dimethyl-1,5-heptadiene with P450 2B4 (39).

From an enzymological viewpoint, the aromatase is of much interest because of the unusual reaction by which,

TABLE 2. *Scope of reactions that may involve a P450 iron-peroxy species as oxygen donor*

Reaction	Substrate tested	Diversozyme
Deformylation of alicyclic xenobiotics	Cyclohexanecarboxaldehyde	2C3>2G1>2B4
Deformylation of aliphatic xenobiotics	Trimethylacetaldehyde	3A6>2B4,2E1>2C3>1A2
Deformylation of terpenoids	Citronellal	2B4
Aromatization of steroid model	ODEC	2B4>2C3
Oxidation of arginine	N-Hydroxyarginine	Murine macrophage
(Z)-9-Tricosene synthesis	(Z)-15-Tetraacosenal	*Musca domestica*

in three sequential oxidative steps, it catalyzes removal of the 10β-methyl group of the androgen nucleus, as shown in **Fig. 4A**. For a study of this reaction with liver microsomal P450s, we synthesized 3-oxodecalin-4-ene-10-carboxaldehyde (ODEC) as an analog of the A and B rings of the 10β-formylandrogen intermediate (see Fig. 4*B*) and found that it undergoes aromatization with P450 2B4 to yield 3-hydroxy-6,7,8,9-tetrahydronaphthalene (40). This product and formic acid were identified by mass spectrometry. In two respects the conversion of the bicyclic model compound to the phenol is similar to the steroid decarbonylation reaction. Deuterium in the formyl group of ODEC was retained in the formic acid that was produced, and with preparations of ODEC containing deuterium in the 1α position or the 1α and 2α positions the desaturation reaction was shown to be specific for removal of the 1β-hydrogen atom, thus demonstrating a stereospecific *cis* elimination of formate.

A proposed mechanism for the aldehyde deformylation reaction is given in **Fig. 5** with 2-methylbutyraldehyde as substrate. In the overall reaction with NADPH present, an oxygen-derived, P450 heme iron-bound hydroperoxide is believed to react with the electrophilic aldehyde carbonyl group to form an enzyme-bound peroxyhemiacetal-like intermediate. Rearrangement would then yield the olefin and formic acid by a concerted mechanism or both the alcohol and the alkane (and formic acid) by a stepwise mechanism involving a transient carbon radical.

The oxidative cleavage reaction with olefin formation appears to be widespread, as judged by the variety of aldehydes that serve as substrates and the number of P450s that serve as catalysts. Whereas oxidative reactions catalyzed by P450 are widely believed to involve a pentavalent oxoiron species or "iron oxene," our findings on aldehyde deformylation implicate P450 iron-bound peroxide as the oxidant, as already indicated. Our present knowledge of the scope of such hydrogen peroxide-supported reactions is summarized in **Table 2**. As with many other transformations catalyzed by microsomal P450s, the individual isozymes have overlapping specificity in the case of aldehyde cleavage. Cyclohexanecarboxaldehyde deformylation was originally studied with 2B4 (38), but 2C3 and 2G1 have recently been shown to be more active. With trimethylacetaldehyde as substrate, 3A6 is the most active, but the other cytochromes tested are also functional, and citronellal has so far been tested only with 2B4 (39). Some selectivity was seen with ODEC, which is aromatized by 2B4 and only at one-tenth of that rate by 2C3, with 1A2, 3A6, 2E1, and 2G1 being inactive. Pufahl et al. (41) have recently reported that the hydroxylation of arginine by NO synthase appears to involve the usual oxenoid mechanism, but that the step in which N-hydroxyarginine is converted to citrulline is supported by hydrogen peroxide. Finally, Reed et al. (42) have found that an insect cytochrome P450 is involved in the removal of carbon-1 of a C_{24} aldehyde to give the C_{23} hydrocarbon 9-tricosene. This product is known to be the major sex pheromone component of the female housefly. Whether this transformation has the same reaction mechanism as the deformylation reactions we have studied with microsomal P450 isozymes remains to be established, but it is evident that P450 iron-bound peroxide is an effective oxygen donor in many biological systems. ☐

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