MULTIPLE ACTIVATED OXYGEN SPECIES IN P450 CATALYSIS

Contributions to Specificity in Drug Metabolism

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ABSTRACT:

A hypervalent iron-oxene species has been widely proposed as the “active oxygen” in cytochrome P450 (P450)-catalyzed reactions. We recently examined the effect of mutation of the highly conserved threonine residue in P450s 2B4 and 2E1 to alanine, a change that is believed to interfere with proton delivery to the active site, and have determined the change in rates of deformylation of aldehydes, epoxidation of olefins, and hydroxylation of various substrates. The results support the concept that three distinct oxidants are functional in P450 catalysis: nucleophilic per-oxo-iron, nucleophilic or electrophilic hydroperoxo-iron, and electrophilic oxenoid-iron. The occurrence of multiple oxidizing species may contribute to the remarkable versatility of the P450 family of isoenzymes in the modification of drugs and other substrates. Furthermore, the relative concentrations of these oxidants in a particular P450 isozyme may contribute to substrate specificity and govern the type of reaction catalyzed.

As is now widely known, cytochrome P450 (P450) is unmatched among biological catalysts in its versatility (Coon et al., 1996). Recent progress in many laboratories has revealed the occurrence, across different species, of numerous P450 isoforms that catalyze a multitude of reactions. The number of organic compounds that serve as substrates was cautiously estimated in the 1980s to be in the hundreds or even the thousands, but currently no one familiar with the field is surprised at the prediction of a million or more. These include physiologically occurring compounds such as fatty acids, steroids, eicosanoids, lipid hydroperoxides, retinoids, and amino acids. Equally unexpected is the very large list of xenobiotic substrates, including drugs (with many new ones being produced each year by the pharmaceutical industry), procarcinogens, antioxidants, solvents, anesthetics, dyes, pesticides, petroleum products, alcohols, and, among plant products that are foreign to animals, flavorants and odorants. “Billions and billions,” the widely quoted expression used by Carl Sagan (1997) to indicate a very large number related to the cosmos, may far exceed the number of potential P450 substrates. However, it emphasizes the ability of this catalyst to metabolize a multitude of organic compounds that can now be produced readily by combinatorial techniques but do not occur naturally on this planet.

Drug metabolism is a particularly interesting example of P450 action, with many compounds undergoing oxidative inactivation and others being activated or, in some unfortunate cases, yielding products that are toxic, teratogenic, or carcinogenic. Much remains to be learned about the factors responsible for the very broad substrate specificity of microsomal P450s toward some drugs and, in other cases, for the remarkably narrow positional and stereochemical specificity. The detailed structure, which is as yet unknown for the mammalian P450 isoforms, undoubtedly contributes to substrate selectivity, and in this article we present evidence for the occurrence of multiple species of “activated oxygen” and propose that they may determine the type of reaction catalyzed.

A hypervalent iron-oxene species is widely considered to be the oxidant in P450-catalyzed reactions, with the protein functioning to orient the substrate (White and Coon, 1980; Guengerich and MacDonald, 1990). This concept of the oxidant has developed in part from the well-known chemical properties of peroxidases and porphyrin model compounds (Groves, 1986), as well as from the need for a species capable of inserting into unactivated carbon-hydrogen bonds (Groves et al., 1978; Son et al., 1996). However, a nucleophilic iron-peroxo species was proposed as an oxidant in the demethylation of androgens by P450arom to give estrogens (Akhtar et al., 1982), and our laboratory found that purified liver microsomal P450s bring about a comparable reaction, the oxidative demethylation of various xenobiotic aldehydes to olefins and formate (Vaz et al., 1991; Roberts et al., 1991; Vaz et al., 1994). An important line of evidence for the iron-peroxo species as the oxidant was our finding that H2O2 supports the P450-catalyzed demethylation of cyclohexene carboxaldehyde to cyclohexene, whereas artificial oxidants such as cumyl hydroperoxide, iodosobenzene, and m-chloroperbenzoic acid do not (Vaz et al., 1991). The scheme in fig. 1 shows the activation of molecular oxygen by P450 in the presence of NADPH and the reductase; two-electron reduction gives peroxo-iron, which, upon protonation, yields hydroperoxo-iron. The addition of a second proton in an irreversible reaction then gives the oxene species and water. Alternatively, the ferric enzyme and free H2O2 could be produced (not shown).
The investigations described here on the effect of site-directed mutagenesis of mammalian P450s 2B4 and 2E1 make use of evidence from other laboratories that the corresponding mutation in bacterial P450s interferes with the activation of dioxygen to the oxenoid species by disrupting proton delivery to the active site. In a report on the crystal structure of the P450cam active site mutant T252A, Raag et al. (1991) discussed oxygen activation in connection with two possible pathways for proton delivery via an internal solvent channel between threonine-252 and glutamate-366 or via a hydrogen-bonding network extending to the heme environment from the surface of the cytochrome. As evidence for such a critical role for the conserved threonine, camphor hydroxylation by P450cam (Martinis et al., 1989; Imai et al., 1989) and fatty acid hydroxylation by P450 BM-3 (Yeom et al., 1995) were greatly diminished by the mutation of this amino acid residue to alanine. Furthermore, replacement of the conserved threonine of P450cam by unnatural amino acids provided suggestive evidence for a role of the oxygen atom of the threonine hydroxyl group in hydrogen bonding with the water that functions as the ultimate proton donor to the peroxo heme-iron complex (Kimata et al., 1995).

Because the oxidative deformylation of xenobiotic aldehydes to yield olefins and formate presumably involves the iron-peroxo intermediate as the active oxidant, we proposed that disruption of the pathway leading to the iron-oxene species (fig. 1) would result in the loss of oxenoid-dependent reactions, such as hydroxylation, and enhancement of deformylation reactions. As described below, such proved to be the case with P450 2B4 T302A (Vaz et al., 1996). Furthermore, an examination of epoxidation reactions with P450 2E1 T303A revealed a likely role for hydroperoxo-iron as an alternative electrophilic oxidant (Vaz et al., 1998).

**Results and Discussion**

Selected results from our recently published articles are summarized in table 1. We chose to examine the effects of mutagenesis on recombinant P450 2B4 with terminal amino acids 2 through 27 deleted (Δ2B4) and recombinant P450 2E1 with residues 3 through 29 deleted (Δ2E1). These truncated proteins, which retain catalytic activity, have been studied in our laboratory for other reasons, including membrane-targeting. Replacement of threonine-302 by alanine in Δ2B4 caused decreased formation of formaldehyde from benzphetamine (ninefold), cyclohexanol from cyclohexane (fourfold), and acetophenone from 1-phenylethanol (twofold). In sharp contrast, the deformylation of cyclohexane carboxaldehyde by the mutant was increased approximately tenfold. On the basis of these findings and our previous evidence that P450-dependent aldehyde deformylation is supported by added H2O2, but not by artificial oxidants (Vaz et al., 1991), we concluded that the iron-peroxy species is the direct oxygen donor. It should be noted that the decreased oxidation of the first three substrates cannot be attributed to poor substrate binding, because the values for the spectral dissociation constant (Ks) were found to be unchanged in the mutant protein. Another line of evidence for distinct oxidants is based on the metabolism of aldehydes by P450 with the concomitant inactivation of the enzyme, apparently due to heme adduct formation (Raner et al., 1996). In the deformylation pathway, which is enhanced with the T302A mutation of Δ2B4, the rate of enzyme inactivation is also increased. In contrast, in the pathway leading to carboxylic acid formation, as with trans-4-hydroxy-2-nonenal (a toxic product of membrane lipid peroxidation), the mutant protein is less effective in inactivation (Kuo et al., 1997).

More recently, we have turned our attention to olefin epoxidation with the T302A and T303A mutants of 2B4 and 2E1, respectively, and have obtained evidence pointing to hydroperoxo-iron as an electrophilic oxidant (Vaz et al., 1998). Although we have studied model compounds that are particularly useful for mechanistic studies, it should be noted that our conclusions about oxidant species are perti-

![Fig. 1. Steps in oxygen activation by P450 involving electron and proton uptake, where Fe represents the heme iron atom.](image1)

![Fig. 2. Steady-state kinetic model for products derived from a single oxidant or from multiple oxidants.](image2)
Component in the presence of NADPH and NADPH-cytochrome P450 reductase. (Source: Vaz et al., 1981), which are associated with teratogenicity. In our own investigation, some substrates were selected because of their easily quantifiable products and, in other instances, for the availability of the cis isomers or the ability to assess both hydroxylation and epoxidation reactions.

As shown in the table, styrene epoxidation, cyclohexene epoxidation, and hydroxylation to give 1-cyclohexene-3-ol, and cis- or trans-2-butene epoxidation (without isomerization) and hydroxylation to yield 2-butene-1-ol were all significantly decreased by the 2B4 T302A mutation. As indicated above, reduced proton transfer in this mutant protein is believed to interfere with generation of the oxenoid species. With the T303A mutant of ∆E1, however, quite different results were obtained: namely, enhanced epoxidation of all of the olefinic substrates and decreased allylic hydroxylation of cyclohexene and butene. Such results suggest that two different species with electrophilic properties, hydroperoxy-iron (FeO_H)\(^{2+}\) and oxenoid-iron (FeO)\(^{-}\), can bring about the epoxidation of olefins.

The allylic carbon-hydrogen bond and the olefinic bond, representing distinct reaction centers, are within a 3-Å spherical radius in cyclohexene and in the isomers of 2-butene. Presumably, as an explanation of the results obtained, the small size and symmetric nature of these molecules obviate consideration of steric constraints within the P450 active site. The scheme in fig. 2 indicates the kinetic results expected with a single oxidant or with multiple oxidants. When a substrate gives two or more oxidation products, such as P\(_1\) and P\(_2\), and the ratio of product formation, for example δP\(_1\)/δP\(_2\), is equal to the ratio of the rate constants, k\(_1\)/k\(_2\). If a mutation were to perturb only proton delivery, the concentration of the active complex could change, but the ratio

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product Determined</th>
<th>Reaction Rate</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>∆2B4</td>
</tr>
<tr>
<td>Benzphetamine</td>
<td>Formaldehyde</td>
<td>27.0 ± 0.4</td>
</tr>
<tr>
<td>1-Phenylethanol</td>
<td>Acetophenone</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>Cyclohexanol</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>Cyclohexene carboxaldehyde</td>
<td>Cyclohexene</td>
<td>0.15 ± 0.0</td>
</tr>
<tr>
<td>Styrene</td>
<td>Styrene oxide</td>
<td>47.8 ± 0.5</td>
</tr>
<tr>
<td>Cyclohexene</td>
<td>Cyclohexene oxide</td>
<td>48.7 ± 4.4</td>
</tr>
<tr>
<td>Cyclohexene</td>
<td>1-Cyclohexene-3-ol</td>
<td>29.3 ± 1.7</td>
</tr>
<tr>
<td>cis-2-Butene</td>
<td>cis-2-Butene oxide</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>trans-2-Butene</td>
<td>trans-2-Butene oxide</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>trans-2-Butene</td>
<td>2-Butene-1-ol</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

* Rates of oxidation of various substrates were determined in a reconstituted enzyme system containing an NH\(_2\)-terminal–truncated P450 or the corresponding mutant enzyme as the limiting component in the presence of NADPH and NADPH-cytochrome P450 reductase. (Source: Vaz et al., 1996 and 1998.)

![FIG. 3. Proposed versatility in P450 oxygenating species.](Image)

The nucleophilic or electrophilic properties and typical reactions catalyzed are indicated under the structures of the peroxo, hydroperoxo, and oxenoid species. (Source: Vaz et al., 1998.)

TABLE 1

Effect of threonine-to-alanine mutagenesis on rates of substrate oxidation by P450 cytochromes.
of the rate constants and of products formed would remain constant. However, if two products—for example, \( P_2 \) and \( P_3 \)—are formed by the action of two different oxidants, then the ratio of the products would be dependent on the concentration of each oxidant. In this case, if a mutation were to alter the steady-state concentration of the reactive complexes, the ratio of products formed would remain constant. Examination of the data in the table indicates that the threo-2E1 resulted in an increase in the ratio from 1.0 to 2.4, which is consistent with the involvement of different oxidants in formation of the two products. Similar results were obtained with cis- and trans-butenes, with the mutation of \( \Delta 2E1 \) resulting in an increase in the epoxide to allyl alcohol ratio from 90 to 460 and from 4.0 to 5.6, respectively.

As shown in fig. 3, we propose that three discrete oxidants—peroxy-iron, hydroperoxy-iron, and oxenoid-iron—contribute to the versatility of P450 in the oxidation of various substrates. The structures of these species are indicated as well as the expected nucleophilic and/or electrophilic properties and typical reactions affected. A summary of reactions that we consider likely to utilize the hydroperoxy-iron species as an alternative electrophilic oxidant in P450 catalysis is given in table 2. These include some examples that are documented in the article presented here, such as epoxidation, and others that are now under investigation. The criterion we have used for inclusion in this list is enhanced activity with P450 \( \Delta 2E1 \) when Thr-303 in this isozyme is replaced by alanine.

Since much remains to be learned about the three-dimensional structure of the mammalian P450s we are studying, a note of caution is in order. The possibility should be considered that a structural change attributable to the threonine mutation is somehow responsible for different rates of epoxidation and hydroxylation of the same substrate. Although various control experiments argue against this possibility, it cannot be entirely ruled out. Another possible explanation is that the rate-limiting steps in epoxidation and hydroxylation are somehow affected differently by the mutation.

In summary, our results suggest that the hydroperoxy complex of P450 is capable of the epoxidation of unactivated olefinic bonds, and we propose that the versatility in oxidative reactions may depend, at least in part, on the ability to utilize as multiple oxidants the peroxy-iron, hydroperoxy-iron, and oxenoid species, depending on the substrate and type of reaction catalyzed. This could add to the scope of reactions catalyzed by the P450 family of isozymes, and, for a particular isozyme, could contribute to substrate specificity and even determine the type of reaction that occurs.

**References**


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