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Oxygen Economy of Cytochrome P450: What Is the Origin of the Mixed Functionality as a Dehydrogenase−Oxidase Enzyme Compared with Its Normal Function?

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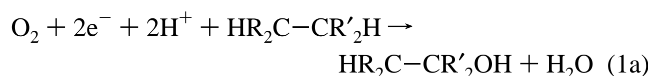
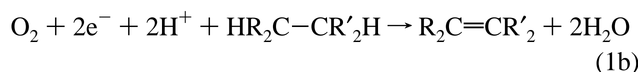
The economy of dioxygen consumption by enzymes constitutes a fundamental problem.[1] The enzyme cytochrome P450 catalyzes the "insertion" of an oxygen atom into substrates by utilizing two reduction equivalents ($2e^-$), 1 mol of O_2, and two proton equivalents; the other [O] equivalent is converted to water. When the stoichiometry of, e.g., a P450-catalyzed C−H hydroxylation follows eq 1a, the monoxygenation reaction is called "tightly coupled", meaning optimal O_2 consumption.[1]



$$O_2 + 2e^- + 2H^+ + HR_2C{-}CR'_2H \rightarrow HR_2C{-}CR'_2OH + H_2O \quad (1a)$$



$$O_2 + 2e^- + 2H^+ + HR_2C{-}CR'_2H \rightarrow R_2C{=}CR'_2 + 2H_2O \quad (1b)$$

Occasionally, the O_2 consumption follows eq 1b,[2] where *the enzyme converts all the dioxygen into water*, thereby acting as an oxidase,[1,3] but at the same time it dehydrogenates the substrate[4] and functions such as nonheme enzymes that catalyze fatty acids metabolism. This mixed function of the enzyme is intriguing, and constitutes the focus of this paper.

A seminal study[5] of C−H hydroxylation of the antiepileptic drug valproic acid showed that the dehydrogenase−oxidase activity and the normal monoxygenation function branch from the same oxidation mechanism that involves the high-valent iron-oxo species, Compound I (Cpd I) of the enzyme (see Figure 1). This[5] and other studies[2,4b,c,6] led to the conclusion that the mechanism involves an initial hydrogen abstraction from the substrate by Cpd I. Subsequently, the resultant radical may be partitioned between a few possible pathways. In the normal route, the radical rebounds[7] to form an alcohol, while in the mixed oxidase−dehydrogenase route, the radical loses one more hydrogen atom (or a sequential loss of electron and proton), thus producing olefin and water. Nevertheless, the mechanistic details are not clear-cut and the desaturation reaction is not very predictable,[2b] so that theory may be helpful to elucidate the mechanism. This work uses density functional theory (DFT) to investigate the mechanism in order to trace its origins and the requirements that lead to the mixed dehydrogenase−oxidase activity of P450.

The calculations used the B3LYP functional coupled with the double-ζ LACVP(Fe)-6-31G(H,C,N,O,S) basis set. JAGUAR 4.2[8] was used for geometry optimization and calculations that include the effect of medium polarity via a dielectric constant, $\epsilon = 5.7$ (specific interactions with the protein are not included). TDDFT[9] calculations ascertained the crossing in Figure 1a. Technical details are given in Supporting Information, while the discussion focuses on the chemical essence.

The model alkane (Alk-H) chosen for the calculations is *trans*-2-phenyl-*iso*-propylcyclopropane (**1**), which was shown before[10]

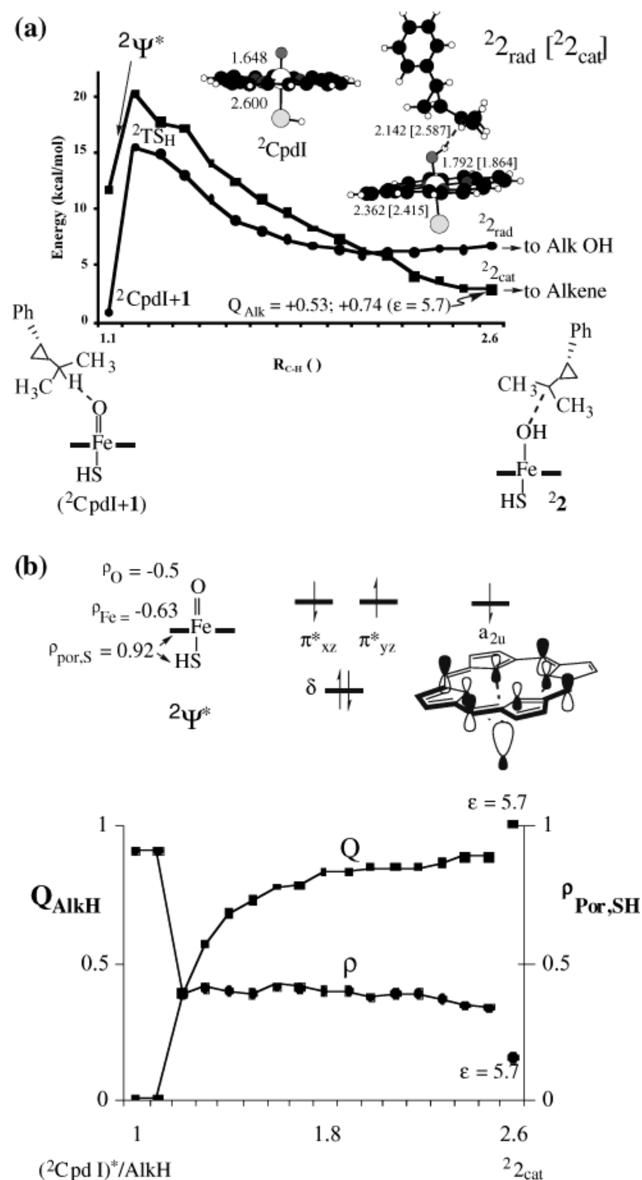


Figure 1. (a) C−H abstraction profiles, in the reaction of ^{2}Cpd I with **1**, leading to the radical and cationic complexes $^2\mathbf{2}_{rad}$ and $^2\mathbf{2}_{cat}$. The arrows near these species indicate the followup processes to yield alcohol (AlkOH) and alkene. (b) The excited state ($^2\Psi^*$) that correlates to $^2\mathbf{2}_{cat}$ and its orbital population. Underneath the description of $^2\Psi^*$ is a plot showing the variations of the spin density in the a_{2u} orbital ($\rho_{por,SH}$), and the charge on the substrate (Q_{AlkH}) as the excited state $^2\Psi^*$ evolves into the cationic complex. The two data points corresponding to dielectric constant (ϵ) of 5.7 show the effect of polarity. The break in the plot shows the initial electron transfer from **1** to fill the porphyrin "hole" in $^2\Psi^*$.

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to lead to normal C−H hydroxylation with high-spin and low-spin manifolds and to give both radical and cationic species. Figure 1a shows the energy profile for the hydrogen abstraction step by Cpd I on the low-spin manifold. The profile exhibits crossing of two states that terminate at two structures, $^2\mathbf{2}_{rad}$ and $^2\mathbf{2}_{cat}$. The alkyl group spin density (ρ_{Alk}) and charge (Q_{Alk}) show that $^2\mathbf{2}_{rad}$ is the LS radical complex made from the alkyl radical ($\rho_{Alk} = -0.94$; $Q_{Alk} = +0.06$) coordinated to the ferryl hydroxo complex. In contrast, $^2\mathbf{2}_{cat}$ has a large positive charge on the alkyl moiety ($Q_{Alk} = 0.53$; +0.74 in $\epsilon = 5.7$) and is hence more akin to the carbocationic complex, PorFe(III)OH^-/Alk^+. While the calculation revealed two-state crossing, the crossing can most likely be weakly avoided,[9b] and as such, electron transfer will induce passage from the radical to the cationic state.

In a previous study,[10] we showed that the $^2\mathbf{2}_{rad}$ species underwent normal rebound and spontaneously produced the alcohol product, AlkOH (arrow in Figure 1a). By contrast, as shown by the arrow near $^2\mathbf{2}_{cat}$ in Figure 1a, $^2\mathbf{2}_{cat}$ undergoes a spontaneous proton transfer from the Alk^+ moiety to the ferric-hydroxo anion to form the corresponding alkene and the ferric−water complex. In fact, the $^2\mathbf{2}_{cat}$ species is on the downhill slope en route to alkene product complex. Indeed frequency analysis of $^2\mathbf{2}_{cat}$ exhibits a single negative eigen-mode that corresponds to the second hydrogen transfer to the heme. *Thus, theory reveals that the dehydrogenase−oxidase activity is not associated with radicals of the substrates but rather with the corresponding carbocations*, formed through electron transfer from the alkyl radical to the iron-hydroxo complex. This is indeed one of the possibilities postulated in the experimental literature.[2a,b,6]

However, where does the carbocationic complex originate from? Figure 1a shows that the LS ferric-hydroxo-carbocation formally originates from an excited state of the Cpd I−substrate complex ($^2\Psi^*$). This excited state is shown in Figure 1b along with key orbitals. The species involves a porphyrin radical−cation (see a_{2u}-type orbital) and two singlet-coupled electrons in the π^* orbitals of the Fe═O moiety, as opposed to the ground state that involves triplet-coupled electrons in this moiety.[7,10,11] Thus, the mother state of $^2\mathbf{2}_{cat}$ is the excited state of ^{2}Cpd I and is analogous to the $^1\Delta_g$ singlet state of dioxygen, save the porphyrin radical−cation. As seen from the plot in Figure 1b, this state gradually loses its porphyrin radical−cationic character ($\rho_{Por,SH}$) by accepting an electron from the alkane (see Q_{AlkH}) and, at the same time, the resultant alkane radical cation transfers an H$^\bullet$ atom to the heme-oxo. It follows that the transformation to the carbocationic surface involves two states with *successive atom and electron transfers* from the substrate to Cpd I.

What is the reason for the oxidase−dehydrogenase mode of $^2\mathbf{2}_{cat}$? Inspection of the geometry of the species in Figure 1a shows that the C−O distance is 2.6 Å compared with a short 2.1 Å for the corresponding radical. The reason for this long C−O distance is rooted in steric bulk and charges of two moieties in $^2\mathbf{2}_{cat}$. The iron-hydroxo is negatively charged, and it maintains O···H^+ interaction with the nearby positively charged hydrogen ends of the CH_3 groups of the Alk moiety. Thus, due to this electro-steric factor, on one hand, the $^2\mathbf{2}_{cat}$ species is not set up for rebound,[10b] to form the alcohol, and on the other hand, the O···H^+ interaction causes a spontaneous proton transfer from the alkyl cation to the iron-hydroxo complex. The combination of these two factors results in the oxidase−dehydrogenase activity.

The electro-steric factor links the process studied here to the "pure" oxidase activity of P450, which converts all the O_2 to water, by using an extra mole of the reductase that provides $4e^-$ in the course of the reaction (i.e., $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$). As shown by experiments,[3] this "decoupling" of oxygen consumption from substrate oxidation occurs when Cpd I accepts two electrons from the reductase. Sligar et al.[1] have further demonstrated that whenever the protein pocket is encumbered, e.g., to prevent approach of the substrate's C−H to the FeO moiety of Cpd I, the result is increased "decoupling" and heightened "pure" oxidase activity. Our present study shows the same trend, namely, whenever the substrate is sterically encumbered, as in **1**, so that the C−O approach is perturbed, the cationic intermediate will lead to a mixed oxidase−dehydrogenase reaction. Thus, the oxidase−dehydrogenase function requires substrates that combine *steric inhibition of rebound and stable carbocationic complexes*. It is further reasonable to speculate that in the protein environment, release of the carbocation, away from the iron hydroxo anion, may compete with dehydrogenation. In such an event, the carbocation will either get hydroxylated (e.g., by rebound from a long C−O distance[10b] or simply by water) or mediate "pure" oxidase activity by accepting electrons from the reductase, while restoring the substrate by abstracting H$^\bullet$.

In sum: the oxidase−dehydrogenase mixed activity *occurs from the cationic intermediate species and requires electro-steric inhibition of the rebound process*. The carbocationic species correlates with excited state of Cpd I, in which the Fe═O moiety is singlet coupled as in the $^1\Delta_g$ state of dioxygen. The crossing of the radical and carbocationic states[10] highlights once again the repeated findings of our group that the reactivity of Cpd I-type reagents generally involves a multistate scenario.

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Supporting Information Available: Three tables and seven figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) (a) E.g.: Stratmann, R. E.; Scuseria, G. E.; Frisch, M. J. *J. Chem. Phys.* **1998**, *109*, 8218. (b) The mixing is very weak due to the long distance between the radical center state and the iron. The crossing could be weakly avoided, but this would require a high-level multireference calculation.

(10) (a) Kumar, D.; de Visser, S. P.; Shaik, S. *J. Am. Chem. Soc.* **2004**, *126*, 1907−1920. (b) In ref 10a, the $^2\mathbf{2}_{cat}$ species was found to rebound in a barrierless fashion after geometry alteration to a rebound conformation. The optimized C−O distance in this conformation is long, 3.2 Å, presumably due to steric repulsion.

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