

On the Mechanism of Cytochrome P450-Catalyzed Oxidations: Reaction of a New Enzyme Model with a Radical Clock

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'625 fsec' dedicated to Bernd Giese on the occasion of his 65th birthday

Abstract: *trans*-2-Phenyl-methylcyclopropane was oxidized using PhIO and a P450 enzyme model carrying a SO_3^- ligand coordinating to iron. Analysis of the product distribution revealed a ratio of 9:1 of the non-rearranged cyclopropyl methanol **18** over 1,1-allyl phenyl methanol **16**. Given the rate of rearrangement $k = 1.8 \cdot 10^{11} \text{ sec}^{-1}$ of the phenyl cyclopropyl methyl radical **14** in solution, the life time of the intermediate radical cluster **I**_{C-H} can be calculated as 625 fsec. The hydroxylation proceeds by concerted non-synchronous 'O'-insertion into the C-H bond of the methyl group.

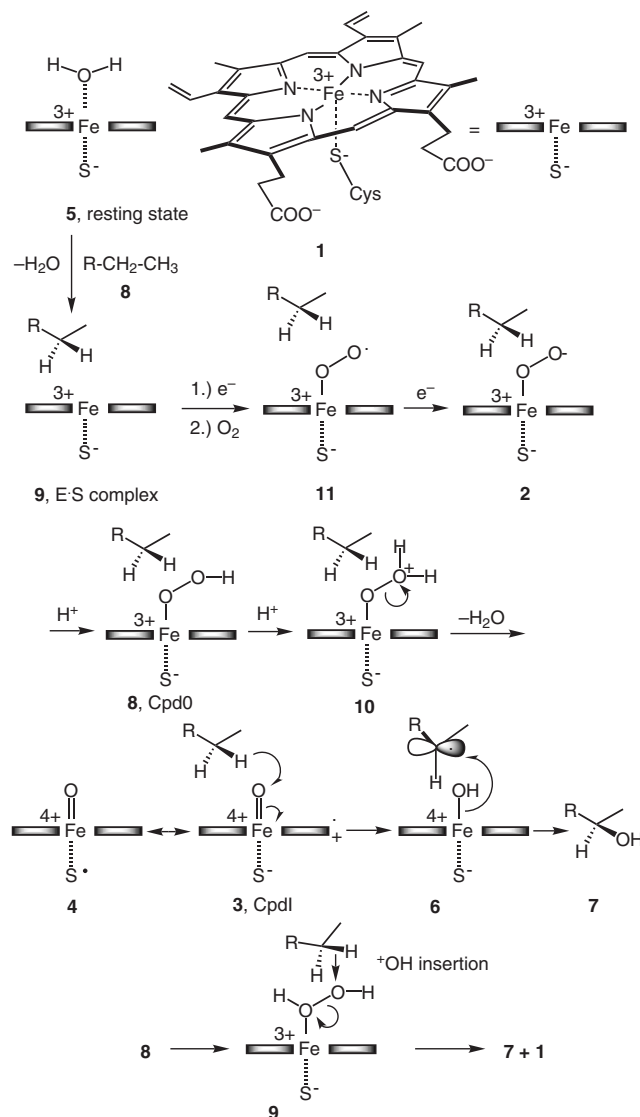
Key words: catalysis, enzyme models, heme-thiolate proteins, iron porphyrins, radical clock, radical rearrangement

Cytochrome P450-catalyzed reactions¹ are one of nature's most sophisticated methods to oxidize endogenous compounds in prokaryotic and eukaryotic cells. P450s can be isolated from bacteria,² plants³ and different tissues of mammals,⁴ where numerous isozymes participate highly specifically in the metabolism of endogenous compounds as diverse as steroids, fatty acids and alkaloids. The same reactivity is also common to those P450s acting on xenobiotics, e.g. in liver microsomes. The membrane-bound hepatic P450s generally accept a broad spectrum of substrates, which is understood as a method of detoxification in order to render lipophilic compounds water soluble and excretable.

A common feature of the P450 molecular structure is an iron(III) protoporphyrin IX cofactor **1** in the active site carrying a unique deprotonated cysteine ligand (S^-) coordinating to iron (Scheme 1). This thiolate ligand is hydrogen-bonded to three amino acids of the protein backbone fine-tuning the redoxpotential, spin state and reactivity of the cofactor. The principal reactivity of the system is quite diverse including double bond epoxidations, aromatic hydroxylations, oxidations at and adjacent to hetero atoms and most interestingly the hydroxylation of non activated positions.^{1,2} Experiments with labelled $^{18}\text{O}_2$ have shown that one of the oxygen atoms is incorporated into the substrates and the other is reduced to water (monooxygenase reaction).

Our understanding of the reaction pathways of these heme-thiolate proteins^{1,5,6} (Scheme 1) rests to a large ex-

tent on investigations of cytochrome P450_{cam}, a cytosolic protein from *Pseudomonas putida*⁷ which catalyzes the hydroxylation of camphor at the 5-*exo* position. Numerous mechanistic studies of P450_{cam}, other P450s¹ and investigation of structurally remote model compounds established a sequence of events in the catalytic cycle of cytochrome P450 which is well defined up to intermediate **2**^{8,9} (Scheme 1).



Scheme 1 Catalytic cycle of cytochrome P450

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The consecutive steps were deduced from studies using point mutated enzymes, by comparing spectroscopic parameters of enzymes/model compounds and measuring isotope effects. In agreement with data from different sources is the formation of a high-valent iron(IV) oxo porphyrin radical cation intermediate **3** formed by protonation of the peroxo complex **2** and subsequent O–O bond cleavage. By analogy with model studies,¹ this intermediate is believed to have the electronic structure **3**¹⁰ rather than **4**.¹¹ For the past two decades, the mechanism of P450-catalyzed hydroxylations was described as a two-step reaction of **3**¹² with the substrate: hydrogen abstraction by the iron-oxo of **3** gives a substrate alkyl radical that is immediately trapped by HO from the iron, with **6**, yielding the hydroxylated substrate **7** and the water-free form of the enzyme's resting state.

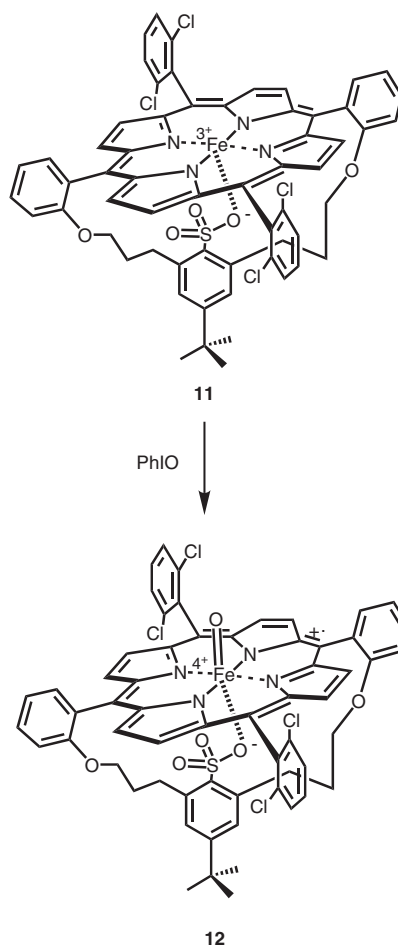
Various DFT calculation addressing the reactivity of Cpdl revealed very different activation energies for C–H activation.^{13,14} Further, a two-state reactivity (TSR) of Cpdl was proposed suggesting two competing pathways on the reaction coordinate, i) a two step, high-spin route and ii) a concerted reaction on the low-spin surface.¹⁵ The former formalism would allow for a short lived radical intermediate (see **6** in Scheme 1) and hence corresponds to the rebound mechanism. Partitioning between both routes seems to depend on substrates and the protein.

Finally, through experiments with 'ultra fast' radical clocks that revealed radical lifetimes in the time frame of vibrations (ca 100 fsec) and complementary investigations of mutated P450s it has been inferred that **8** (Cpd0), a precursor of Cpdl (Scheme 1), or its protonated form **9** are competitive oxidants reacting by ⁺OH insertion into C–H bonds.¹⁶ Because of this controversy regarding the significance of Cpdl and its possible dual reactivity we decided to employ a synthetic P450 enzyme mimic for a radical clock reaction.

Recently we have synthesized some new P450 enzyme mimics which carry a SO₃[−] ligand coordinating to iron (see **11** in Scheme 2).¹⁷ The replacement of the natural thiolate ligand for SO₃[−] was envisaged to mimic electronically the proximal coordination sphere which according to X-ray analysis of e.g. P450_{cam} contains a thiolate H-bonded to three amino acids of the protein backbone.¹⁸ This sophisticated arrangement fine-tunes the redox potential of the cofactor and favours spin density in the porphyrin MO rather than at sulfur.¹⁹ The complex **11** indeed shows E₀ = −134 mV, a redox potential very similar to the resting state of P450_{cam}, and displayed good reactivity towards olefin epoxidation with turnovers up to 1800.¹⁷ DFT calculations further revealed that one oxygen atom of the SO₃[−] group coordinates to iron and that **12**, the Cpdl equivalent of **11** shows reaction profiles for allylic hydroxylation indistinguishable from the corresponding thiolate coordinating complex and only minor differences in the reaction pathway for epoxidation of propene.¹⁷

To focus on the reactivity of the O=Fe(IV) porphyrin radical cation and circumventing the problem of alternative

oxidants¹⁶ we used PhIO which in a 'shunt pathway' reacts with **11** to generate **12**, an equivalent of Cpdl (see **3** in Scheme 1) which could be identified through UV and EPR spectroscopy. By using a P450 mimic under these conditions any cationic pathways originating from **8** or **9** can be excluded. As a radical clock we chose substrate **13** for which the corresponding radical **14** is known to rearrange²⁰ with $k_{\text{rearr.}} = 1.8 \cdot 10^{11} \text{ s}^{-1}$ to yield **15** and subsequently the alcohol **16** (Scheme 3). Further, we reasoned that $k_{\text{rearr.}}$ of **14**, determined in organic solvents, is comparable to our reaction conditions, i.e. the catalyst does not display interactions with the substrate that could change the rate of rearrangement.

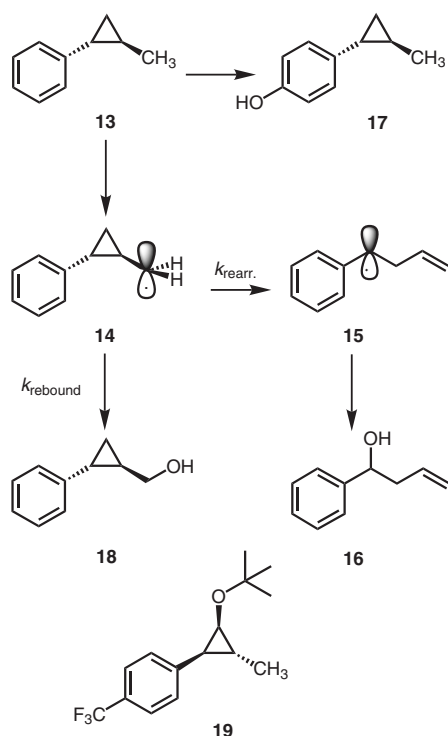


Scheme 2 P450 enzyme model for catalytic reactions

It is important to note that the effective rate of ring opening of the radical **14** in the active site of a P450 enzyme might be smaller due to steric constraints originating from substrate protein interactions.^{21,22}

Under carefully controlled conditions the P450 mimic reaction employing **13** gave the phenol **17** (Scheme 3) as the main product and the alcohols **18** and **16** (Scheme 3) reproducibly in the ratio 9:1 (four independent experiments, see experimental section). Accordingly $k_{\text{rebound}} = 1.6 \cdot 10^{12} \text{ s}^{-1}$ and the life time, $\tau = 625 \text{ fsec}$, of the intermediate 'radical' can be calculated. These values are identical to those

reported for the product distribution determined on incubation of **13** with liver microsomes from barbiturate-treated rats.²¹ In contrast τ is longer for a norcaranyl radical ($\tau = 16\text{--}52$ psec; various P450 enzymes)²³ and is even longer for an allyl radical that appears to be an intermediate in the stereospecific hydroxylation of a chiral methyl group of geraniol catalyzed by a P450 hydroxylase from plants.²⁴ Due the stability of these radicals and their electron-donating character this trend is expected.



Scheme 3 P450-catalyzed oxidation of radical clock **13**

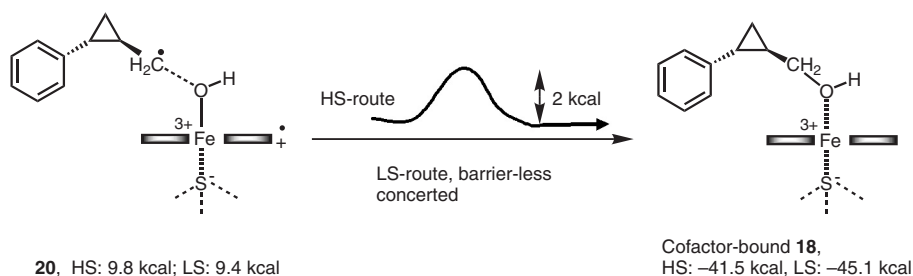
The formation of the rearranged alcohol **16** proves that this reaction does not only proceed by a concerted 'O' insertion into the C–H bond. Further, the apparent life time of the intermediate is well above 170 fs, the 'life time' of a transition state, and is also ca 6 times higher than the corresponding value calculated from product distribution of the P450 2B1 catalyzed oxidation of **19**.¹⁶ The discrete, albeit short-lived intermediate is certainly not a 'free' carbon radical but rather a non separated ensemble, i.e. a radical cluster, see below.

Since there have been comments on the validity of radical clock experiments²⁵ it seems useful to discuss our result with respect to data from recently published advanced DFT calculations of the reaction of **13** with CpdI.²⁶ This TSR scenario, in principle, has two distinct pathways; one on the high-spin route (HS) the other, on the low-spin route (LS), usually on a lower energy surface. However, the calculated high-spin and low-spin routes gave the same energy levels well within <1 kcal, i.e. within experimental error, for all (but one) transition states and intermediates.²⁶ Thus, apparently there is no difference between the HS- and LS-energy states except for the transformation of the key intermediate **20** that can either yield the 'rebound' product **18** by H' abstraction (HS route) or by concerted 'O' insertion into the C–H bond (LS route). For these two pathways it has been computed that formation of the cofactor adduct of **18** happens to be quasi barrier-less on the LS-route while the HS-pathway encounters a transition state roughly 2 kcal above the intermediate **20** (Scheme 4).

Thus, these DFT calculations suggest a bias in favor of the non-synchronous, concerted oxygen insertion into the C–H bond leading finally to **18**. On the other hand the calculations also revealed that the HS-/LS-intermediate (see **20** in Scheme 4) has only an extremely small activation energy, i.e. 0.2 kcal, to rearrange en route to the open-chain radical which is subsequently trapped in a 'rebound' step to yield the cofactor-bound product **16**.

Figure 1 displays our view of the sequence of events which may be valid both for the model reaction and the enzymatic hydroxylation of **13** taking into account the reported relative energies of all states²⁶ and the 'fact' that their HS- and LS-forms are energetically not well separated.

Accordingly C–H activation of the substrate complex CpdI-**13** passes through the transition state $\text{TS}_{\text{C-H}}$ leading to intermediate $\text{I}_{\text{C-H}}$. Energy levels of the high-spin form (HS) and the low-spin form (LS) of both $\text{TS}_{\text{C-H}}$ and $\text{I}_{\text{C-H}}$ are indistinguishable, and hence LS- and HS-routes are identical. For $\text{I}_{\text{C-H}}$ we favour a 'side-on' approach of the oxygen atom to the C–H bond rather than a linear H-abstraction²⁶ yielding **20** (Scheme 4) since this would allow for both concerted 'O' insertion and H' abstraction. The C–H of the methyl group is elongated creating spin density at CH_2 ; one of the cyclopropyl C–C bonds may be already weakened. Hence $\text{I}_{\text{C-H}}$ is prone either to rear-



Scheme 4 The relative orientation of molecules and the relative energies for the high-spin and low-spin states are taken from ref.²⁶ Dotted lines at sulfur indicate that hydrogen bonding has been included in DFT calculations.

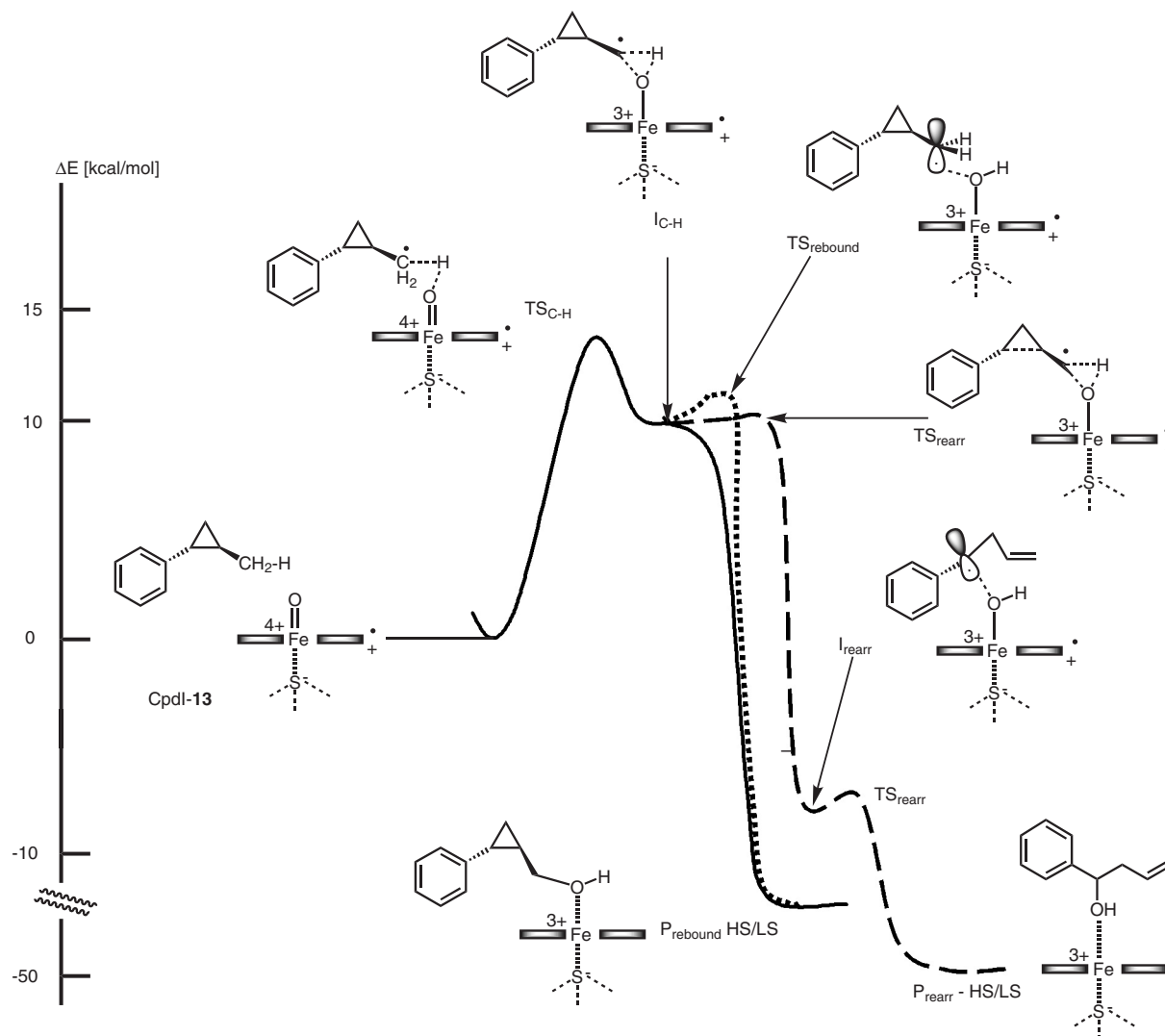


Figure 1 Reaction pathways in the oxidation of **13** by cytochrome P450 (CpdI). The straight and the dashed curves refer to both the high-spin (HS) and the low-spin (LS) route; the dotted curve is a HS pathway. The ZPE-corrected relative energies (ΔE) were taken from ref.²⁶

rearrangement or direct hydroxylation. The activation energy of ca 2 kcal for the HS-rebound process exclude this pathway to be significant with respect to rearrangement ($\Delta H^\ddagger = 0.2$ kcal), i.e. a ratio of 9:1 of non-rearranged **18** to rearranged product **16** exclude any participation of the high-spin route leading to **18** since this would lead to a preference of the rearranged product which is not observed. On the other hand the quasi barrier-less, concerted low-spin pathway to **P_{rebound}** can compete with rearrangement yielding **P_{rearr}** via **TS_{rearr}** and **I_{rearr}**.

Since the high-spin and the low-spin form of the crucial intermediate **I_{C-H}** are equal in energy the observed product distribution **18/16** = 9:1 does not reflect the yield of HS- **I_{C-H}** over LS- **I_{C-H}** as stated previously²⁶ but is rather a matter of the respective activation energies of the forward reactions. A difference of $\Delta H^\ddagger = 1$ –2 kcal between the two routes leading to **18** and **16**, respectively, may be sufficient to account for the observed product ratio. Note that the rearrangement of a cyclopropyl methyl radical has an activation energy of about 1 kcal in solution.

In conclusion, our experiments with a P450 enzyme CpdI-model **12** and the radical clock **13** in organic solvents, excluding possible substrate protein interactions, account for a very short lifetime, $\tau = 625$ fs, of the intermediate **I_{C-H}** which certainly is not a ‘free’ radical but rather a cluster containing a CH_2 group carrying spin density coupled to the spin system of the $\cdots\text{H}\cdots\text{O}-\text{Fe}(\text{III})$ porphyrin radical cation. Departing from **I_{C-H}**, formation of the so called rebound product **18** is effectively concerted, whereas the rearranged product is obtained via either the high-spin or the low-spin pathway.

General Procedure for Catalytic Oxidations

In a 1.5 mL glass vial *trans*-2-phenyl-methylcyclopropane (**13**; 3.3 mg, 25.0 μmol >99.5% GC-pure) was dissolved under Ar in previously degassed and anhyd CH_2Cl_2 (250 μL). Iron(III) porphyrin **11** (0.75 mg, 0.65 μmol , 2.5 mol%) and subsequently PhIO (5.5 mg 25.0 μmol , 1 equiv) were added under Ar. The reaction mixture was then stirred vigorously for 10 min at r.t. After this time an aliquot was taken and centrifuged in order to precipitate the undissolved PhIO. The supernatant was directly analyzed by GC-FID (Focus

Finnigan GC-FID/SUPELCOWAX 10/gradient 100–260 °C). Compounds were identified by distinct retention times: 3.9 min (**13**), 10.3 min (**16**), 13.0 min (**18**), 14.9 min (**17**); and by co-injection with authentic material; >99% GC-pure **13**, **16**, **17** and **18** were prepared according to slightly modified published procedures.²⁷ The following product ratio was obtained: **17**/(**16** + **18**) = 2:1 and **18**/**16** = 9:1. Oxidation experiments were repeated four times and standard deviation of $\pm 5\%$ was determined for product ratios. Further, control experiments showed that **18** does not rearrange to **16** in the presence of PhIO.²⁷

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