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An ultimate species in the substrate oxidation process by cytochrome P-450

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We propose a structure for the ultimate species which gives a straightforward explanation of the major results in the oxidation of hydrocarbons by cytochrome P-450 where the stereochemistry is retained and the isotope effect is small.

In the substrate oxidation process by cytochrome P-450 it was believed that the ultimate species is compound **I** [(heme)Fe=O] (Fig. 1).[1] Recently, Harris and Loew[2] showed that the spin-density of compound **I** in the spin doublet state (the ground state of this compound) is localized at the oxygen atom that is bound to the Fe atom of the heme, *i.e.* the spin-density is 0.92, indicating that the oxygen atom has the character of a free radical. The summation of the spin-densities shared by the heme and the fifth ligand gives a value of 0.08. We re-confirmed this spin-density distribution of compound **I** by performing similar calculations using density functional theory (DFT).[3]

It is well known that a free radical abstracts a hydrogen atom from hydrocarbons.[7] We found that compound **I** abstracts a hydrogen atom from hydrocarbons and produces Fe–OH with an activation energy of 15.5 kcal mol^{-1}. The hydrogen abstraction from hydrocarbons has been considered to be one of the plausible mechanisms caused by the ultimate species, compound **I**, and the substrate oxidation process by cytochrome P-450 should be as in the two-step mechanism shown in Fig. 1(a).[1,8] This two-step mechanism has the characteristics that (*i*) the stereochemistry is lost, and (*ii*) an isotope effect is clearly observed.[1,8]

The hydroxylation of a hydrocarbon by cytochrome P-450, however, generally occurs with retention of stereochemistry and a small isotope effect.[1,8] The concerted insertion of an oxygen atom into a hydrocarbon C–H bond [Fig. 1(b)] is therefore, favorable from this viewpoint. In order to examine the possibility of the one-step mechanism [Fig. 1(b)], a theoretical study has been preformed.

Firstly, we carried out the molecular dynamics (MD) simulations for 300 ps at 310 K on oxy-ferrous P-450cam in water, and showed that the substrate–oxygen molecule interaction is maintained in the heme pocket of cytochrome P-450cam, *i.e.* the distance between the C5 atom of d-camphor and the oxygen atom which is not directly bound to the Fe atom of the heme (the distal oxygen atom, O2) is *ca.* 3.0–3.5 Å (3.22 Å in Fig. 2). It is considered that the interaction between the substrate and the oxygen molecule bound to the Fe atom of the heme is always effective throughout the substrate oxidation process by cytochrome P-450. Experiments by Tuckey and Kamin[14] support the existence of the substrate–oxygen molecule interaction in the heme pocket, *i.e.* the 1-electron-reduced–oxygenated complex of cytochrome P-450 is kinetically stabilized by the binding of the substrate.

From Fig. 2 it is natural to consider that the distal oxygen atom is inserted into the C5–H bond of d-camphor. From this the revised ultimate species shown in Fig. 3 is proposed. Because it exists at a minimum in the potential energy hypersurface, the structure of this compound is stable. The potential energy of the structure is 59.8 kcal mol^{-1} higher than the sum of the potential energies of compound **I** and H_2O. However, it is considered that formation of the revised ultimate species could occur, because reduced oxy-ferrous heme is stabilized by > 700 kcal mol^{-1} upon binding of two protons to form each ultimate species. For the revised ultimate species

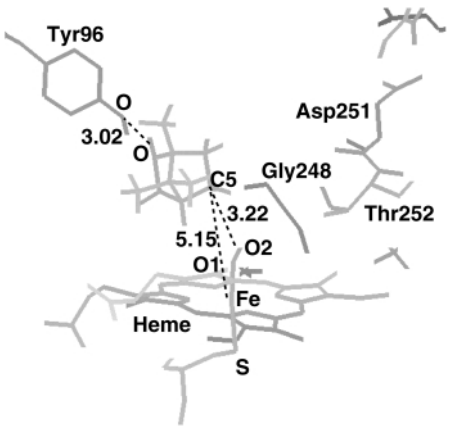

Fig. 2 Schematic of oxy-ferrous P-450cam obtained by 300-ps MD simulation at 310 K. Numerals are inter-atomic distances in Å. Computational program is AMBER 4.1.[9] A united-atom force field[10] was applied except for d-camphor, for which an all-atom force field[11] was used. Calculations of the non-bonded term were accelerated by the use of a hardware accelerator (MD Engine).[12] A cutoff distance (8 Å) was applied for computation of van der Waals forces (r^{-6} and r^{-12}). The electrostatic term (r^{-1}) was calculated with no cutoff, taking full advantage of the MD Engine. The SHAKE constraint,[13] where all of the bonds are kept at equilibrium distances, was used.

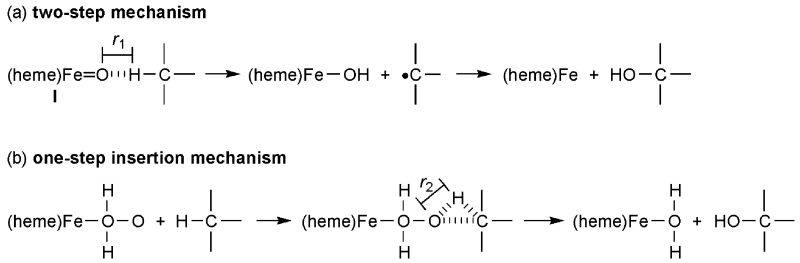

Fig. 1 Two processes in the substrate oxidation by cytochrome P-450: (a) two-step mechanism by the currently believed ultimate species, compound **I**, which has an active oxygen atom in the spin doublet state; (b) one-step insertion mechanism by the newly proposed ultimate species which has an active oxygen atom in the spin singlet state. The existence of these two processes gives complicated results in the oxidation products by cytochrome P-450.

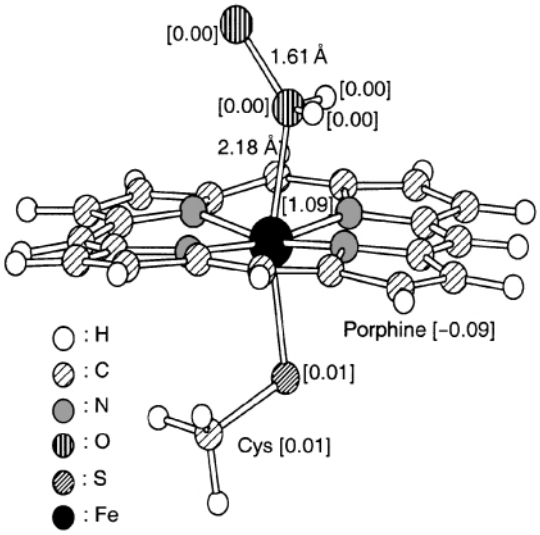

Fig. 3 Spin-density distribution of the stable structure of the newly proposed ultimate species for the mono-oxygenation reaction by cytochrome P-450. Numerals in parentheses are the spin-densities. The total electronic charge of the model compound is neutral and the total spin multiplicity is that of a doublet.

(Fig. 3) it should be noted in the spin-density distribution that the distal oxygen atom is in the spin singlet state, and it has already been suggested that a spin singlet state oxygen atom ^{1}D operates a one-step insertion mechanism.[15] The activation energy of the insertion reaction of this singlet oxygen atom to the C5–H bond of d-camphor was calculated to be 4.2 kcal mol^{-1}. This value is very small, compared with the activation energy (13 kcal mol^{-1}) of singlet oxygen atom ^{1}D insertions into hydrocarbons,[15] suggesting the important role of the heme-protein in the substrate oxidation. It is clear that this revised ultimate species gives a straightforward explanation of the major results in the oxidation of hydrocarbons by cytochrome P-450, where the stereochemistry is retained and the isotope effect is small. The formation process of the ultimate species may be followed by the same experiment which support the formation of compound **I** except in the final process, where the conventional mechanism postulates the formation of the compound **I** and a free water molecule, but the new mechanism considers the oxygenated hydrocarbon and a water molecule which binds to the Fe atom of the heme. Provided that the interaction between a substrate and the distal oxygen in the heme pocket is weak enough that the interaction is not maintained throughout the substrate oxidation process, the conventional ultimate species (compound **I**) may be produced. It is well known that compound **I** is produced in the substrate oxidation process by peroxidase where the isotope effect is clearly observed.[16]

The mechanism shown in Fig. 1 is clearly explained by the existence of these two ultimate species. The two kinds of the ultimate species generated in the substrate oxidation process by cytochrome P-450 explains in a straightforward manner the reasons why a variety of products have been reported in the literature.[1,8]

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