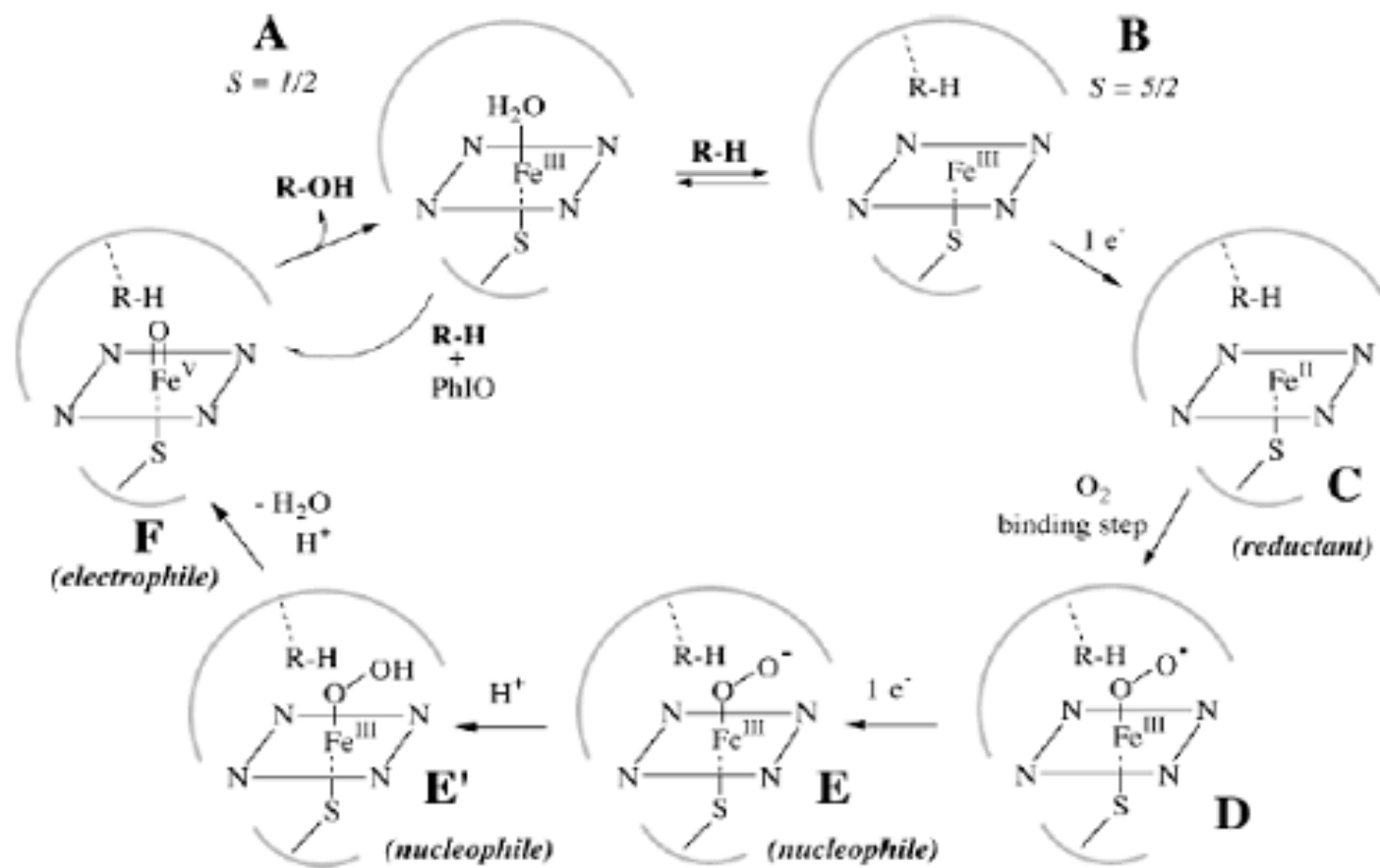


Cytochrome P450 Mechanism
Major Types of P450 oxidation
Reactions

Med Chem 527 Winter 2013
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The P450 Cycle



Main reaction catalyzed by P450 enzymes is mono-oxygenation resulting in the net insertion of one atom of oxygen from molecular oxygen into product

Cytochrome P450

Camphor is substrate for P450cam

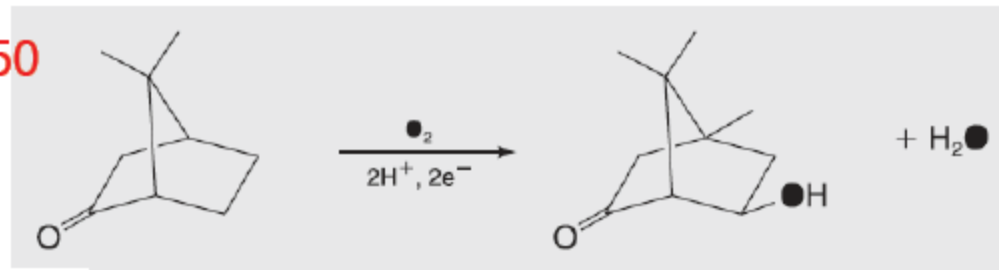
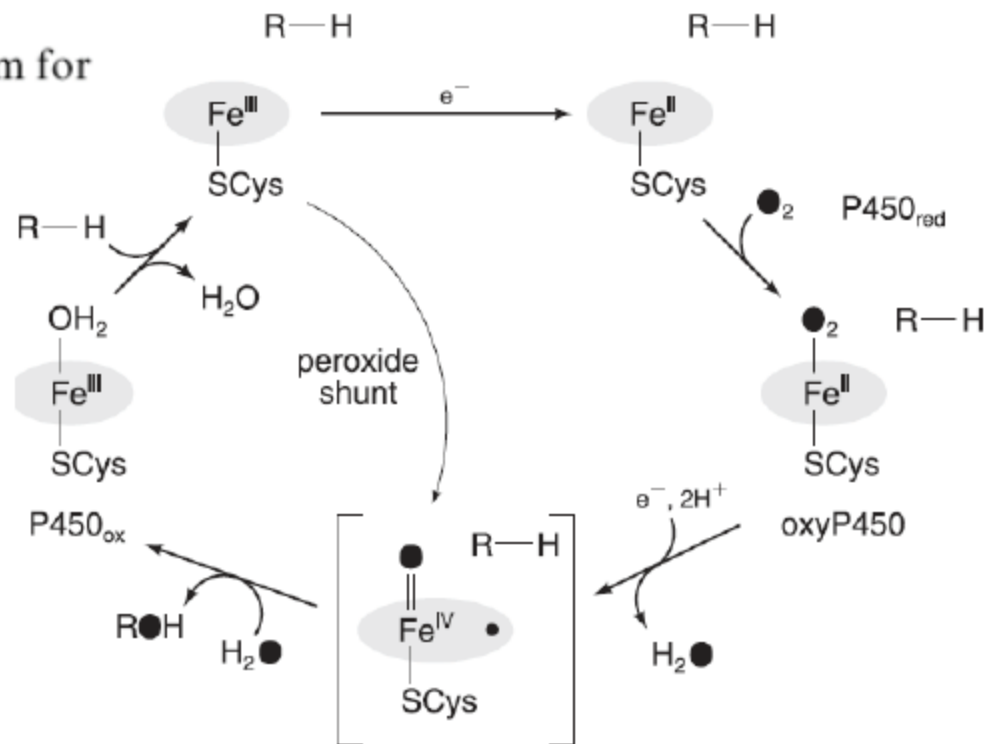


Fig. XI.5.3.
Catalytic mechanism for cytochrome P450.



Questions to answer: What is the mechanism of oxygen insertion? What are the intermediates generated? Is the reaction concerted or stepwise? What is the nature of the reactive oxidant? Is a single oxidant species involved in this reaction or multiple species?

General References:

Guengerich, F. P. Common and Uncommon Cytochrome P450 reactions Related to Metabolism and Chemical Toxicity. *Chem. Res. Tox.* (2001) 14: 611-650.

Meunier, B., De Visser, S. and Shaik, S. Mechanism of Oxidation Reactions Catalyzed by Cytochrome P450 enzymes *Chem. Revs.* (2004) 104: 3947-3980.

Hlavica, P. Models and Mechanisms of O-O Bond Activation by Cytochrome P450: A Critical Assessment of the Potential Role of Multiple Active Intermediates in Oxidative Catalysis. *Eur. J. Biochem.* (2004) 271: 4335-4360.

Groves JT. High-valent iron in chemical and biological oxidations. *J. in org. chem.* (2006) 100, 434-447.

Emre M. Isina and F. Peter Guengerich Complex reactions catalyzed by cytochrome P450 enzymes *Biochimica et biophysica Acta* 2007 vol 1770, 314-329.

Studying the chemical mechanisms of P450 reactions:

Parameters of mechanism:

Stereospecificity: The retention, inversion or loss of stereoselectivity. A reaction is stereospecific if stereoisomeric reactants give stereoisomeric products.

Stereoselectivity: Selection among several substrates. (e.g. *d* vs. *l*, pro-R vs. pro-S, etc) or among several products (enantiomers, diastereomers, geometric isomers)

Regioselectivity (or chemoselectivity): Relative reactivity towards different functional groups in the same molecule or different molecules.

Intermediates: True intermediates in a chemical reaction always give the same products regardless of how the intermediate species is generated. They can sometimes be trapped and stabilized for examination. (e.g. spin traps: intermolecular traps or radical clock groups: intramolecular traps)

Kinetic Isotope effects: Ratio of two rate constants for the identical reaction of two compounds differing only in isotopic substitution. Deuterium isotope effects arise when enzyme discriminates between H and D based on differential reactivity of C-H vs. C-D.

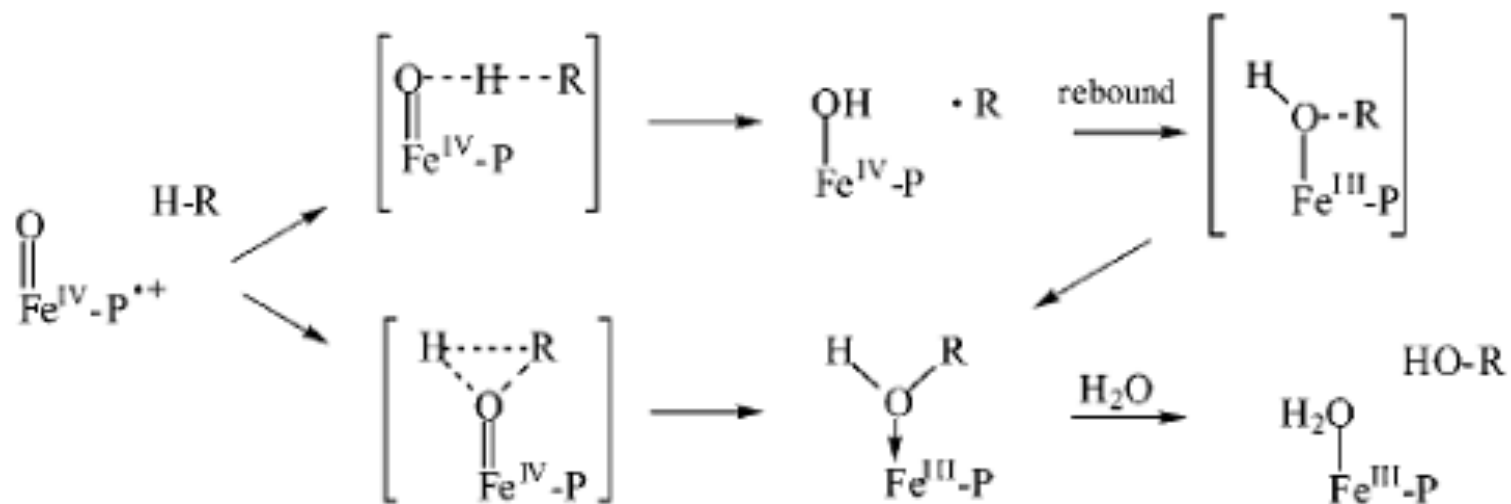
Isotope Tracers: Detect rearrangements, source of atoms in products (e.g. $^{18}\text{O}_2$, D_2O). (only net change is isotope loss or introduction)

Hydrocarbon Hydroxylation

The net reaction is an insertion of the perferryl iron bound oxygen atom into a C-H bond (sp^3) to produce an alcohol. The ease with which P450 enzymes perform aliphatic hydroxylation in unreactive substrates as hydrocarbons indicates a highly reactive enzyme intermediate capable of mediating such chemistry.

Early work on the mechanistic nature of P450 concentrated on the formation of reactive oxygen species such as an oxene (a carbene like) that would insert directly into a C-H bond. This model was replaced with the reactive iron-oxo ($Fe^{IV}=O$) complex and a two step mechanism: hydrogen atom abstraction to form a carbon radical and a hydroxyl recombination (oxygen rebound) to form the alcohol.

Hydrocarbon Hydroxylation



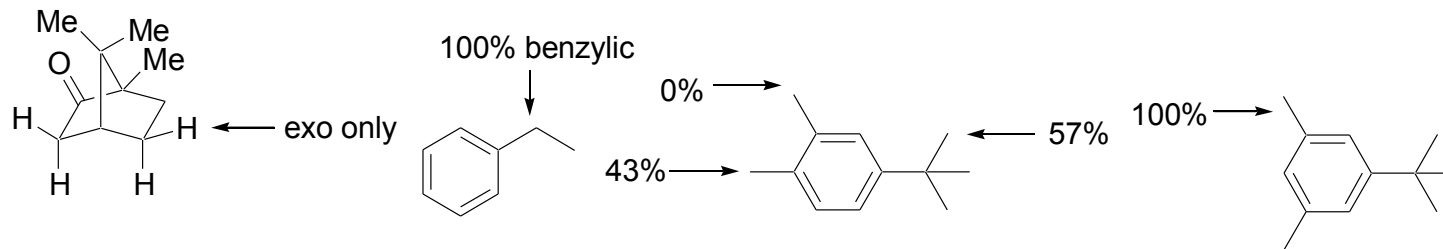
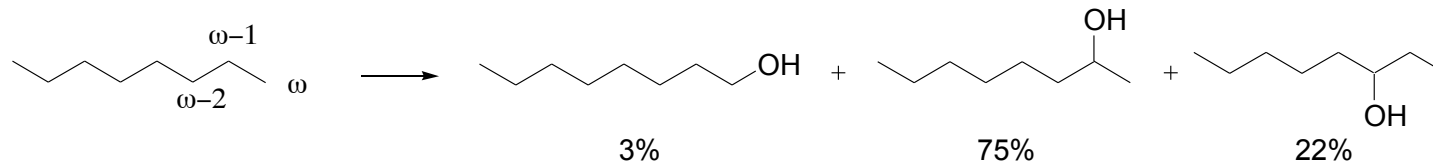
Auclair, K., Hu, Z., Little, D., Ortiz de Montellano, P. and Groves, J. **JACS**. VOL. 124, NO. 21, 2002

A major determinant in the regioselectivity of hydroxylation is the relative activation energy for the hydrogen atom abstraction and the stability of the radical intermediate being formed.

In aliphatic compounds the ease of hydroxylation follows the trend 3° (benzylic and allylic) $> 2^\circ > 1^\circ$. Of course the pattern of hydroxylation is highly controlled by binding orientation in the active site. (see Arch. Biochem. Biophys. 1998 p 285 for an example with P450 cam and camphor.

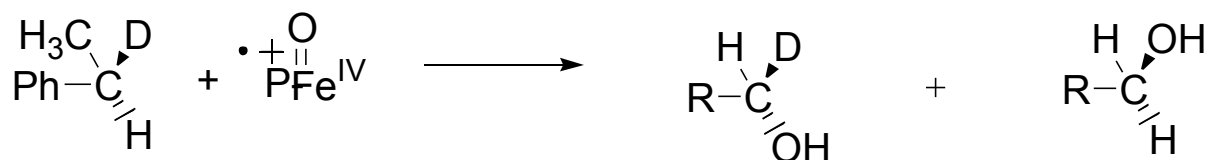
Regioselectivity of octane (and other substrates) oxidation by P450.

Terminal hydroxylation of octane:



The Abstraction Recombination Mechanism

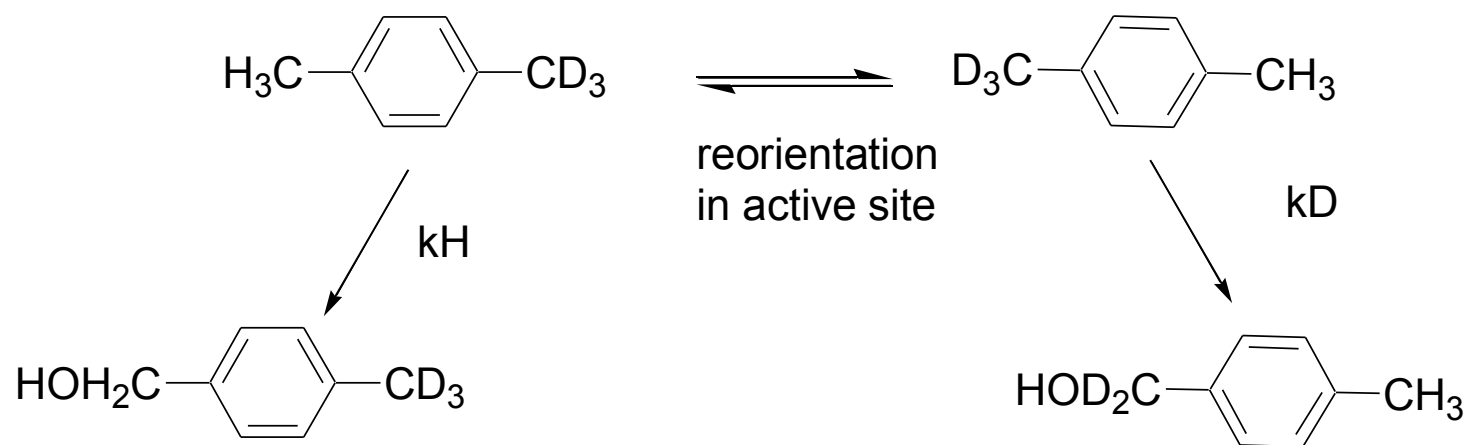
- Evidence supporting the abstraction/recombination mechanism came from experiments measuring intrinsic isotope effects. When alkyl substrates are deuterated in such a way to expose the intrinsic isotope effect. Now the perferryl oxygen can choose to attack either a C-H or C-D on the same compound.



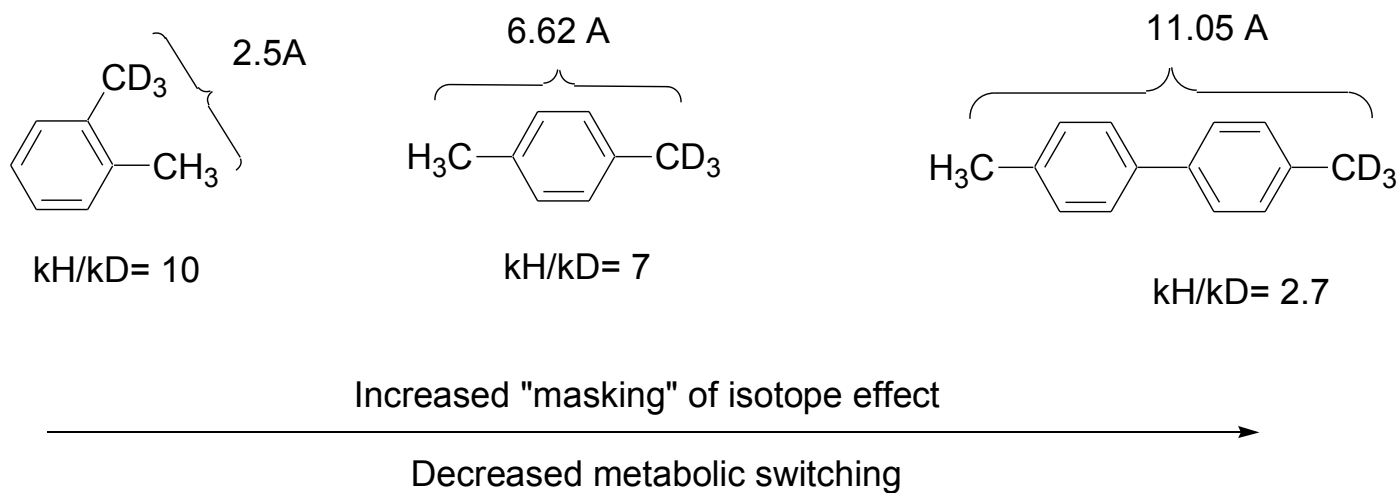
$$k_{\text{H}}/k_{\text{D}} \text{ intra} = 6.7$$

High deuterium isotope effects ($k_{\text{H}}/k_{\text{D}} > 6$) suggest that the oxygen-hydrogen-carbon transition state is reasonably linear ruling out a concerted mechanism. In this case, we have one substrate, one isotope effect and the H/D discrimination occurs only in the product formation step (the one we are interested in.) Note that the magnitude of the intrinsic isotope effect does not tell how the electrons of the C-H bond are distributed so that we can't distinguish between $[\text{C}\cdot, \cdot\text{H}]$, $[\text{C}^+, \text{H}^-]$ or $[\text{C}^-, \text{H}^+]$.

Deuterium isotope effects are useful in telling us something about the dynamics in the active site when the distance is changed between labeled and unlabeled sites of a molecule. For references on isotope effects in P450 reactions see Korzekwa et al. *Biochemistry* 1989, 28: 9012-9018, Hanzlik et al. *JACS* 1993, 115: 9363-9370 and *JACS* 1999 121: 41-47.



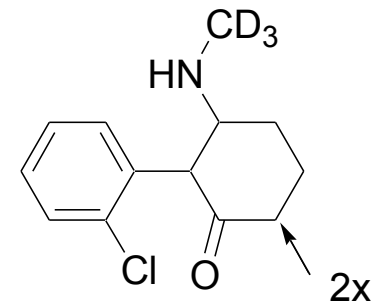
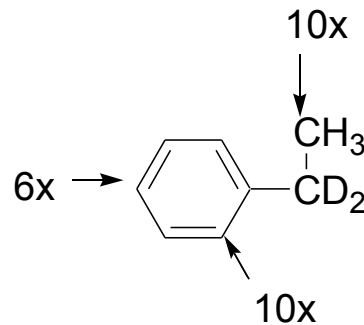
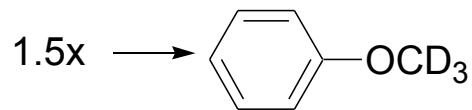
As the distance between chemically identical sites increases, the ratio of metabolites due to hydrogen vs. deuterium abstraction decreases. High switching means that the lifetime of the perferryl intermediate is much longer than the rate of reorientation in the active site. Low metabolic switching means slow substrate reorientation is slow before the perferryl oxygen reacts (commitment to catalysis) which leads to suppression of the isotope effect.



Metabolic Switching:

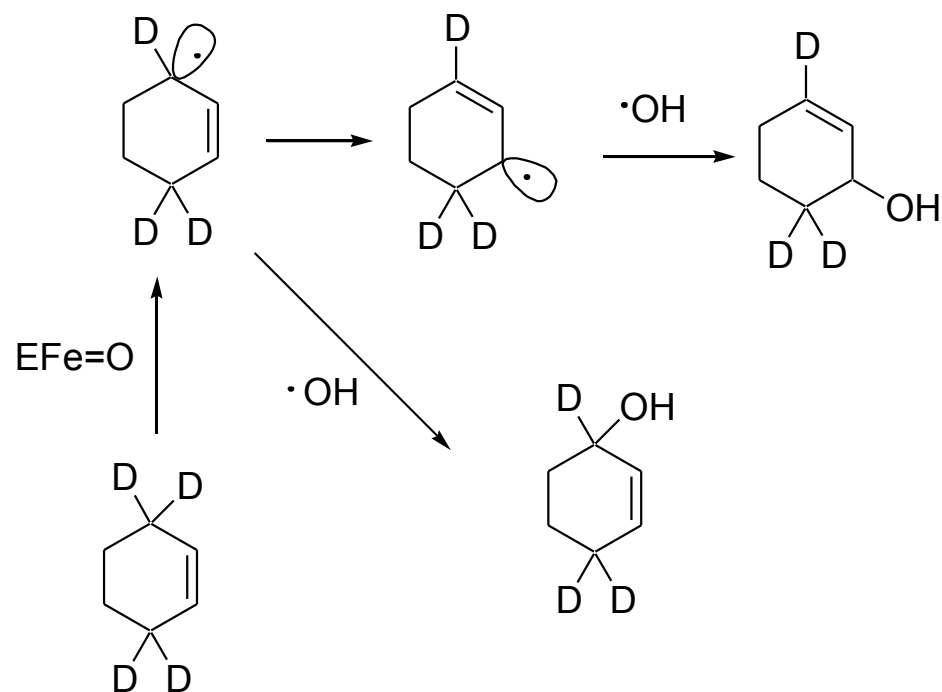
Sometimes upon deuteration of a major site of attack metabolism switches to other sites of the molecule (and to different type of reaction) and minor products become more significant.

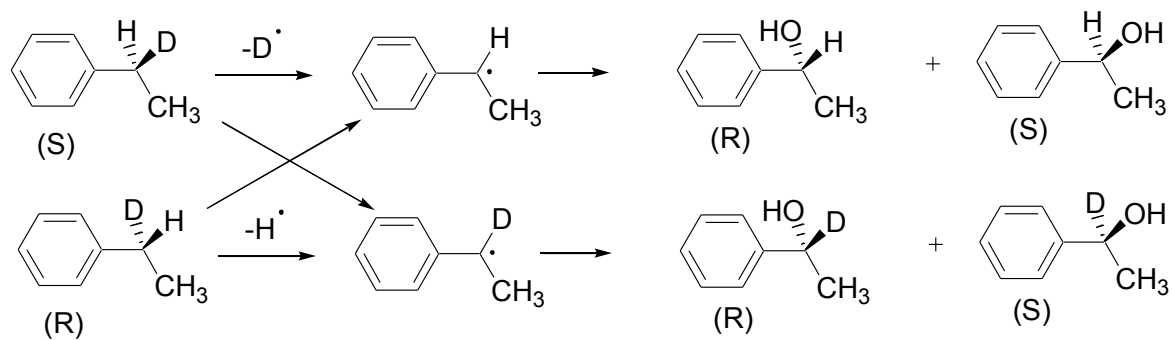
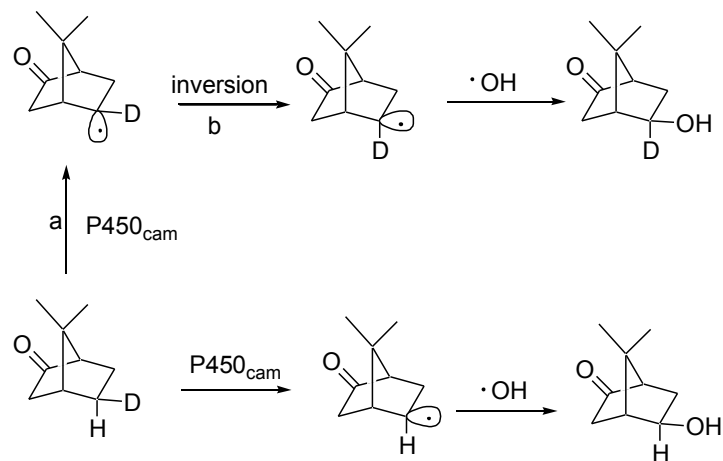
Rate of oxidation increase
when other site is deuterated



What is the nature of the intermediate formed in aliphatic hydroxylations?

- Groves *et al.* (Groves, T.; Subramanian, D. V. *J. Am. Chem. Soc.* **1984**, *106*, 2177-2181.) observed allylic scrambling in the allylic alcohols formed when deuterated cyclohexanes were incubated with P450 enzymes. So when the radical is dispersed over a three carbon π system, the carbon accepting the hydroxyl group may not be the carbon undergoing abstraction.
- The 5-*exo* alcohol expected from the oxidation of camphor *exo*-5-monodeuterated-D-camphor by P450cam retained a significant amount of deuterium located in the 5-*endo* position. (Groves, J. T.; McClusky, G. A. *J. Am. Chem. Soc.* **1976**, *98*, 859-861.) *Here we are using deuterium as labels to trace reaction progress only.*





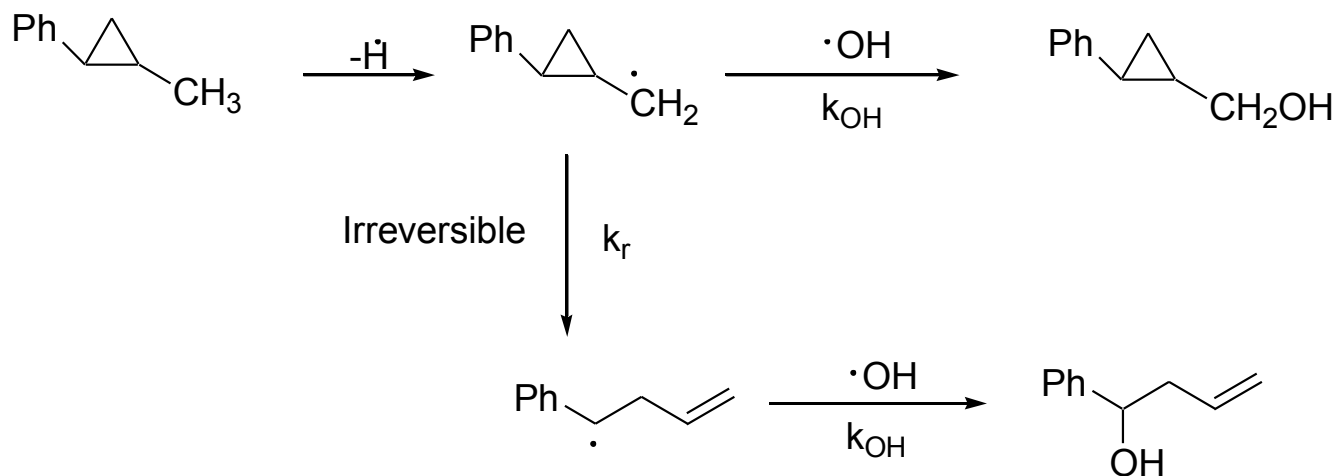
These results, along with the large intrinsic kinetic isotope effects, strongly suggest that a discrete radical is formed during aliphatic hydroxylation reactions and that it has a finite lifetime, which allows it to rearrange or invert prior to the C-O bond-formation step.


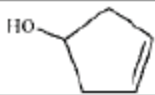



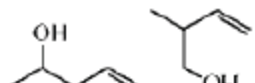


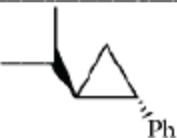
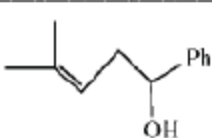
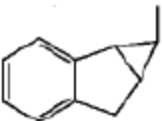
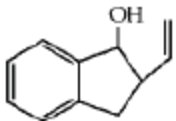
“Radical clocks” to trap radical intermediate formed in aliphatic hydroxylation and measure the rate of oxygen rebound

Radical clocks contain a highly sensitive functionality (e.g. a cyclopropane ring) that will rapidly rearrange if a radical is formed during the reaction.

These functionalities have to be fast to divert the reaction of the radical before it is trapped in the “normal” reaction. Product distribution (rearranged vs. unrearranged) would report on the formation of a discrete radical and determine the rate of trapping if the rate for the radical clock rearrangement (k_r) is known independently.

The unimolecular Cyclopropylcarbinyl radical ring opening rate constant provides a clock for oxygen rebound along with the ratio of rearranged vs. unrearranged products. The rate of oxygen rebound for radical clock substrates have been estimated from 10^{10} to 10^{11} s⁻¹.



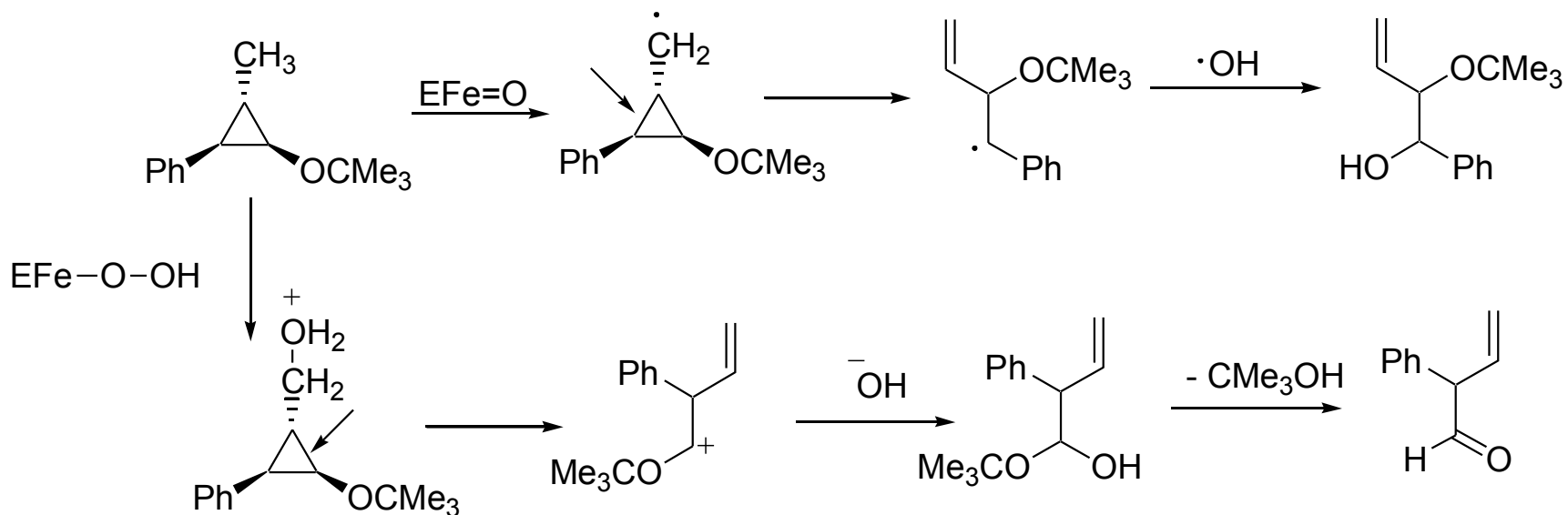
"Normal" radical clocks				
Clock structure	Rearranged product	k_r (s ⁻¹)	P450 system	k_t (s ⁻¹)
		2.1×10^9	Rat liver microsomes	2.2×10^{10}
	ND	1.2×10^8	Rat liver microsomes	ND
	ND	0.8×10^8	Rat liver microsomes	ND
		1.6×10^8	Rat liver microsomes	1.5×10^{10}
		1.8×10^8		1.6×10^{10}
		8.0×10^8	Rat liver microsomes	1.9×10^{10}
		2.3×10^8		1.8×10^{10}
"Fast" radical clocks				
		1.2×10^{11}	CYP2B1	$>1.2 \times 10^{13}$
		3×10^{11}	Rat liver microsomes, CYP2B1	1.4×10^{13}

Paul R. Ortiz de Montellano; *Chem. Rev.* 2010, 110, 932-948.

As work in this field developed less rearrangement was observed with faster clocks. Studies with hypersensitive radical clocks indicate that the lifetime of a radical in P450 hydroxylation is too short (< 100 fs) which is too fast for a “true intermediate”. This was interpreted to mean that the hydroxyl recombination step (k_{OH}) was sufficiently rapid to preclude formation of rearranged products. Even as radical probes became *ultrafast*, little or no rearranged products were observed. One issue to consider was that clocks can't differentiate between radical and cation intermediate. Newcomb *et al.* studied the mechanism of hydroxylation using probes that are capable of differentiating between radical and cation intermediates by rearranging to give separate products depending on the intermediate formed.

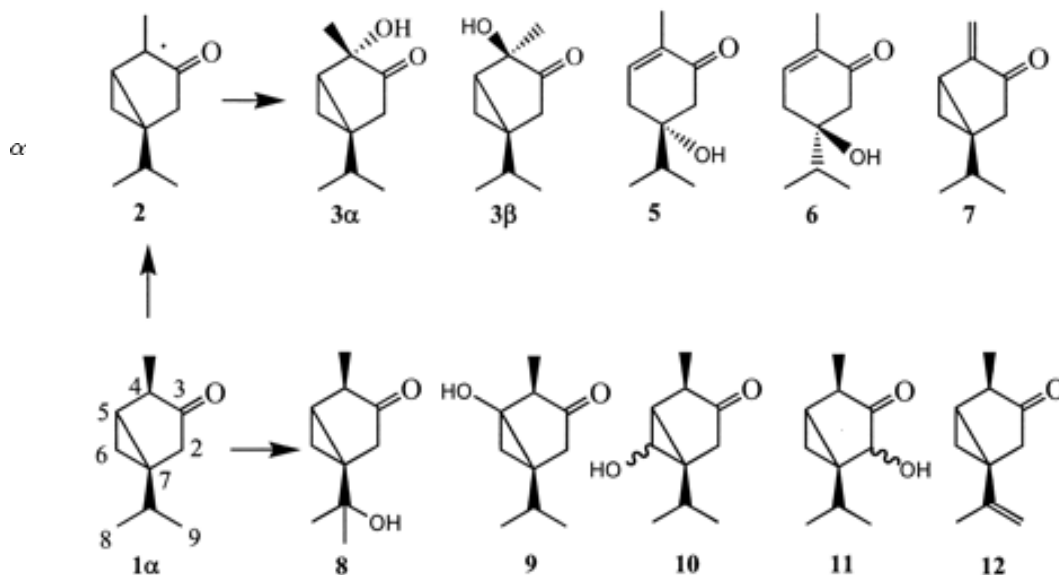
Newcomb *et al.* observed rearrangement products resulting from cationic intermediates as well as products from radical rearrangement. The measured rate of oxygen rebound measured at 10^{13} s^{-1} (too fast for a true intermediate). Newcomb has interpreted these results to suggest that a discrete carbon radical intermediate cannot exist in P450-catalyzed hydroxylations. Instead he has proposed a concerted “non-synchronous” mechanism involving in effect the insertion of the element of HO^+ into a C-H bond by a hydroperoxo-iron intermediate. *JACS*. 1995,117, 12085-12091

Clocks that differentiate between intermediates formed



Auclair K, Hu, Z., Little, D., Ortiz de Montellano, P., and Groves, J. 2002 JACS, 124 2806-2817. Results agree with earlier studies implicating a radical mechanism rather than an OH⁺ mechanism and suggests radical lifetimes from 16 to 50 ps. Only trace amounts of cationic rearrangement products were detected.

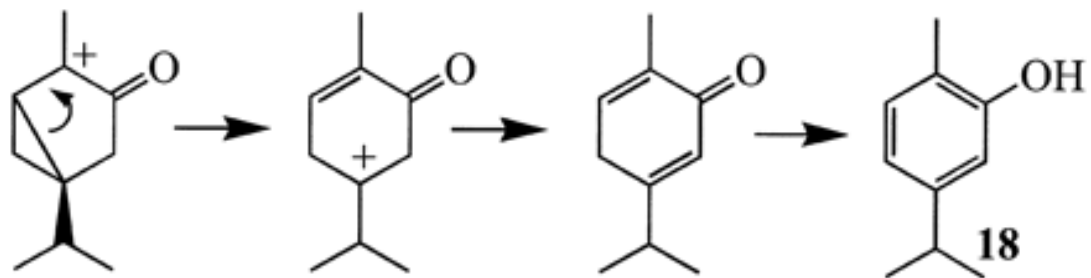
Most recently a study Ortiz de Montellano *et al* (JBC, 2004: 279, 39479-39484) used α - and β -thujone to study the radical rebound mechanism of P450 hydroxylations. α - and β -Thujone are radical clocks that give different products if intermediate is a radical or a cation. When oxidized by P450 enzymes (P450cam and CYP BM3) give rise to products consistent with a radical mechanism. The phenol product expected if a cationic intermediate is formed was not detected. Rate of oxygen rebound ranges from 0.2×10^{10} to 2.8×10^{10} s⁻¹.



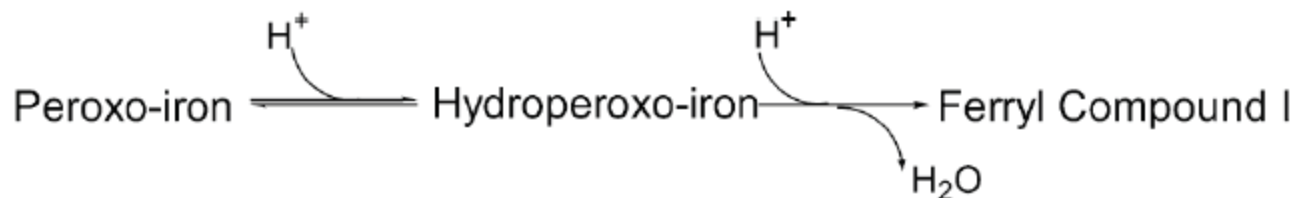
Products resulting from formation of a radical intermediate upon P-450-catalyzed oxidation of α -thujone

Carvacrol, the product expected from formation of a thujone C-4 cationic intermediate and the sequence of steps that result in its formation. Carvacrol was not observed initially in the reaction but with improvement of analytical methods was later detected.

Jiang et al. *Biochemistry* 2006, 45, 533-542.



P450 – Multiple Oxidants? – “Two-state” Mechanism



Nucleophilic

Deformylation

Electrophilic

Epoxidation
Sulfoxidation?
Hydroxylation
(inserts OH⁺)?

Electrophilic

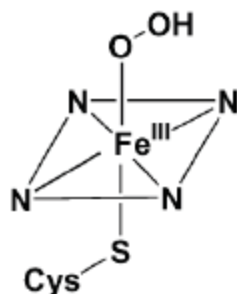
Epoxidation
Sulfoxidation
Hydroxylation
(inserts O)?

Electrophilic

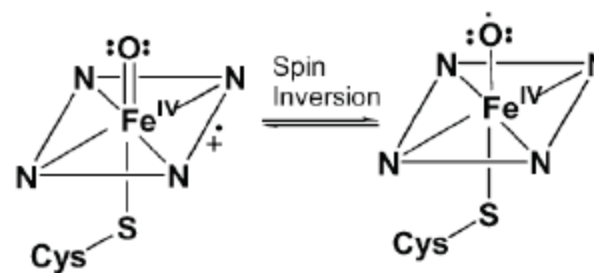
Hydroxylation
(abstracts H)



Ferric peroxo anion



Ferric hydroperoxo



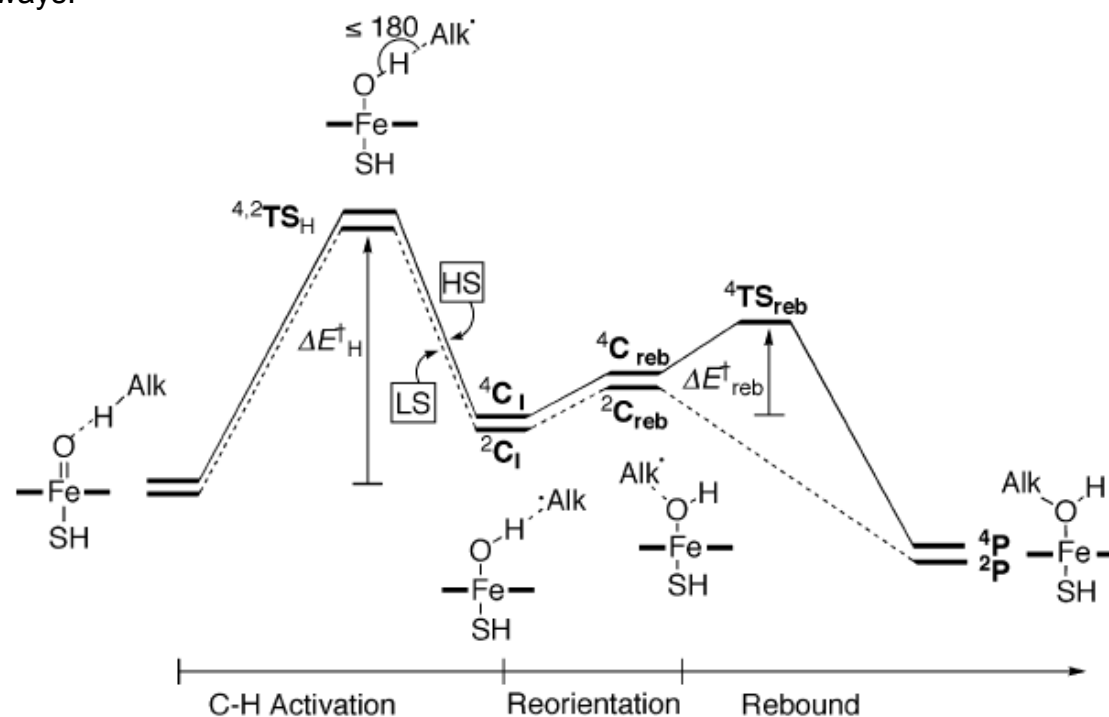
Low Spin

High Spin

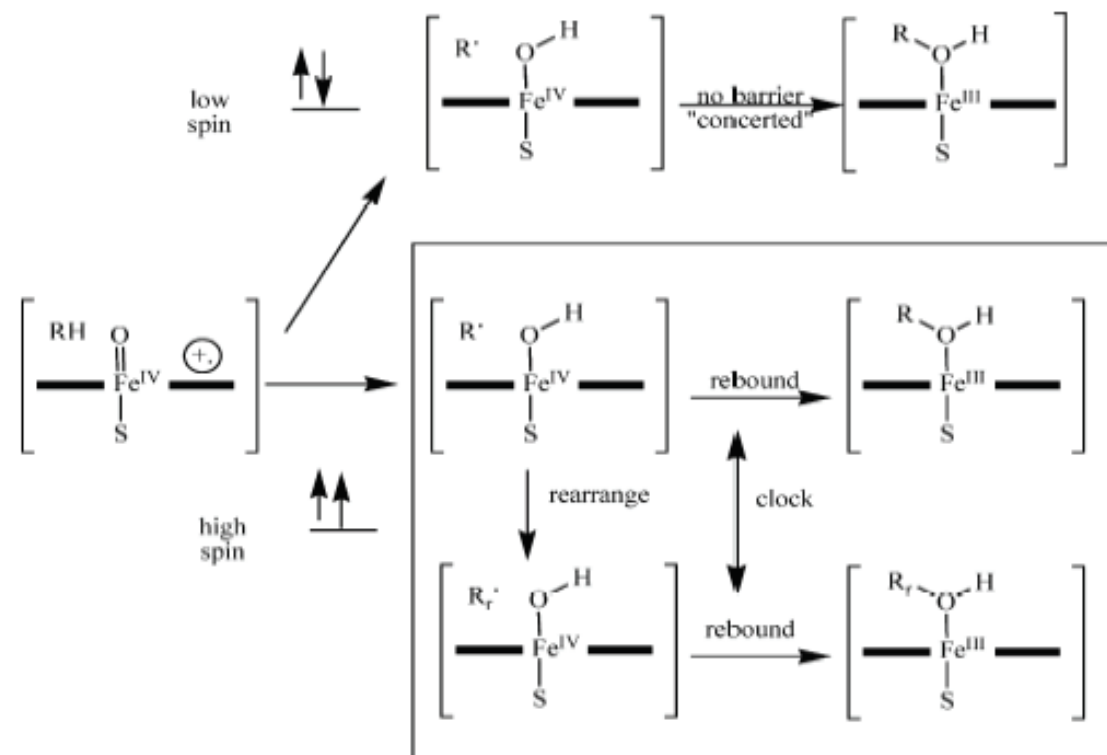
Density Functional Theoretical Studies and the Two Spin State Reactivity Model for aliphatic hydroxylation

References: J. Biol Inorg Chem (2004) 9: 661-668 and Chem Rev (2004) 109 3947-3965 and references within.

Theoretical studies by Sason Shaik implementing the density functional theory offers a solution to the mechanistic dilemma posed by studies of C-H hydroxylation. The theory proposes a two state reactivity (TSR) of the perferryl species (compound I) alone. That is one reagent with two different reaction pathways.



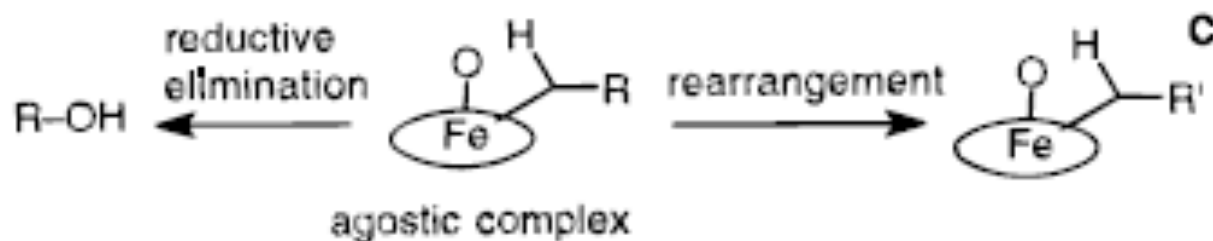
- Basically the perferryl oxidant has two electromeric states (doublet 2, low spin and quartet 4 high spin) very close in energy. In the two state reactivity (TSR) model, during aliphatic hydroxylation the low spin form undergoes a barrier free hydroxyl recombination step which is fast and allows for no rearrangement. The high spin intermediate faces an energy barrier during the recombination step allowing the carbon radical to undergo rearrangement (or other radical chemistry, such as inversion of configuration epimerization and allylic scrambling) as well as recombination.
- Both the high and the low spin should exhibit high isotope effects.
- Density functional theoretical calculations for the hydroperoxy species (compound 0) so far indicated that it is a poor electrophile that can't compete with compound I. So the two oxidant scenario where the two oxidants co-exist and compete is not supported.



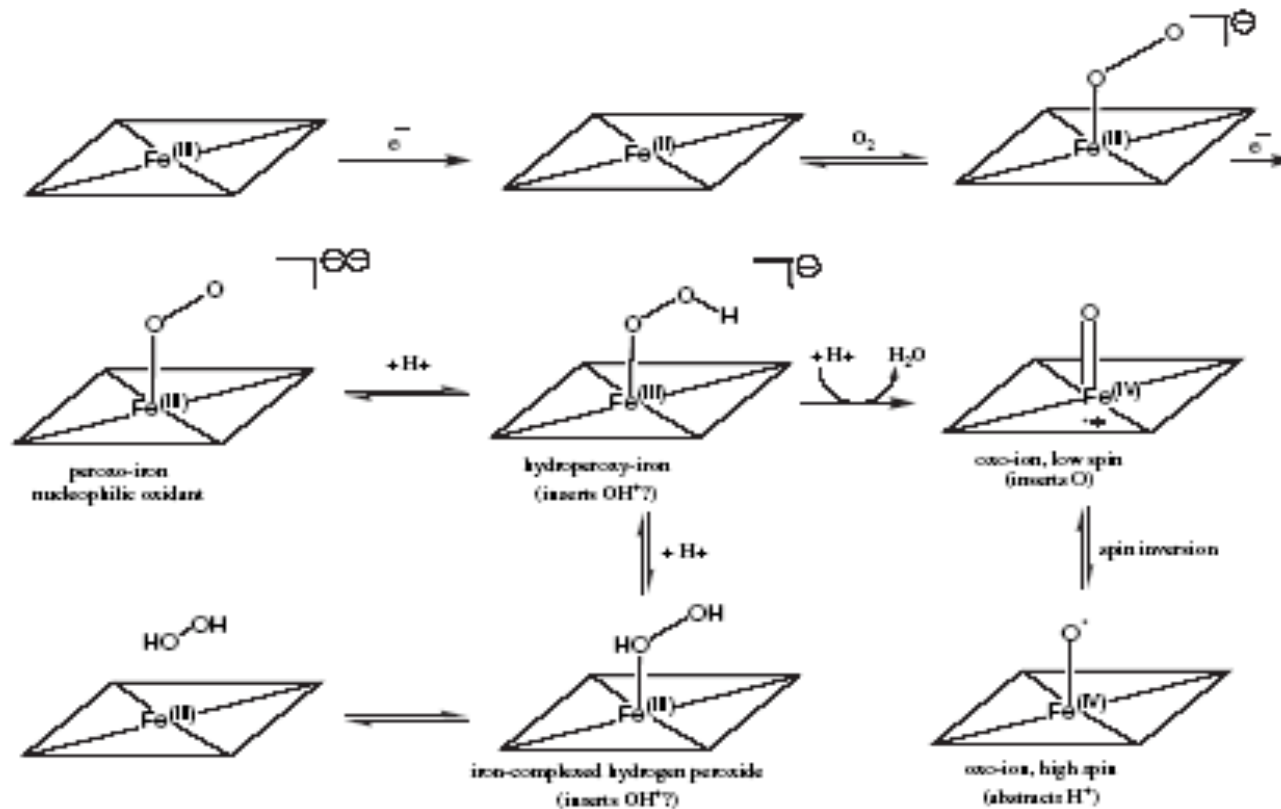
A schematic showing the rationalization for the observation of extremely fast radical recombination times with some radical clock substrates provided by the two-state hydroxylation hypothesis. The doublet (low)-spin transition state collapses in a barrierless manner and is not able to undergo radical rearrangements, whereas the quartet (high)-spin transition state (in the box) has a barrier to the recombination reaction that allows the radical rearrangement to occur. The radical clock thus only operates in the lower (high-spin) trajectory, but the overall product ratios reflect the sum of the two pathways. As a result, the final product ratio has a deceptively large proportion of the unrearranged product.

Agostic alternative

- **Colleman et al (1998) An agostic alternative to the P450 rebound mechanism JACS 120: 425-426**
- Agostic σ -complex between substrate and the perferryl species was proposed to arrange in competition with the hydroxylation reaction.
- So far the two spin state model seems to be a viable model that explains the experimental data.



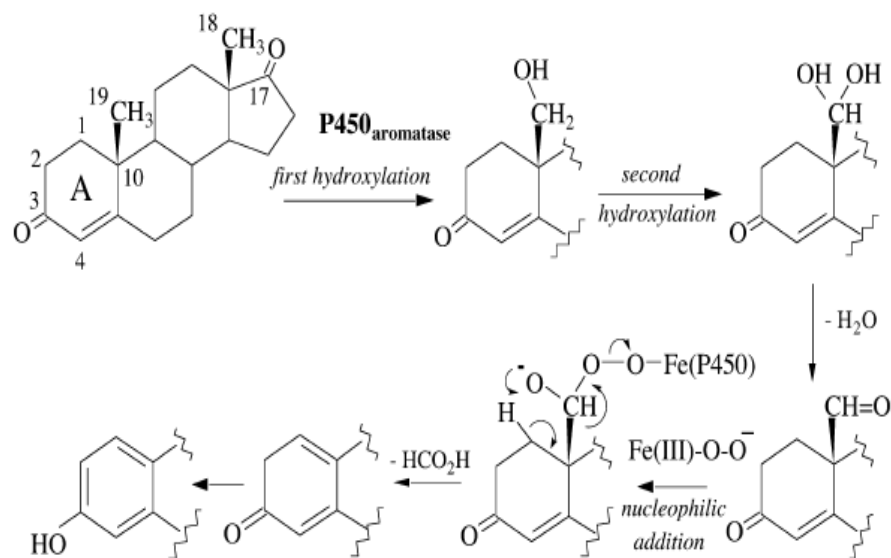
Putative iron-oxygen intermediates in P450 and possible roles as oxidants



Other candidates FeIV-O (Compound II), the one electron reduced form of compound I (remember peroxidase chemistry), the Ferric hydrogen peroxide complex FeIII(H₂O₂) and the tautomer of Cpd 0, FeIII(HO-O)

Evidence for reactions involving alternative oxidant species

Peroxo-iron acting as a nucleophilic oxidant in CYP19 (aromatase) catalyzed biotransformation of androgens to estrogens via oxidative deformylation of the 19-aldehyde to release formic acid and estrone.

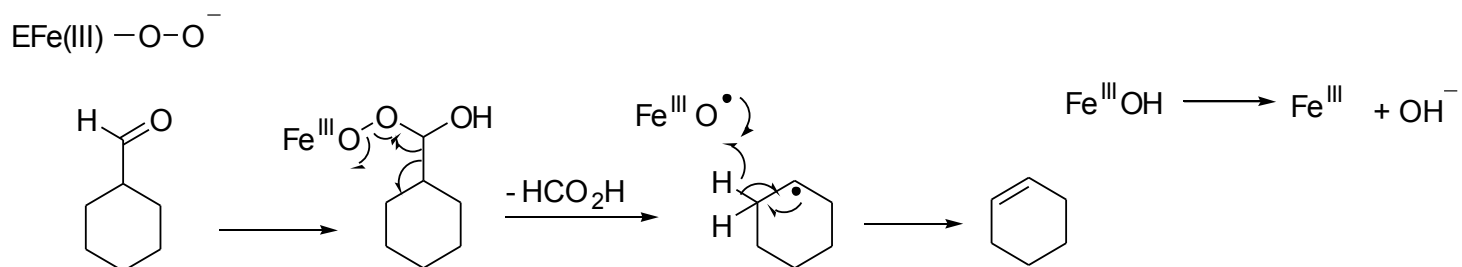


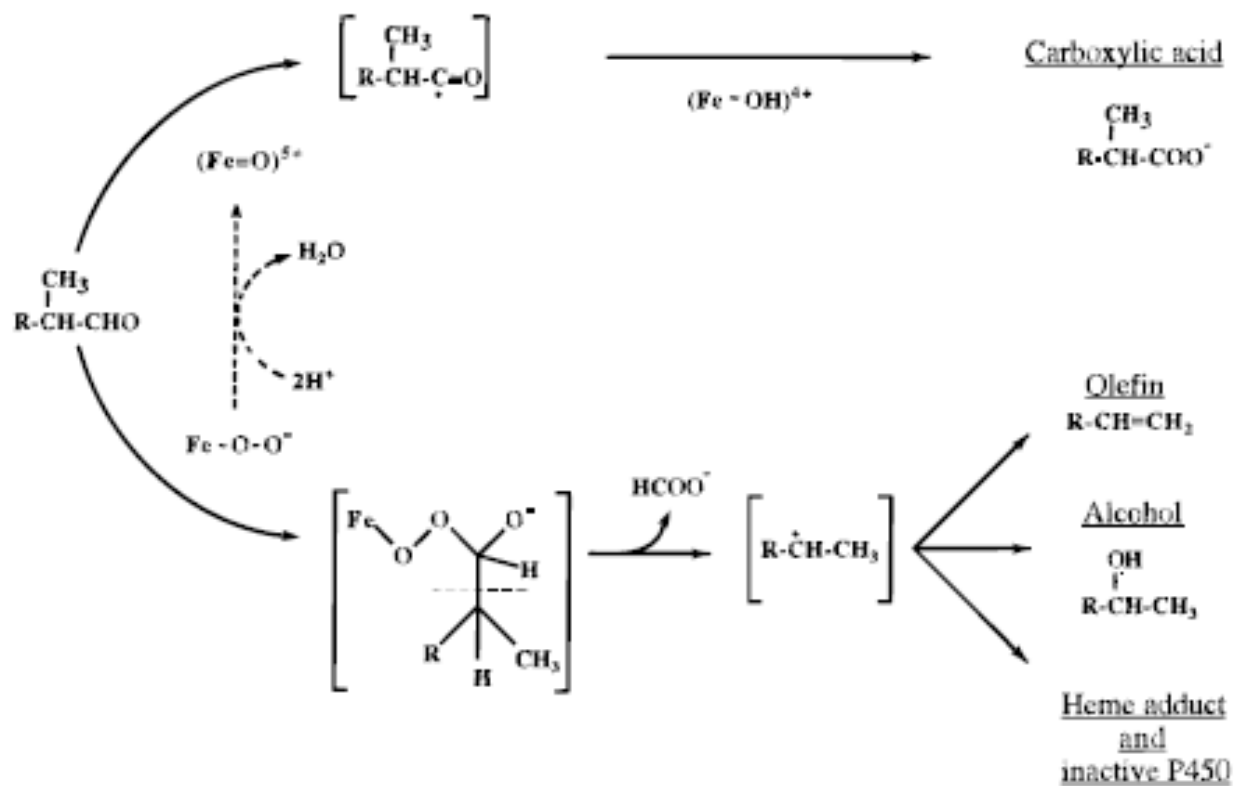
The peroxy-hemiacetal like complex was shown to be a strong contender to explain the C-C bond scission in experiments using deuterated starting material and ¹⁸O₂. Formic acid also enriched with ¹⁸O. Also synthetic peroxo-metalloporphyrins are able to catalyze this deformylation step. (see Inorg. Chem 1997, 36 p 979 and JACS 1998 120p 2652)

A mechanism of the aromatization of the A-ring of androst-4-ene-3,17-dione to estrone catalyzed by humanplacental aromatase. From Meuner et al. Chem Rev. 2004, 104 3947-3980.

Evidence for alternative oxidants in drug metabolizing P450 enzymes

- Vaz and Coon invoked the iron-peroxo intermediate to explain the deformylation of aldehydes to olefins and formate by liver microsomal P450. They used cyclohexylcarbaldehyde as a probe and observed the oxidative deformylation to formic acid and cyclohexene.
- **References:** Proc Natl Acad Sci U S A. 1996 May 14;93(10):4644-8. Mol Pharmacol. 1997 Jan;51(1):147-51. Biochemistry. 1997 Apr 22;36(16):4895-902.
- Support for this proposal came from experiments showing that hydrogen peroxide supported the deformylation of cyclohexane carboxyaldehyde while iodosylbenzene did not.





The covalent modification of heme moieties by aldehyde substrates was seen as a direct involvement of the peroxy species in the deformylation reaction.

Ortiz de Montellano, P.R. De Voss, J. Oxidizing Species in the Mechanism of cytochrome P450, (2002) Nat. Prod. Rep. 19, 477-493.

Evidence from site engineered proteins mutating the conserved Threonine residue within hydrogen bonding distance to the peroxy-iron unit.

Mutations were considered to disrupt a key step in H⁺ delivery, presumably the second proton to hamper the O-O scission.

Effect of Thr to Ala mutation on the ratio of the rates of epoxidation and hydroxylation of olefins

Substrate	Ratio of initial rates, epoxidation/hydroxylation			
	Δ2B4	Δ2B4 T302A	Δ 2E1	Δ 2E1 T303A
Cyclohexene	1.7	1.7	1.0	2.4
<i>cis</i> -butene	43	ND*	90	460
<i>trans</i> -butene	35	ND*	4.0	56

* ND, not determined (only a trace of the alcohol was formed by this enzyme).

Increase in the ratio of epoxidation vs. hydroxylation was interpreted as involvement of the peroxy intermediate in the epoxidation pathway and the perferryl intermediate in the hydroxylation pathway.

Site directed mutagenesis studies with P450cam and P450 BM3:

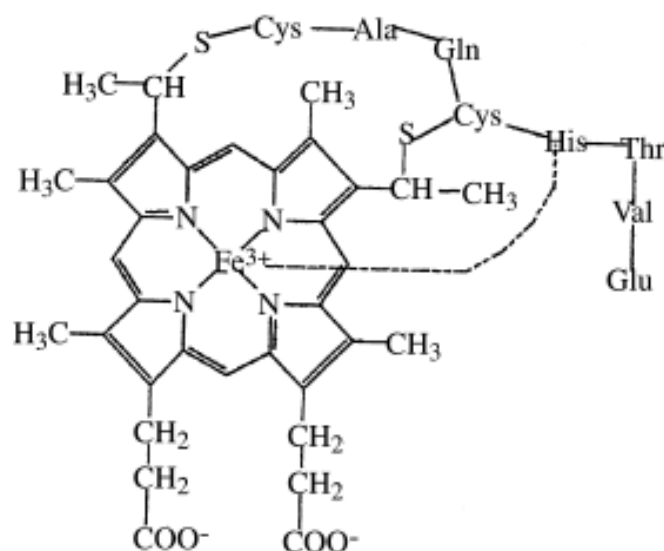
The T252A variant of P450cam was found to accept electrons from NADH and reduce O₂ to H₂O₂ probably through the intermediacy of hydroperoxo-intermediate. T252A was able to epoxidize camphene but not hydroxylate camphor. However, the double mutant D251N/T252A was able to hydroxylate camphor??

In BM3, T268A was highly uncoupled and lauric acid hydroxylation was impaired. However in a recent paper by Cryle and De Voss (ChemBioChem 2008, 9, 261-266) found that the effect of T268A mutation differs with fatty acid substrate used. Whether T268A reduced, activated or had no effect on the hydroxylation was substrate dependent.

Moral of the story: Don't do work with one isozyme and one substrate and try and generalize to all P450s.

Use of non-P450 hemoproteins and metalloporphyrin models to study the mechanism of P450 mono-oxygenation.

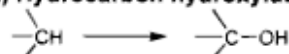
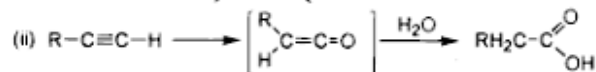
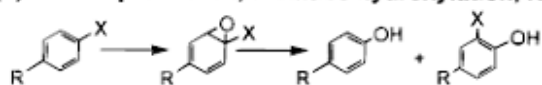
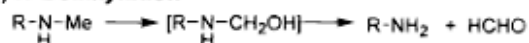
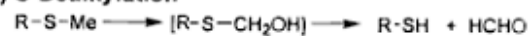
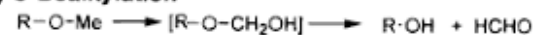
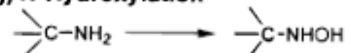
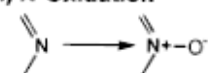
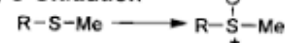
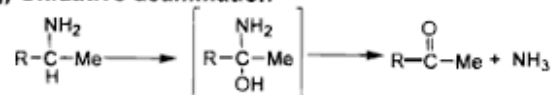
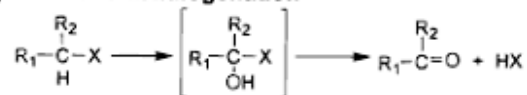
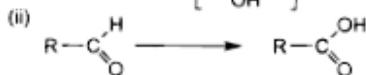
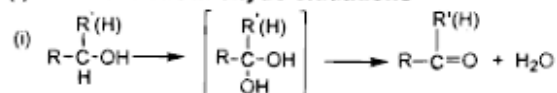
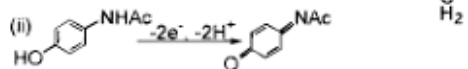
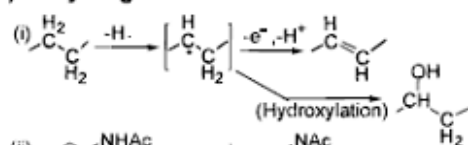
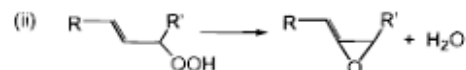
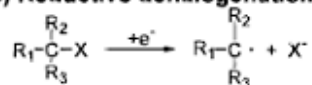
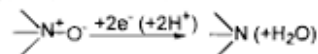
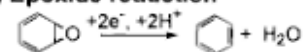
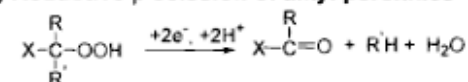
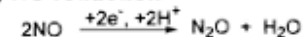
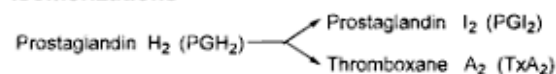
Hemoglobin, Myoglobin as well as heme oxygenase were used to study mono-oxygenation reactions of P450. Synthetic metalloporphyrins were selectively tailored as models of P450 active site and allow detailed insight into the mechanistic basis of oxygen transfer reactions. You can read about those systems and others in Eur. J. Biochem 271, 4335-4360 (2004) and references within.



Microperoxidase-8 (MP8)

MP-8 is a mini-enzyme (octa peptide) obtained by peptic and tryptic digests of horse-heart cytochrome c. It has been depicted as a model for P450-type oxygen transfer reactions.

The MP-8 based (hydro)-peroxo-iron (III) species was identified by optical absorption and rapid-freeze EPR methods.

(a) Hydrocarbon hydroxylation**(b) Alkene epoxidation / Alkyne oxygenation****(c) Arene epoxidation, aromatic hydroxylation, NIH shift****(d) N-Dealkylation****(e) S-Dealkylation****(f) O-Dealkylation****(g) N-Hydroxylation****(h) N-Oxidation****(i) S-Oxidation****(j) Oxidative deamination****(k) Oxidative dehalogenation****(l) Alcohol and Aldehyde oxidations****(m) Dehydrogenation****(n) Dehydrations****(o) Reductive dehalogenation****(p) N-Oxide reduction****(q) Epoxide reduction****(r) Reductive β-scission of alkyl peroxides****(s) NO reduction****(t) Isomerizations****(u) Oxidative C-C bond cleavage**