New Insight into Reaction of Iron(III)-peroxide Adduct with Alkanes: an Alternative Model for Cytochrome P-450 and Methane Monoxygenase

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Iron(III)-peroxide Adduct, Alkane Functionalization, Activation of Peroxide Adduct, Electrophilicity of Metal-peroxide

A new mechanism for alkane functionalization by iron(III)-peroxide adducts was proposed, and the importance of electrophilic nature of the metal-peroxide adduct with η^1-coordination mode was emphasized. This idea suggests that formation of high-valent iron-oxo species occurs most likely when the metal-peroxide intermediate is activated through electronic interaction with both the peripheral organic group and substrate; the latter two act as an electron donor to the peroxide adduct.

One of the remaining frontiers in organic chemistry is the direct functionalization of saturated hydrocarbons. Considerable progress has been made in understanding the chemical requirements for transition metal “activation” of carbon-hydrogen bonds (Hill, 1989). The catalytic cycle that oxidizes a hydrocarbon RH to an alcohol ROH employing P-450 is also a well-established reaction (Montelano, 1986; Sono et al., 1996).

Cytochrome P-450s constitute a large group of enzymes and contain a conserved cysteine, the thiolate group of which is ligated to the haem iron (Poulas et al., 1987). Cytochrome P-450CAM (P-450CAM, CYP101) is a monoxygenase that catalyzes the hydroxylation of camphor in the bacterium Pseudomonas putida (see Table I). It was the first member of a ubiquitous family of P-450 isoenzymes for which a crystal structure was reported. Extensive experimental investigation has helped to elucidate the steps in the P-450 enzymatic cycle thought to be common to all isozymes. Fig. 1 indicates (Groves and Nemo, 1983; Gunter and Turner, 1991) these steps schematically and shows cytochrome P-450’s hydroxylate substrates via an enzymatic cycle which involves (i) entry of the substrate, (ii) displacement of most or all of the product formed per O2 consumed 5-OH-CAM* H2O2 in %

Table I. Oxygen and NADH consumption rates of wild-type and mutant cytochrome P-450CAM in a reconstituted system and the amount of products formed (Imai, et al. 1989).

<table>
<thead>
<tr>
<th>Mutant site-residue</th>
<th>Rate of consumption</th>
<th>Product formed</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Oxygen (µmol/min x µmol heme)</td>
<td>NADH (µmol/min x µmol heme)</td>
</tr>
<tr>
<td>252-Thr(wild)</td>
<td>1350</td>
<td>1380</td>
</tr>
<tr>
<td>252-Ala</td>
<td>1150</td>
<td>1180</td>
</tr>
<tr>
<td>252-Gly</td>
<td>1090</td>
<td>1090</td>
</tr>
<tr>
<td>252-Ser</td>
<td>830</td>
<td>830</td>
</tr>
<tr>
<td>252-Val</td>
<td>260</td>
<td>250</td>
</tr>
</tbody>
</table>

* 5-OH-CAM: 5-exo-hydroxycamphor.

Fig. 1. Mechanism of oxygen activation in cytochrome P-450 by Groves and Nemo (1983).

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substrate cavity water, (iii) a change of ferric heme spin state upon displacement of the sixth ferric heme water, (iv) a change in the redox potential toward one-electron reduction, via a complex electron transport system, (v) a one-electron reduction to a ferrous heme followed by (vi) entry and binding of molecular oxygen to the ferrous state, (vii) a second one-electron reduction, (viii) formation of the putative reactive intermediate, high-valent iron-oxo species, and (ix) hydrogen radical abstraction by the ferryl oxygen from the substrate followed by radical combination to produce the hydroxylated products and regeneration of the resting state of the enzyme. In addition to the nature of the reactive intermediate itself, its mechanism of formation from the ferrous dioxygen species is also still under active investigation. Among the major unresolved questions are the (1) the role and identity of the binding residues, if any, involved in the formation of the reactive intermediate and (2) the concertedness of the second electronic reduction and O–O bond cleavage to form the ferryl oxygen species. One common hypothesis is that hydrogen bonding and/or proton donation stabilizes the initial ferrous dioxygen complex and facilitates the O–O bond cleavage in the twice reduced state. However, a high-valent iron(V)-oxo species in Fig. 1 has not been yet identified or characterized for the cytochrome P-450's (Harris and Loew, 1994).

Sequence alignments of P-450's indicate that the threonine residue is a highly conserved residue at sequence location 252. In P-450CAM, this threonine forms part of the dioxygen binding groove in the substrate binding site. Imai et al. have reported that the efficiency in conversion of camphor to 5-exo-hydroxycamphor drops to only 5–6% and electrons are channeled to produce hydrogen peroxide and water, when the highly conserved active site Threonine-252 is replaced with alanine or glycine (see Table I) (Imai et al., 1989). The crystal structure of a P-450CAM site-directed mutant in which the active site Thr252 has been replaced with an alanine(Thr252Ala) has been determined by Raag et al. (1991). This has revealed that a solvent molecule not present in the native enzyme is positioned in the dioxygen-binding region of the mutant enzyme active site, and thus solvent protons appear to be much more accessible to dioxygen in the mutant than in the wild-type enzyme, factor which may promote hydrogen peroxide and/or water production instead of substrate hydroxylation. These facts suggest that promoted O–O bond cleavage by hydrogen bonding and/or proton donation in the twice reduced state (see Fig. 1) is unlikely. Above consideration may be supported by the fact that a mutant enzyme with a methoxy group in place of the hydroxy group of threonine-252(OMe-mutant) retains a considerably high monoxygenase activity, yielding a stoichiometric amount of the product to that of the oxygen consumed (Kimata et al., 1995).

Harris et al. (1994) have performed the molecular dynamics simulations on the ferrous bound form of wild type P-450CAM, and the results indicate a time-dependent bimodal interaction of Thr252 with both Gly248 and the terminal oxygen of the bound dioxygen. The hydrogen bonding interaction of Thr252 with these two moieties is "anticorrelated" in the sense that the breaking of the Thr252–Gly248 hydrogen bond is concurrent with formation of the Thr252-dioxygen interaction. Because it seems quite unlikely that proton is related with the cleavage of O–O bond as described above, the bonding of Thr252-dioxygen should have another important meaning in dioxygen activation in P-450. In order to investigate the function of Thr252 and its O-methyl derivative in the oxygenation reaction, we have compared the reactivities towards alkane functionalization of the iron(III)-peroxide adducts where the peroxide ion can interact with the organic group nearby; examples are illustrated in Fig. 2 (Nishida et al., 1995; Ito et al., 1996a). The figure in the left side of Fig. 2 is our model for P-450CAM, which may be represented by the complexes with (etapy) and also with (bbimae), where in the latter complex the coordination of alcohol group to an iron(III) is confirmed (Takahashi et al., 1985). The peroxide adduct shown in the right side is indicating our model for the OMe-mutant.

Fig. 2. Interaction between Fe(III)-peroxide adduct and peripheral group.
Materials and Methods

Materials

We have prepared linear (μ-oxo)diiron(III) compounds with the ligands shown in Fig. 3 (Nishida et al., 1995); the compound with (etapy) was obtained in this study. Crystal structure determinations of several compounds have revealed that the structural features of the compounds are essentially the same as those reported for the (tpa)-complex (Leising et al., 1993; Kojima et al., 1993) with linear μ-oxo bridge; as an example the ORTEP drawing of the (epy)-complex is illustrated in Fig. 4 (Nishida et al., 1995).

Fig. 3. Chemical structures of the ligands cited in this paper and their abbreviations.

Catalase-like function of complexes

All reactions were performed at 20 °C in a 10 cm³ reactor containing a stirring bar under air (Ito et al., 1996a). The flask containing an iron(III) complex (10 μmol) solution (5 ml, acetonitrile) was closed with a rubber septum. Hydrogen peroxide solution (1 ml, commercial 30% aqueous solution) was diluted to 1 ml solution by acetonitrile) was injected through the septum with a syringe. The reactor was connected to a graduated burette filled with water and dioxygen evolved was measured at appropriate time by volumetry. The theoretical quantity of dioxygen molecule, which should be evolved under our experimental conditions, is ca. 16 ml, which was exemplified by the authentic experiment by the use of MnO₂ as a catalyst.

Oxygenation reaction of cyclohexane in the presence of iron(III) complex and hydrogen peroxide

In a typical run, an acetonitrile solution (20 ml) containing iron(III) complex with (etapy) and/or (bbimae) (0.05 mmol) and cyclohexane (840 mg) was added to an acetonitrile solution (10 ml) containing hydrogen peroxide (1.13 g of commercial 30% aqueous solution), and was kept to stand for at room temperature, and the oxygenated products were determined by GC. Cyclopentanone was used as an internal standard (Ito et al., 1996a).

Molecular orbital calculations

The MNDO/AM1 calculations were performed for methylethylether, and the positional parameters of the compound was optimized by the use of the AM1 program (Ito et al., 1996a). MO calculations for Fe(NH₃)₄(dimethylether)(HO₂⁻) and Fe(NH₃)₄(methanol)(HO₂⁻) were performed by the use of EHHMO method reported by Hoffmann et al. (1977), where the parameters used for the iron(III) ion are the same as those used in the literature.

Results and Discussion

It is well known that some binuclear iron(III) compounds with μ-oxo bridge exhibit high catalase-like function, decomposition of hydrogen per-
oxide. We already reported that the diiron(III) compounds with μ-oxo bridge are divided into two classes, A and B, and class-A complexes exhibit high catalase-like function, whereas that of the class-B complexes, negligible as shown in Table II. The class-B compounds exhibit high activity for hydroxylation of cyclohexane in the presence of hydrogen peroxide, compared with that of class-A compounds. Here it is noteworthy that Fe(III) complex with (etapy) and (epy), which are considered to be a model for P-450 and its OMe-mutant, respectively, show high activity for hydroxylation of cyclohexane.

Table II. Turn-over numbers (TN) of cyclohexanol catalyzed by iron(III) compounds.

<table>
<thead>
<tr>
<th></th>
<th>Cyclohexanol</th>
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<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>2 hours</td>
<td></td>
</tr>
<tr>
<td>Class-A complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-(tpa)</td>
<td>0.06</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Fe-(mopy)</td>
<td>0.3</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Class-B complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-(epy)</td>
<td>3.2</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Fe-(etapy)</td>
<td>1.1</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Fe-(tfpy)</td>
<td>1.2</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Fe-(bbimae)</td>
<td>1.3</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

a See ref. Takahashi et al. (1985); see Fig. 3 for abbreviations.

At present it is generally accepted that catalase-like function by the diiron(III) compounds may proceed through formation of an intermediate with μ-η:η'-peroxo adduct (adduct-I), as shown in Fig. 5a (Ito et al., 1996a; Menega et al., 1994). This implies that formation of an another peroxide-adduct, whose structure is different from μ-η:η'-coordination mode occurs in the solutions of compounds with (epy), (etapy) or (tfpy), and this should be an intrinsic active species for alkane functionalization. One of the possible structures in shown in Fig. 5b, adduct-II (Ito et al., 1996a) where peroxide ion coordinates to an iron(III) ion in the (dpks)-complex with η'-coordination mode (Nishida et al., 1995; Nishida and Ito, 1995a). In the peroxide adduct with η'-coordination mode, both α*- and π*-orbitals of the peroxide ion mix with d-orbital which directly interacts with the peroxide ion (Ito et al., 1996a). EHMO calculations revealed that the adduct-II shows electrophilic reactivity at both the O1 and O2 positions, as indicated by arrows in Fig. 5c (Nishida et al., 1994; Nishida and Ito, 1995b).

The electrophilicity at O2 position has been confirmed by several experiments (Nishida and Ito, 1995b, Ito et al., 1996b) especially in heme-oxygenase reaction (Torpey et al., 1996). Thus, it has become apparent that interaction between O2 and ligand system of (dpks)-ligand can contribute to the stabilization of adduct-II in energy. In Fig. 6, presence of orbitals A and B indicates that stabilization of the peroxide adduct occurs via interaction with the (epy)-ligand system, and also suggests that remarkable stabilization of energy of HOMO of (epy)-ligand, in this case pz-orbital of ethereal oxygen, occurs through interaction with the peroxide ion; this effect is specific for the ligand which contains a non-conjugated oxygen atom such as ethereal or alcoholic oxygen, for examples (epy), (etapy) and (tfpy). Stabilization in energy as described for the (dpks), (epy), (etapy) and (bbimae)-ligand systems is not detected for the cases of (tpa) and (mopy)-ligands, which may be due to both steric and electronic reasons (Ito et al., 1996a). These are suggesting that electronic in-
Fig. 6. EHMO calculation for Fe(NH₃)₄(O₂H)(CH₃OCH₃) as a model for intermediate of OMe-mutant. (In the equations below, (C) denotes the carbon atom of methyl group close to the O₂ atom; pₓ(C) and pₓ(O₂) denote the p-orbitals of the carbon and oxygen atom, respectively; for details, see Hoffmann et al. (1977).

Orbital A: -0.230pₓ(C)+0.273pₓ(C)+0.232pₓ(O₁)
-0.245pᵧ(O₁)-0.388pₓ(O₂)+0.548pᵧ(O₂)
Orbital B: 0.529pₓ(O)-0.263pₓ(C)-0.389pₓ(O₁)
+0.604pᵧ(O₂).

The interaction between the peroxide in and organic moiety, which may originate from the electrophilic nature of d-orbital containing peroxide ion in the adduct-II, plays an important role to induce facile formation of adduct-II. In addition, it should be noted here that in the interaction between the d-orbital and the organic moiety, electron flows from the organic moiety to peroxide ion, and thus the peroxide ion coordinated to the iron(III) atom is more activated, because the electron flow to σ*-orbital of the peroxide occurs in this process (Ito et al., 1996a).

If we assume that substrate approaches to the adduct-II, there should be electronic interaction between the HOMO of substrate and O₁, since O₁ atom of the adduct-II is also of electrophilic nature, as suggested in the papers (Nishida et al., 1994; Ito et al., 1996a). As the approach of substrate also donates electron to the adduct-II, the peroxide ion is more activated, leading to facile cleavage of O–O bond. There are two possible ways in the O–O bond in the peroxide adduct; i.e., homolytic and heterolytic cleavage; in the latter case, H₂O₂ is cleaved to OH₂ and a metal-O(atomic oxygen) (Schroder et al., 1996; Bach and Su, 1994) and the latter species may be alternately formulated as a high-valent metal-oxo species. In the adduct-II, peroxide ion is unsymmetric, which may lead to facile heterolytic cleavage. Thus, activation of the peroxide ion through interactions with both the peripheral group and approach of substrate promote formation of a high-valent iron-oxo species, giving the oxygenated product (Bach et al., 1995; Newcome et al., 1995) as illustrated in Fig. 7a).

Fig. 7. a) Assumed scheme for reaction of methane with an Fe(III)-(epy)-peroxide adduct with η¹-coordination mode.
b) Reaction between (peroxo)iron(III) species with organic compounds containing carbonyl group.

This is meaning that interaction between the peroxide adduct and the peripheral organic group, and the approach of substrate play an important role in formation of an iron-oxo species; this consideration is completely different from that reported in the previous papers (see Fig. 1). Present discussions clearly explain several questions in P-450 reaction; that is, the bonding in the Thr252-dioxygen possesses important role in activation of peroxide ion, and may give an answer to the fact
that a compound I radical action perferryl oxygen species has not been identified for P-450's.

The importance of the electrophilicity at O2 atom of the peroxide adduct with \( \eta^1\)-coordination mode is also observed in several P-450's; aromatase and 14-demethylase catalyze not only the conventional hydroxylation reaction but also the oxidation of an alcohol into a carbonyl compound. These include a more important acyl-carbon bond cleavage reaction (see Fig. 7b)) (Vaz et al., 1996; Pratt et al., 1995) and degradation of amide-compounds (Ming et al., 1995) and proteins (Rana and Meares, 1991).

It should be noted here that when target substrate contains an oxygen atom, the iron peroxide is trapped, producing an adduct which may decompose by one of several closely related pathways. These facts are also comprehensively explained by our model, i.e., when substrate contains an oxygen atom, similar to the ligand system of (epy), (tfpy) or (etapy), the electrophilic nature of O2 atom promotes the binding of O2 atom with the carbon atom of the substrate, which contributes to the stabilization of the system, and also to enhanced reactivity of the peroxide adduct, leading to facile hydroxylation or oxidation of the substrate. Our consideration may be applied to elucidate the reaction mechanism in non-heme oxygenases, such as in methane monooxygenase.

In the case of methane monooxygenase, it has been postulated that the intermediate Q reacts with methane directly, giving methanol (Lipscomb, 1994; Waller and Lipscomb, 1996). Until now, many reports have been published on the structure of the intermediate Q (Liu et al., 1995). Very recently Que et al. have proposed that this intermediate has an Fe(IV)\(_2\)O\(_2\) diamond core structure (see Fig. 8a)), which seems to be consistent with the results of Mossbauer Spectra and EXAFS data (Shu et al., 1997). They have considered that the Fe(IV)\(_2\)O\(_2\) diamond core forms via a Fe(III)-peroxide adduct, but this may be inconsistent with the recent result on the Ribonucleotide reductase, where formation of a Fe(IV) state is promoted by donation of one electron (Sturgeon et al., 1996). Thus, it seems most likely that the interaction between the peroxide adduct and peripheral carboxylate group (Glutamate 243) (Feig and Lippard, 1994) should enhance heterolytic cleavage of the peroxide adduct(intermediate P), leading a Fe(III)-O(atomic oxygen)-Fe(III) species (see Fig. 8 b)), which can be written as a Fe(IV)-O(oxo)-Fe(IV).


Nishida Y. and Ito S. (1995b), Comparison on reactivity of Fe(III) and Al(III) compounds in the presence of hydrogen peroxide; its relevance to possible origin for central nervous system by aluminium ion. Z. Naturforsch. 50e, 571–577.


