Heme Degradation

Porphyrin Traps Its Terminator! Concerted and Stepwise Porphyrin Degradation Mechanisms Induced by Heme-Oxygenase and Cytochrome P450**

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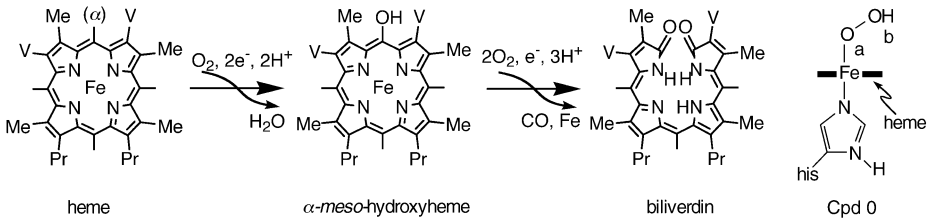
Dedicated to Don Truhlar on the occasion of his 60th birthday

The enzyme heme-oxygenase (HO) traps heme species and induces their degradation.[1] The degradation process (Scheme 1) is thought to serve three key functions: a) maintenance of iron homeostasis, b) creation of CO in the brain that acts as a neurotransmitter akin to NO, and c) generation of products (e.g., biliverdin) that play a role in the defense mechanism against oxidative stress. Structural studies by Poulos and co-workers[2] of the human form (hHO-1) of the enzyme-heme complex show that the trapped heme undergoes ligation by an imidazole ring of a histidine side chain and a water molecule. Upon dioxygen uptake, followed by two-electron reduction and protonation, which activate the O=O bond, the heme unit undergoes hydroxylation at the α-*meso* position and subsequently opens up, releasing iron and carbon monoxide.

Decisive mechanistic studies by Wilks et al.[3] and Davydov et al.,[4] showed that the active species of the enzyme is the ferric hydroperoxide, Compound 0 (Cpd 0 in Scheme 1), which is the competent reaction intermediate that leads to biliverdin is the α-*meso* hydroxyheme species (Scheme 1). Furthermore, Wilks et al.[3] and Ortiz de Montellano,[1a] considered two viable mechanisms for the hydroxylation of the *meso* carbon atom. The first one involves homolytic O–O bond dissociation of Cpd 0, followed by OH· attack at the *meso* carbon atom, while the second one is an acid-catalyzed concerted electrophilic attack of the OOH moiety on the *meso* carbon atom, that is, a formal "OH^+" transfer. Ortiz de Montellano[1a] and Wilks et al.[3] preferred the electrophilic mechanism over the homolytic alternative primarily because a free OH· radical would be expected to attack the heme in a nonselective manner, unlike the regioselective reaction of Cpd 0 in HO. However, as pointed out by Poulos and co-workers,[2] there are no specific acid or basic residues close enough to the FeOOH moiety to take part directly in the reaction. One wonders, therefore, how an electrophilic mechanism can be acid catalyzed. Could there, alternatively, be a homolytic scenario with a regioselective OH· attack on the *meso* position?

Another intriguing issue is the reactivity of Cpd 0 in HO, which is unusual for a heme enzyme. In most other heme enzymes the primary active form is the high-valent iron–oxo species, Cpd I,[2,5] whereas Cpd 0 is either inactive or reacts by default in the absence of Cpd I.[6] Indeed, theoretical calculations show that Cpd 0 in the enzyme P450 is a poor electrophilic species.[7] Could the change of the proximal ligand from thiolate (in P450) to imidazole (in HO) be really so dramatic to endow Cpd 0 with appreciable reactivity? Eventual resolution of these mechanistic questions may require the assistance of theory. To this end, and with an eye on promoting interplay between experiment and theory, it was deemed necessary to study computationally the mechanism of the initial step of heme activation by Cpd 0 of HO, by comparison to the putative process in P450.



Scheme 1. Degradation of heme catalyzed by heme-oxygenase. V = vinyl.

Following established procedures,[7a] we used hybrid density functional B3LYP calculations; the double zeta LACVP(Fe)/6-31G(H,C,N,O,S) basis set (hereafter, LACVP) was employed for geometry optimization, followed by single-point calculations with the LACVP** (Fe)/6-31G**(H,C,N,O,S) basis set (hereafter, LACVP**).[8] The two model systems involve an imidazole (ImH) ligand for HO and an HS^- ligand for P450; in both cases iron porphine represented the heme. For the reactions of Cpd 0(HO), we

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[**] This research is supported by an Israel Science Foundation (ISF) grant. Discussions with P. R. Ortiz de Montellano, T. L. Poulos, S. Sligar, J. D. Dawson, and W.-D. Woggon are acknowledged.

Supporting information for this article (16 Tables and 28 Figures) is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200352943

also explored the effects of the environment on the reaction. Following the findings of La Mar and co-workers[9] for buried water molecules, we calculated the reaction in the presence of two water molecules hydrogen bonded to the proximal oxygen atom of Cpd 0 (O_a, Scheme 1). As a limiting situation, we studied the reaction in the presence of a proton source, $H_2O{\cdot}H_3O^+$, hydrogen bonded to the proximal oxygen atom. The effect of pocket polarity was mimicked by a dielectric constant, ε, of 5.7.[7a] Finally, the structure of the heme complex in HO,[2] reveals a significant steric crowding above the would-be site of the FeOOH moiety. Indeed, the electron paramagnetic resonance (EPR) spectrum of Cpd 0 of HO[4] suggests that the FeOOH moiety possesses an acute Fe-O-O angle, unlike analogous species in other systems. The most likely candidate for this steric pressure is the methylene group of the residue Gly143, which points toward the FeOO moiety, and was found by NMR spectroscopy to move 0.5 Å towards the iron center and cause bending of the Fe-CN unit in the corresponding Fe^{III} cyanide complex.[9a] We mimicked the effect of this methylene group by placing a CH_4 molecule initially at the position coresponding to Gly143 in the crystal structure, and subsequently letting it slide 0.5 Å closer to the heme. The data generated by the calculations are summarized in the Supporting Information, while herein we present the key data.

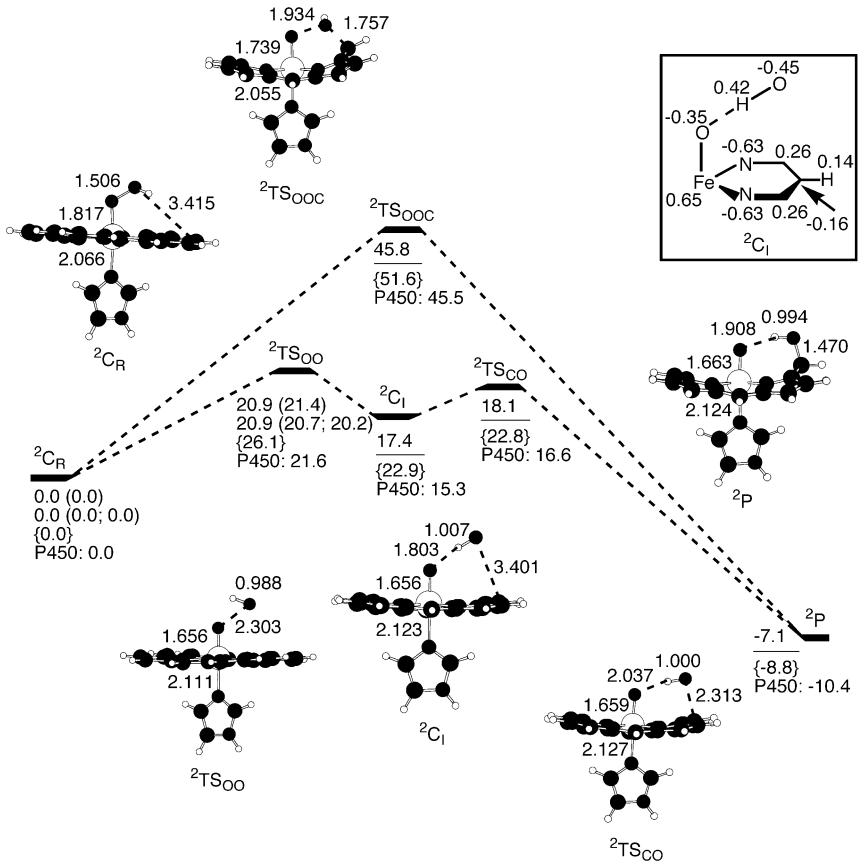

Figure 1. Energy profiles for a concerted and a stepwise reaction mechanism for the hydroxylation of the *meso* carbon atom of porphine by Cpd 0 of heme-oxygenase model (^2C_R) versus a P450 model. The energies are reported, relative to ^2C_R, as follows: the first line gives the LACVP energies in the gas-phase (with hydrogen bond to the proximal oxygen atom by two H_2O molecules in $\varepsilon = 5.7$). The second line relists the LACVP energies in the gas phase (with CH_4 at the distance of Gly143 in the crystal structure; with CH_4 translated 0.5 Å closer to the heme iron atom). The third line shows the LACVP** energies in braces. The fourth line shows the corresponding relative energies (relative to ^2C_R of P450) for the reaction with Cpd 0(P450). ε is a dielectric constant. The hydrogen-bond distance with two H_2O molecules is 2.0 Å. The C···O distance of the CH_4 molecule from the hydroperoxo oxygen atom is 3.85 Å.

Figure 1 displays the two located mechanisms of *meso* hydroxylation by Cpd 0(HO). In the first mechanism the hydroxy group is transferred to the *meso* position in a concerted reaction, akin to the proposed electrophilic mechanism.[1a,b,3] However, the computed spin densities and charge distribution of $^2TS_{OOC}$, show that there are virtually no charge and no spin on the migrating OH group, in line with its synchronous bonding situation. The second mechanism is a stepwise one, initiated by O–O homolysis followed by OH· attack on the *meso* position of porphine. Note that the barrier for the second step is extremely small (< 1 kcal mol^{-1}), as would be expected for an OH· attack on a double bond. The O–OH bond energy was also calculated and found to be 28.8 kcal mol^{-1} (32.3 kcal mol^{-1} with LACVP**), significantly higher than the energy of the intermediate, ^2C_I. As such, the ^2C_I intermediate is bound by approximately 11 kcal mol^{-1} (10 kcal mol^{-1} with LACVP**), and the energy cost of OH· escape would be much higher than the barrier for OH· attack. Thus, although formally part of a stepwise mechanism, once formed, the OH· radical will immediately attack the *meso* position.

To understand the origins of the binding of the OH· radical in the ^2C_I intermediate, we verified that a gradual increase of the O···O separation indeed takes this intermediate to the dissociation limit (Cpd II + OH·). The inset in Figure 1 shows that the OH· radical in ^2C_I is bound by hydrogen bonding and electrostatic stabilization to the Fe^{IV}-oxo group and to the porphyrin nitrogen atoms; it is fixed in space above the *meso* position. Thus, the OH· radical is trapped by the heme-oxo moiety by noncovalent interactions that lower the homolytic-cleavage barrier, the energy of the intermediate, and the barrier for the subsequent regioselective addition to the *meso* position. Comparison of the two mechanisms (Figure 1) shows that the truly concerted mechanism is highly unfavorable compared with the homolytic mechanism, most likely because of the poor bonding of the migrating hydroxyl radical (note the long O···O and O···C separations in Figure 1) and the severe heme deformation.

The homolytic mechanism involves a doublet triradicaloid species, since it results in a Fe^{IV} (FeO) unit that has two

triplet-coupled electrons, and a radical in the porphine moiety, in an orbital of a_{2u} origin. To rule out lower barriers in other spin states, we also calculated the corresponding quartet and sextet surfaces. The resulting $^{6,4}TS_{OO}$ species were found to be higher in energy than the corresponding $^{2}TS_{OO}$ species (see Supporting Information). Thus, the first step of the heme activation is dominated by a single spin state.

The data in Figure 1 (see more details in the Supporting Information) shows that the hydrogen bonding of two water molecules to the proximal oxygen atom (O_a) raises the barrier. The resulting hydrogen bonding effect is in line with experimental evidence, which indicates that the chain of water molecules, which was found buried in the enzyme, serves to stabilize Cpd 0(HO).[9] Apparently, this stabilization is greater than the corresponding one in the transition state (TS).

Applying a steric pressure to the FeOOH moiety, by using the CH_4 unit pointing towards the proximal oxygen atom (as a mimic of the effect of the Gly143 methylene group), did not change the barriers significantly. However, the actual steric pressure in the enzyme may be more severe than our simplified modeling suggests.[2] A series of calculations showed that the energy of Cpd 0 is raised by approximately 4–8 kcal mol^{-1} when the Fe-O-O angle is reduced from 115 to 100–95°. If such a pressure[2,4,9] indeed exists in the pocket, it will lower the barriers for both concerted and homolytic mechanisms. Thus, in any event the homolytic mechanism would prevail.

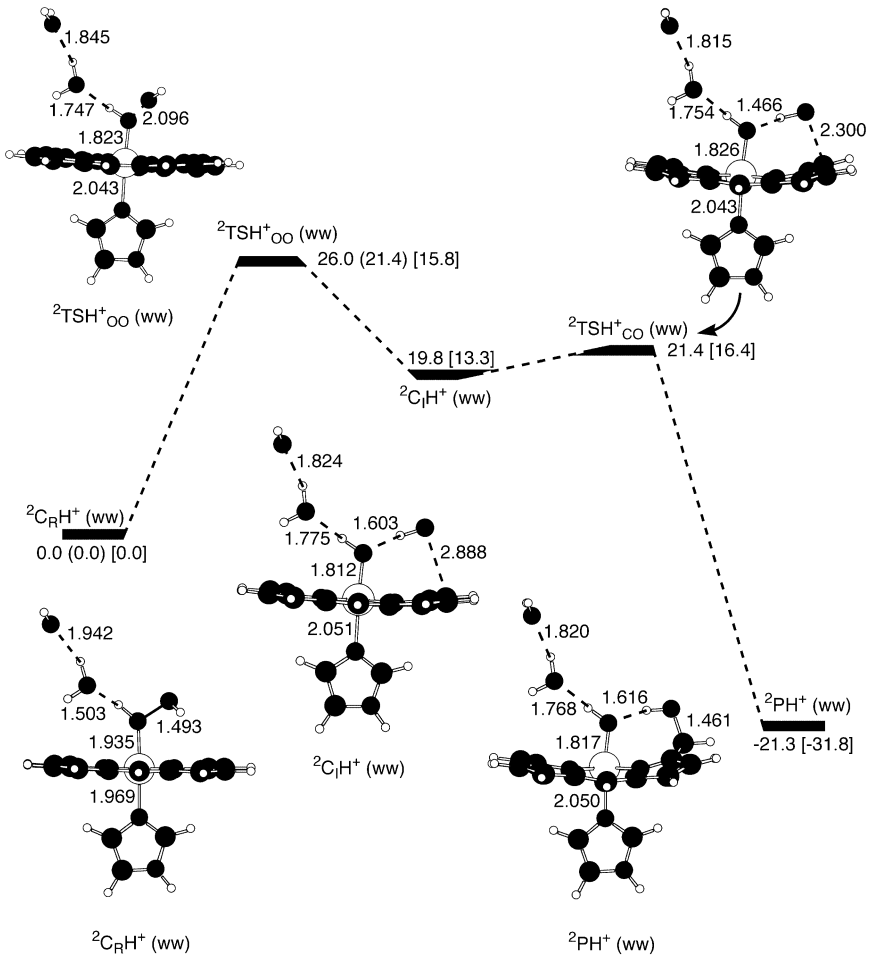

Figure 2. The mechanism of *meso* carbon atom hydroxylation initiated from the protonated Cpd 0 species ($[Fe^{III}(H_2O_2)]^+$) which is coordinated by two water molecules, ($^{2}C_RH^+$ (ww)). Energy values correspond to LACVP. The energy values in parentheses include the effect of a dielectric constant ($\varepsilon = 5.7$), while the values in square brackets correspond to the reaction of the bare $[Fe^{III}(H_2O_2)]^+$ complex ($^{2}C_RH^+$).

To explore an acid–base assisted mechanism, we used an $H_2O{\cdot}H_3O^+$ cluster coordinated to the proximal oxygen atom (O_a) by a hydrogen bond donated by the water molecule (see Supporting Information). Optimizing the structure of Cpd 0 in the presence of this cluster resulted in a spontaneous proton transfer, in a Grotthuss-type mechanism,[10] to give the iron hydrogen peroxide complex, $[Fe^{III}(H_2O_2)]^+$, coordinated by two water molecules and indicated in Figure 2 as $^{2}C_RH^+$(ww). The mechanism remains, however, stepwise with O–O homolysis leading to the intermediate $^{2}C_IH^+$(ww) in which the OH$^{\cdot}$ radical (spin density 0.91) is trapped by the heme hydroxo complex. Subsequently, the radical collapses by attacking the *meso* carbon atom. The barrier for the acid-catalyzed process, in the presence of a dielectric medium ($\varepsilon = 5.7$) is comparable to that of the pristine Cpd 0 in Figure 1 ($\Delta E^{\neq} = 21.4$ and 21.4 kcal mol^{-1}; $\Delta G^{\neq}(298\ K) = 20.8$ (16.2 with $\varepsilon = 5.7$) for the proton-assisted mechanism versus 18.5 kcal mol^{-1} for the pristine). Removal of the two water molecules and repeating the process, starting with the $[Fe^{III}(H_2O_2)]^+$ complex, lead to the same homolytic mechanism, and a significantly lower barrier (Figure 2). The lower barrier in the absence of the water molecules may not be physically significant since it is not apparent that the water molecules would detach from the $^{2}C_RH^+$(ww) complex after protonation. However, the homolytic mechanism prevails, and the remaining question is whether the reaction is proton assisted or not. This question may be settled by experimental means (such as equilibrium or kinetic solvent isotope effect) or by QM/MM calculations within the native protein. Should the proton-assisted mechanism prove to be the path taken by HO, the QM/MM calculations would be expected to reveal the reason why Cpd 0 is protonated only on the proximal oxygen atom, rather than forming Cpd I by distal protonation.

The homolytic mechanism can account for some key experimental findings.[1a,b,3] Firstly, since the OH$^{\cdot}$ radical is locked by the heme moiety, the OH$^{\cdot}$ species will not behave as a nonselective free radical, but on the contrary, its subsequent reaction will be regioselective. Secondly, since the OH$^{\cdot}$ radical is held by polar interactions, there will be a competition between the heme and the amino acid residues on the OH$^{\cdot}$ species. Thus, in some cases, a small fraction of the OH$^{\cdot}$ radicals will be trapped by the surrounding protein and will oxidize one of these residues or abstract a hydrogen atom from it, which generates a protein radical along with a Cpd II

heme species (for example, ImH-PorFeO). These are indeed the experimental observations.[1a,b,3]

Finally, comparison of the data in Figure 1 to that of Cpd 0 of P450 shows very small quantitative differences; the LACVP barrier for the concerted reaction (45.5 kcal mol^{-1}) is virtually identical to that of HO (see Figure 1) while the barrier for homolytic cleavage (21.6 kcal mol^{-1}) is marginally higher than that for Cpd 0 of HO. Clearly, there does not seem to be anything unique about Cpd 0(HO) as such. Indeed, as a rule, heme enzymes have a protonation machinery that protonates the distal oxygen atom and converts Cpd 0 rapidly[5,10] into Cpd I, thereby avoiding HO-type activity. HO appears to lack such protonation machinery.[2] Thus, if our calculations are correct, it might be expected that upon mutation of the amino acid residues that convert Cpd 0(P450) into Cpd I(P450), the enzyme should exhibit some HO activity. In such a case, if some OH$^{\bullet}$ radical escapes the porphyrin cage, either the radical or Cpd II,[11a] may be responsible for the observation of residual epoxidation reactivity in some of the P450 mutants (for example, the T252A mutant in P450$_{cam}$).[6,11]

Note added in proof: A recent mechanistic study[12] shows solvent kinetic isotope effect (KIE) and C_{meso}–D KIE which point to a proton-catalyzed concerted mechanism. KIE calculations carried out by us will be reported elsewhere.

Received: September 24, 2003 [Z52943]

Keywords: density functional calculations · enzyme catalysis · enzyme models · heme proteins · oxygenation

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[11] a) Cpd II-epoxidized olefins: J. T. Groves, Z. Gross, M. K. Stern, *Inorg. Chem.* **1994**, *33*, 5065; b) O–O homolysis of Cpd 0(P450) into Cpd II + OH$^{\bullet}$ is preferred over heterolysis to Cpd I + OH^{-}, because the electron affinity of OH is much smaller than that of Cpd II (see, F. Ogliaro, S. P. de Visser, S. Shaik, *J. Inorg. Biochem.* **2002**, *91*, 554). TD-DFT calculation (see Supporting Information) shows that the heterolytic state is 38.5 kcal mol^{-1} higher than the homolytic state at the geometry of the $^{2}C_{I}$ intermediate in Figure 1. Hydrogen-bonding and polarity effects will reduce the energy difference between the dissociation modes, but may not reverse it, at least not at the intermediate geometry. The heterolytic species will be more stable at the dissociation limit.

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