Cytochrome P450 Mechanism
Major Types of P450 oxidation Reactions

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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:

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

Sligar, Stephen G. "Glimpsing the Critical Intermediate in Cytochrome P450 Oxidations Science 330, 924 (2010)

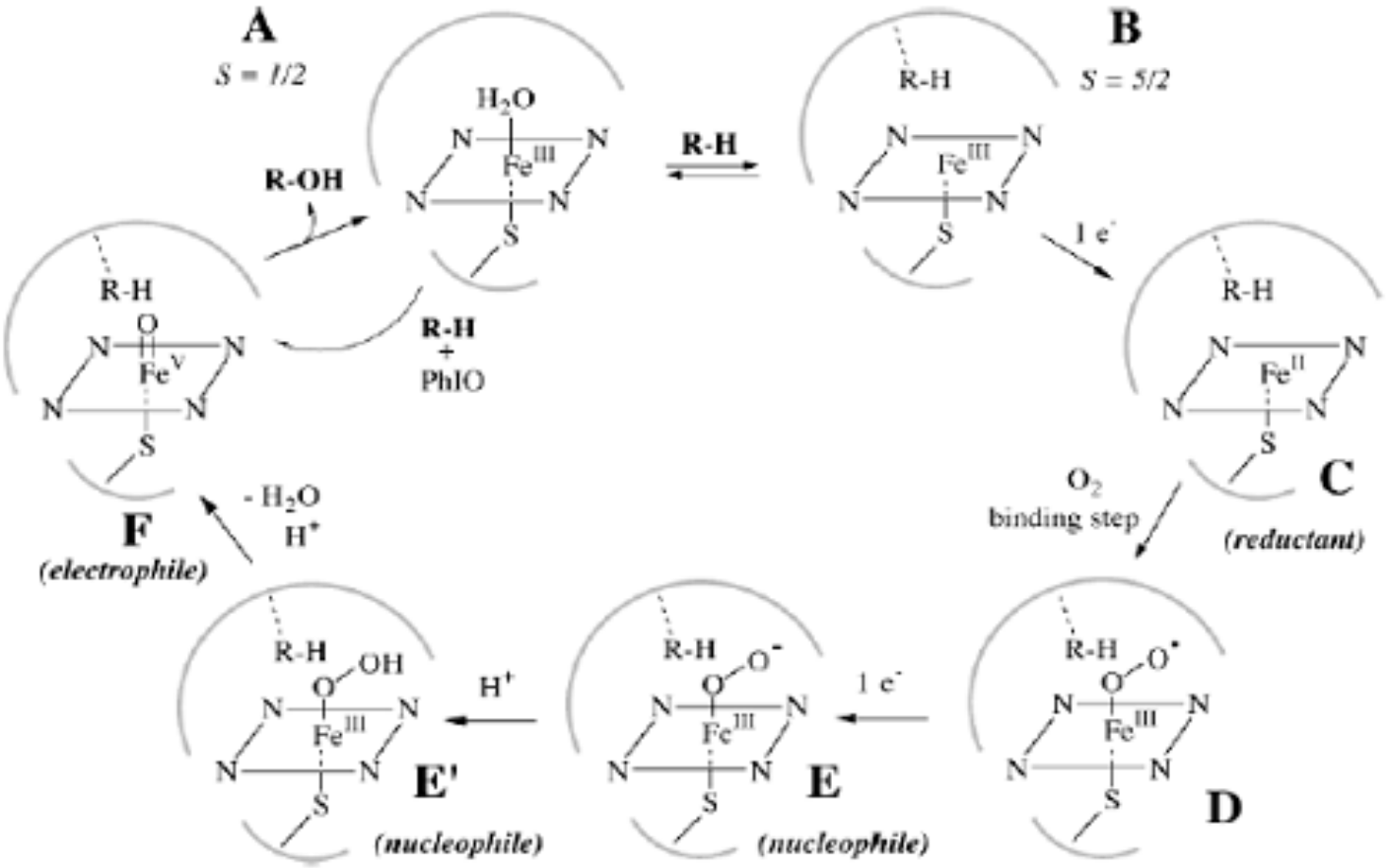
Shakunthala N. (2010). New cytochrome P450 mechanisms: implications for understanding molecular basis for drug toxicity at the level of the cytochrome. *Expert opinion on drug metabolism & toxicology*, *6*(1), 1-15.

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

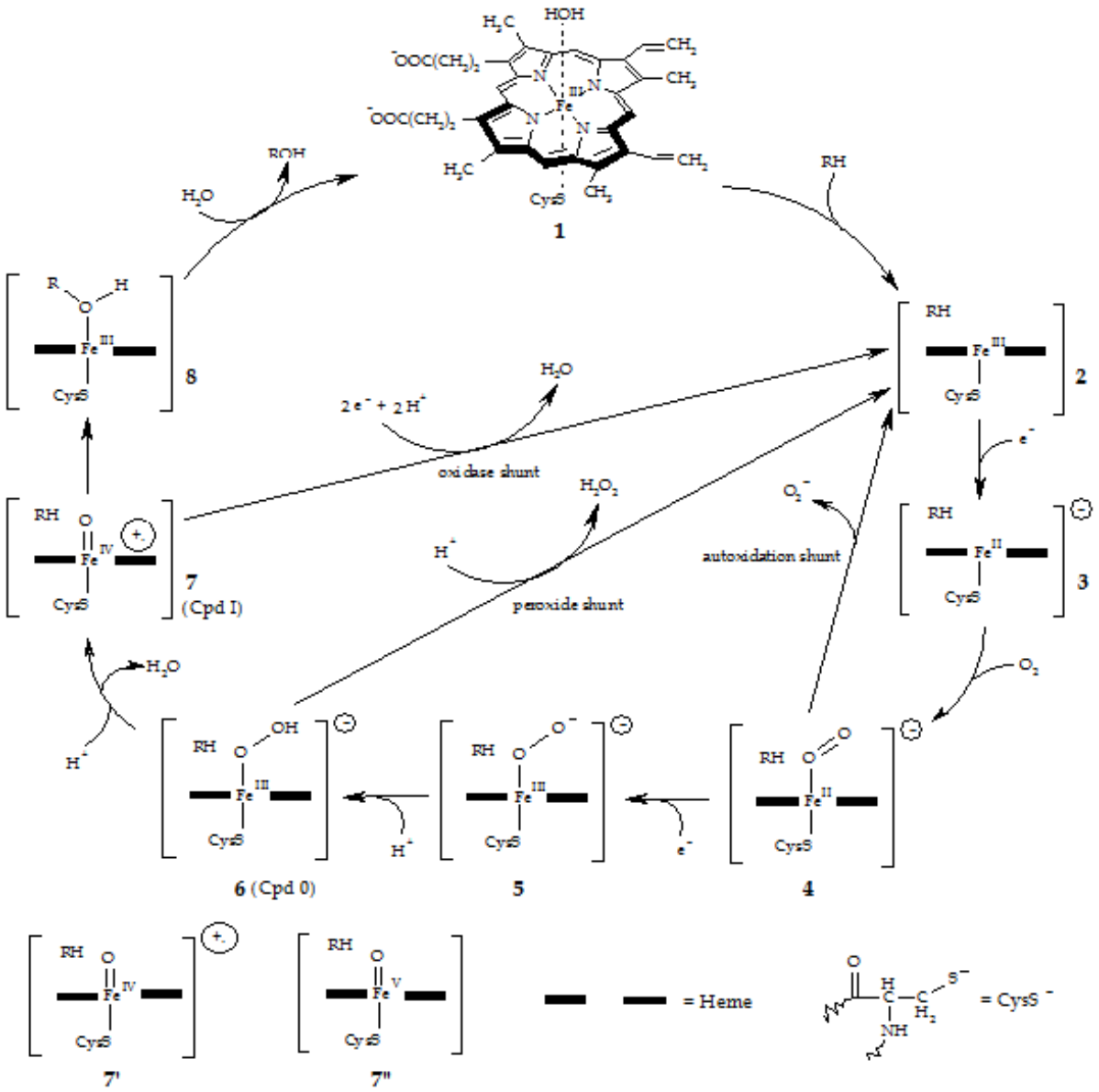
50 years of P450 research (2014) edited by Hiroshi Yamazaki (book)

What makes P450s Tick? Andrew Munro et al. Trends in Biochemical Sciences March 2013, Vol. 38, No. 3 (Review).

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



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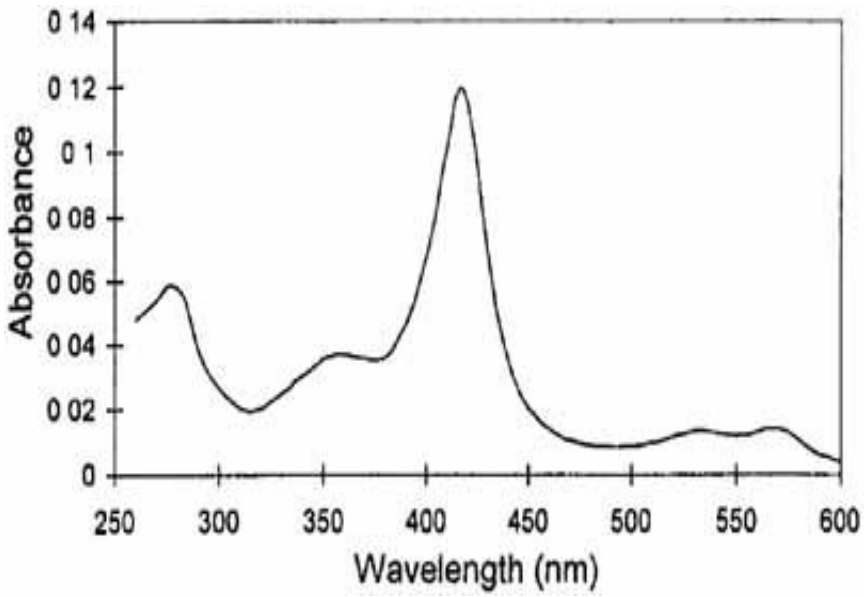
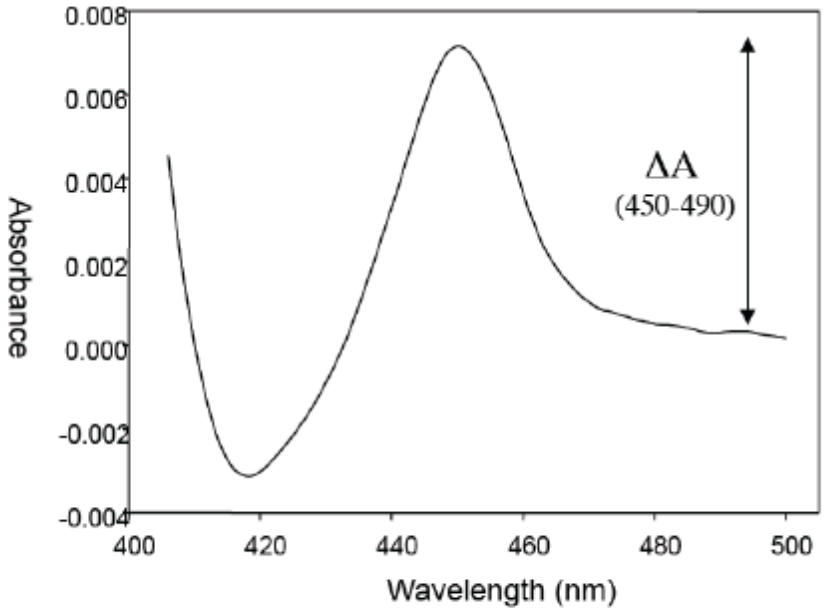
Quantifying P450s using UV vis. Taking advantage of the heme chromophore.





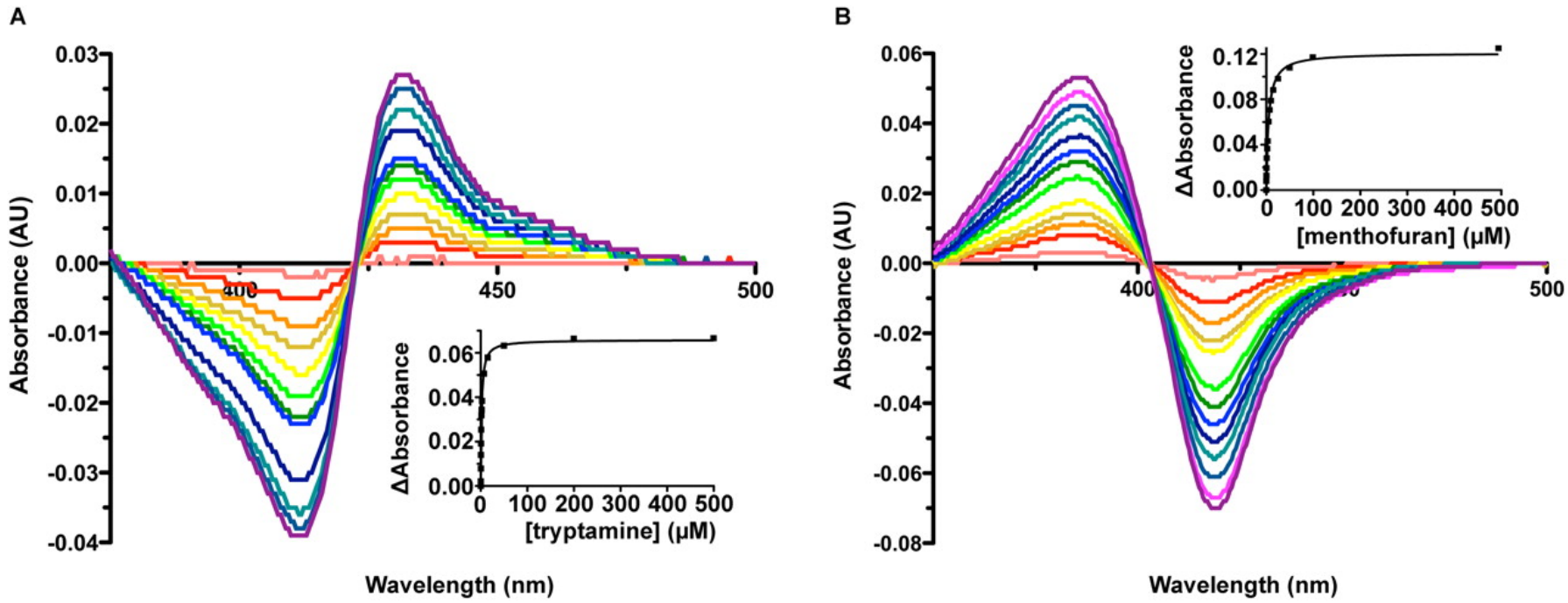
Using a dual beam UV instrument and measuring the difference spectrum of: Fe^{+2}-CO vs Fe^{+2} to obtain the soret band at 450nm.

Difference spectrum is used to quantitate P450s by applying the extinction coefficient

$\varepsilon = 91\ mM^{-1}\ cm^{-1}$ and the following equation:

$$[P450]\ (\mu M) = \frac{\Delta A\ (450\text{-}490) \times 1000}{91 \times 1} = 0.007 \times 11 = 0.08\ \mu M$$

Representative binding spectra for the type II ligand tryptamine (A) and the type I ligand (R)-(+)-menthofuran (B), both with CYP2A6.



Type I ligands usually have alcohols.

Inset shows binding curve to measure kd values.

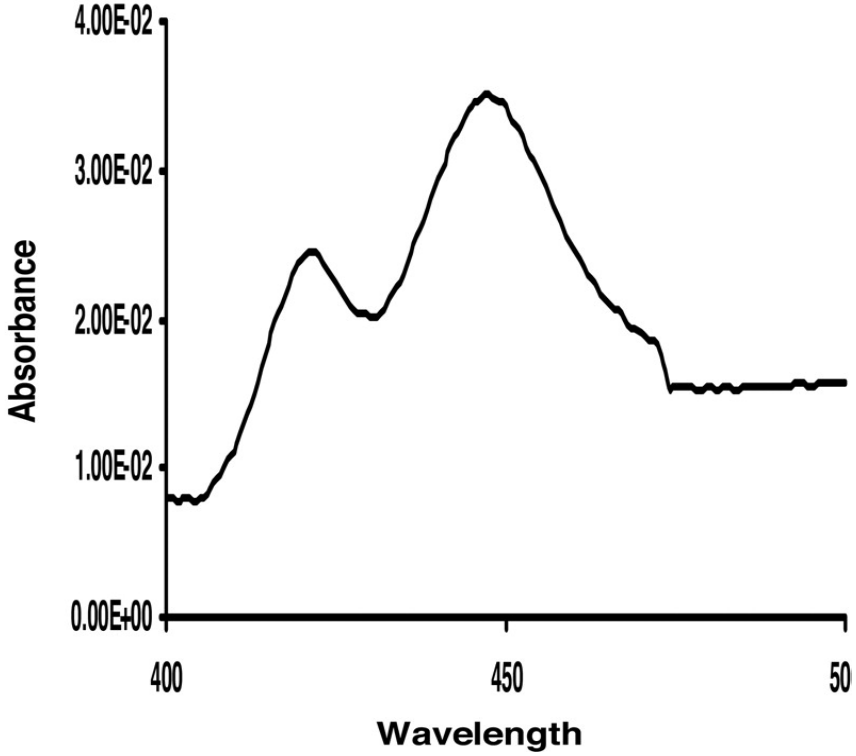
Type II ligands usually have an amine with a free lone pair to bind to the heme iron. Type II ligands can be inhibitors.

Eva S. Stephens et al. Drug Metab Dispos 2012;40:1797-1802

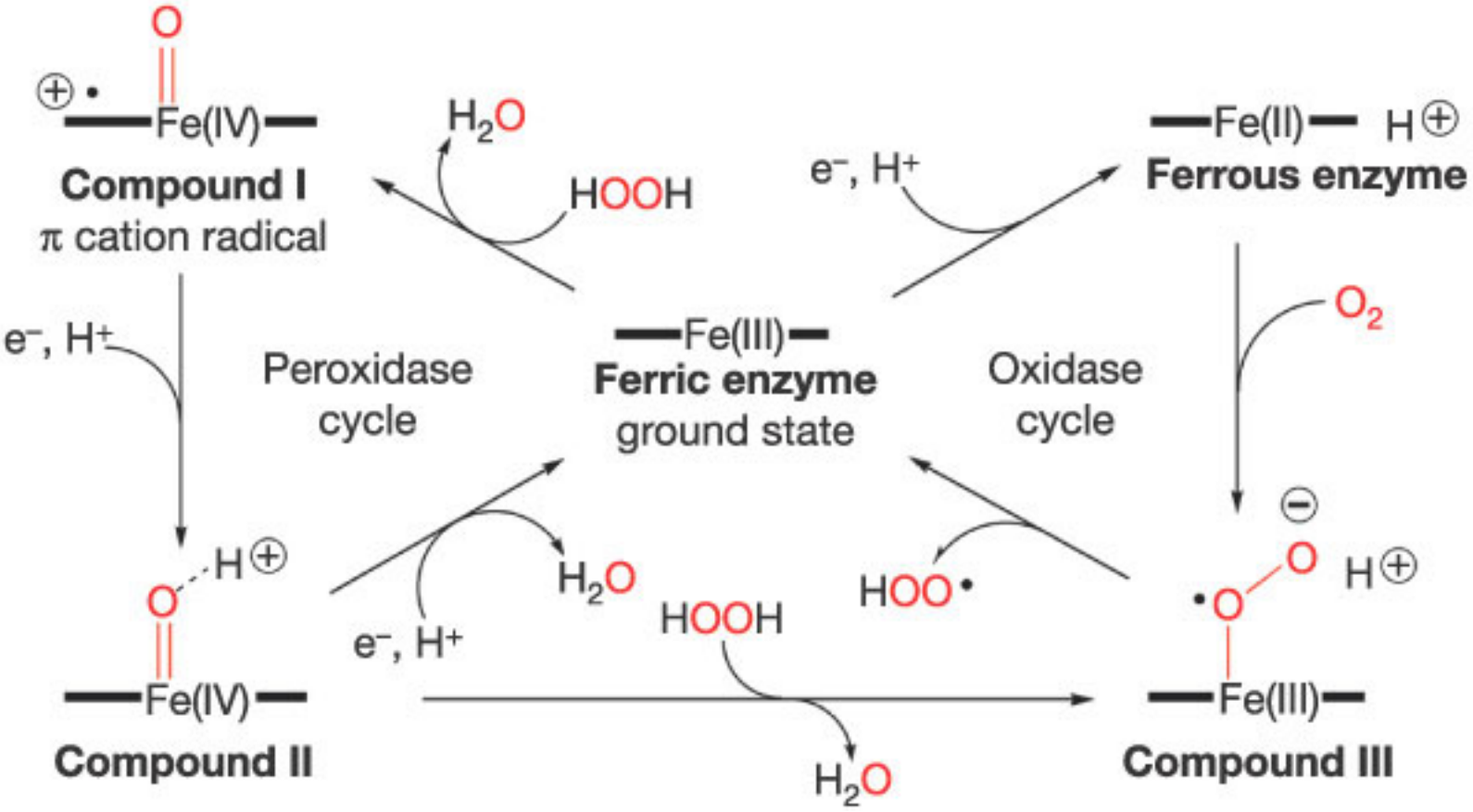
Pros and Cons of UV vis method:

Pros:
Fast easy, cheap, can differentiate between active heme with abosrbance at 450nm vs. inactive protein with absorbance at 420nm. See figure.

Cons:
-Does not work well for dilute systems
-Does not work for turbid systems
-Can only be used to measure total P450 present in a biological sample, if sample turbidity allows.
-Not suitable for a mixture of P450s in a solution.



Peroxidases, reaction and use as model systems for P450s

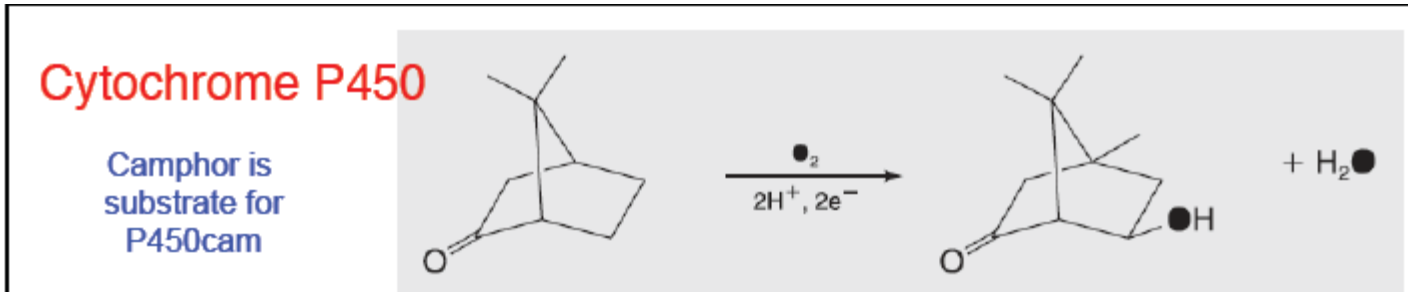


HRP, classical model for one electron abstraction by P450s.
Several human peroxidases participate in drug metabolism.

Diversity of Redox Partners and Mechanism

- Class I P450 systems. Prokaryotic P450s are reduced by small iron-sulfur proteins (ferredoxins), which in turn are reduced by NAD(P)H-dependent FAD-binding reductases. Classic example P450101A1 (P450cam 2Fe–2S putidaredoxin and NADH-putidaredoxin reductase).

- Mitochrondrial P450s use a similar system 2Fe–2S adrenodoxin (AD) and the NADH-adrenodoxin reductase.



- The class II system comprise the remaining eukaryotic microsomal P450 enzymes that are reduced by the diflavin (FAD- and FMN-binding) membrane protein NADPH-CPR.
- Role of cytochrome b5 most likely involves transfer of the second electron or could be allosteric in nature. Various P450 isozymes respond differently to cytochrome b5.

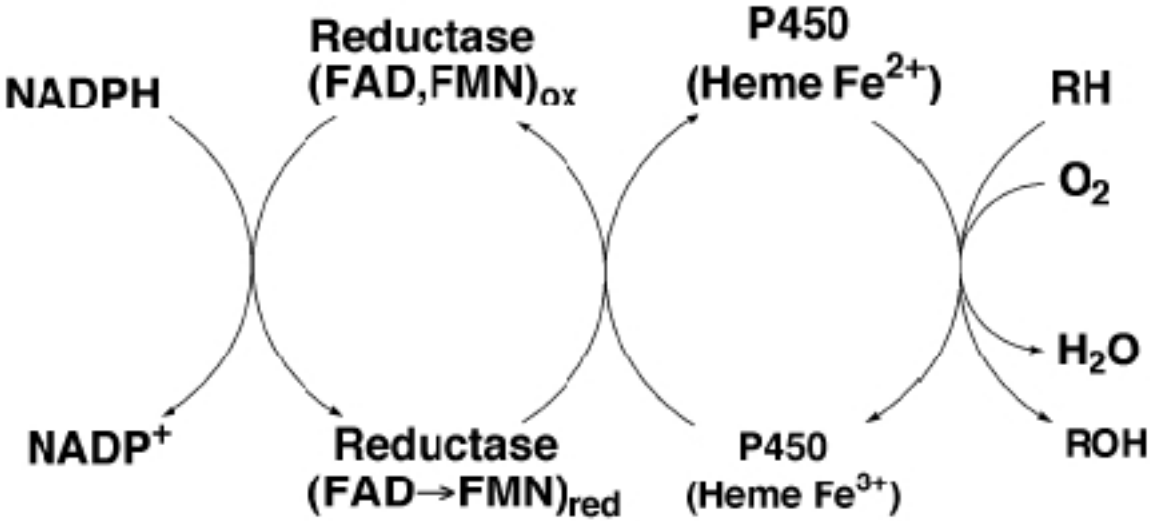


Table 1. Diverse natural and man-made P450 redox systems for biocatalysis[a]

Electron donors	P450s	Notes and applications	Ref
FdR + Fdx + NAD(P)H	Mitochondrial, various bacterial (e.g., CYP101 family)	FAD-containing reductase and Fe/S Fdx	[42]
CPR + NADPH	Eukaryotic (non-mitochondrial)	FMN- and FAD-containing reductase	[42]
CPR fusion + NADPH	CYP102A family (e.g., BM3), CYP505 family (P450 foxy)	P450–CPR natural fusion	[42]
Cyt b_5 + cyt b_5 Red/CPR + NADH	Eukaryotic (non-mitochondrial)	Cytochrome b_5 and FAD-containing reductase	[44]
PDOR fusion + NAD(P)H	CYP116B family	P450–(Fe/S- and FMN-containing) reductase natural fusion; engineered P450–PDOR fusions used to make catalytically self-sufficient P450s	[56]
FLD fusion + FLDR/FdR + NAD(P)H	CYP177A1 (XplA, *Rhodococcus rhodochrous*)	P450–FLD (FMN-binding) natural fusion	[55]
FLD + FLDR + NAD(P)H	CYP176A1 (P450cin, *Citrobacter braakii*)	FMN–binding FLD and FAD–binding FLDR; *Escherichia coli* FLD and FLDR also used as redox partners for human and other P450s	[42]
Fdx fusion + FdR + NAD(P)H	CYP51B1FX (*Methylococcus capsulatus*)	P450–Fdx (Fe/S) natural fusion	[54]
Fdx + 2-oxoacid:FdR (OFOR) + Pyruvate + CoA	CYP119 family (*Sulfolobus* spp.)	Thermostable class of P450s (e.g., CYP119A2, P450st from *Sulfolobus tokadaii*)	[42]
NAD(P)H	CYP55 family (P450nor), CYP119 family	Direct NO reduction from NADH (P450nor) or NAD(P)H (CYP119A2)	[14]
H_2O_2	CYP152 family (e.g., BSβ, SPα)	Peroxide shunt used as surrogate donor to avoid redox partners and NAD(P)H utilization with various P450s	[59,60]
Fatty acid hydroperoxide	CYP74 (allene oxide synthase), CYP5 (thromboxane synthase), CYP8A (prostacyclin synthase)	Fatty acid peroxides used to supply both substrate and oxygen activator, bypassing the catalytic cycle to Cpd II	[15,42]
Peroxidase fusion	CYP6001 family (e.g., Ppo, *Aspergillus nidulans*)	P450–peroxidase/dioxygenase natural fusion	[61]
Hydrolase fusion[b]	CYP631B5 (*Penicillium brevicompactum*)	P450–(Zn) hydrolase natural fusion involved in mycophenolic acid formation *in vivo*	[100]
m-CPBA	CYP119A1 (*Sulfolobus acidocaldarius*), CYP2B4 (human)	*m*-CPBA (surrogate donor of oxygen and hydrogen) used to identify CYP119A1 Cpd I and drive P450 catalysis	[31]
Fdx + PSI + DCPIP + NaAsc	CYP79A1 (*Sorghum bicolor*), CYP124A1 (*Mycobacterium tuberculosis*)	Surrogate donor system using plant PSI, DCPIP and NaAsc	[91]
Electrode system	Various (e.g., CYP101A1, CYP2D6, CYP3A4)	Assembly of P450s on electrodes (e.g., glassy carbon-, PDDA-coated)	[98]
Metal powder + mediator	CYP102A1 (BM3, *Bacillus megaterium*)	Use of Zn dust and Co(III) sepulchrate as electron transfer mediator	[99]
Electron tunneling wires + light + quencher	CYP101A1 (P450cam, *Pseudomonas putida*)	Laser flash photoreduction of wire conjugates bound to the P450 (ruthenium complexes, e.g., Ru–C_{11}-ethylbenzene)	[99]

[a]Abbreviations: FdR, ferredoxin reductase; Fdx, ferredoxin; CPR, cytochrome P450 reductase; PDOR, phthalate dioxygenase reductase type protein; FLD, flavodoxin; FLDR, flavodoxin reductase; *m*-CPBA, *meta*-chloroperbenzoic acid; PSI, photosystem I; DCPIP, dichlorophenolindophenol; NaAsc, sodium ascorbate; PDDA, polydiallyldimethylammonium chloride.

[b]Identified in whole cells; other factors may be required.

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 stereoisometric 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.

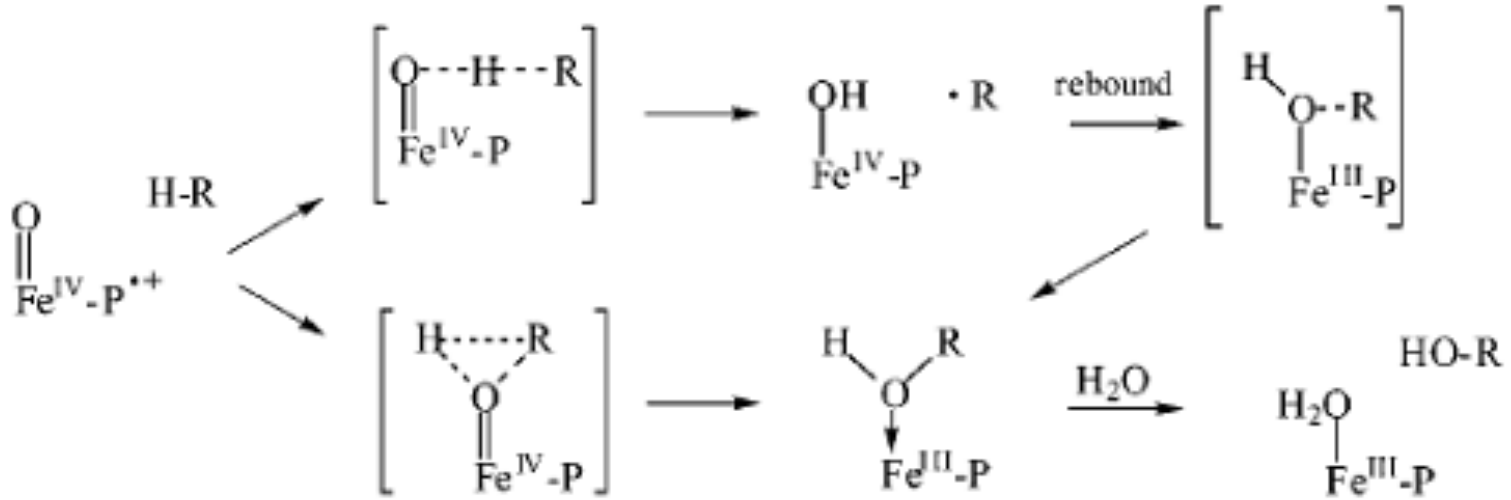
Isotope Tracers: Detect rearrangements, source of atoms in products (e.g. $^{18}O_2$, D_2O). (only net change is isotope loss or introduction)

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



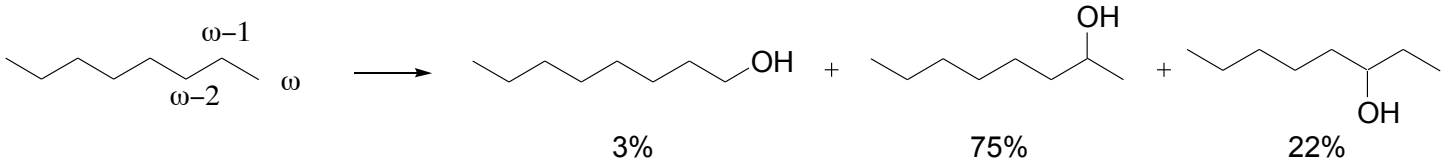
Auclair, K., Hu, Z., Little, D., Ortiz de Montellano, P. and Groves, J. **JACS.** VOL. 124, NO. 21, 2002

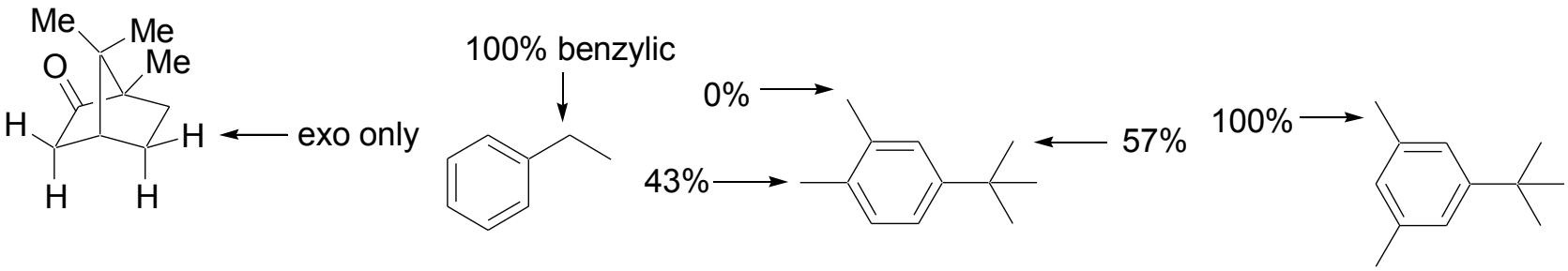
A major determinant in the regioselectivity of hydroxylation is the relative activation energy for the hydrogen atom abstraction and the stability of the radical intermediate being formed.

In aliphatic compounds the ease of hydroxylation follows the trend 3°(benzylic and allylic)>2° >1°. 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.

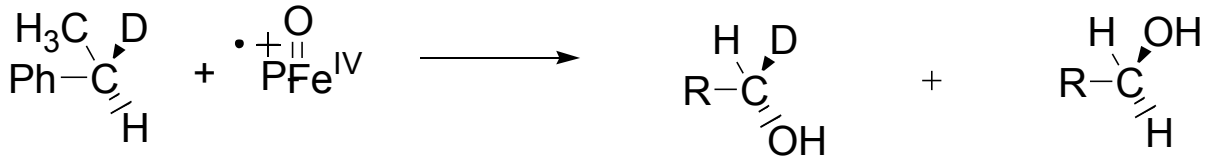
Terminal hydroxylation of octane:





The Abstraction Recombination Mechanism

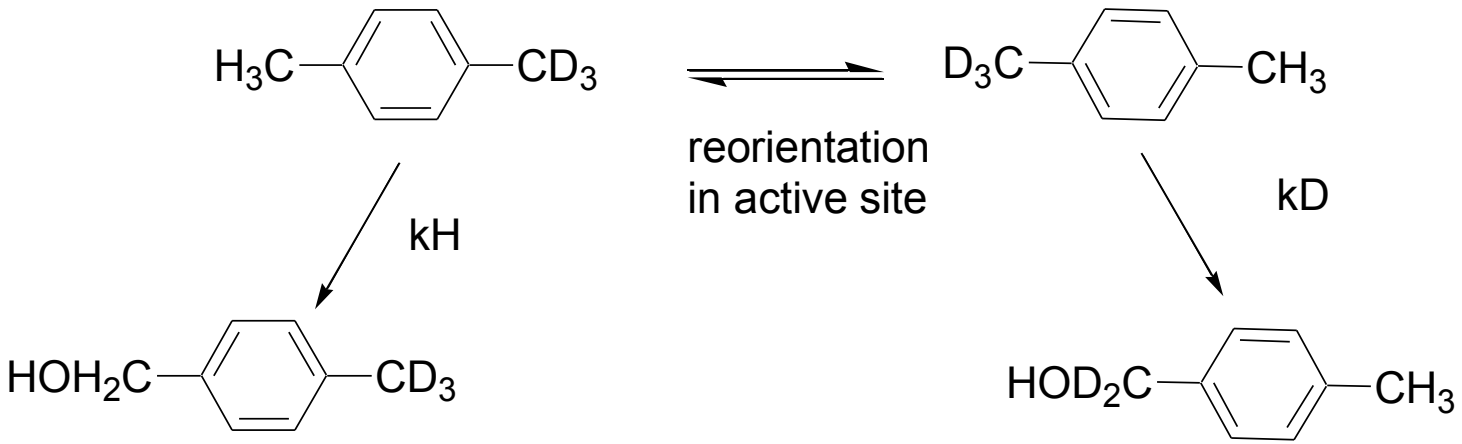
- Evidence supporting the abstraction/recombination mechanism came from experiments measuring intrinsic isotope effects. When alkyl substrates are deuterated in such a way to expose the intrinsic isotope effect. Now the perferryl oxygen can choose to attack either a C-H or C-D on the same compound.



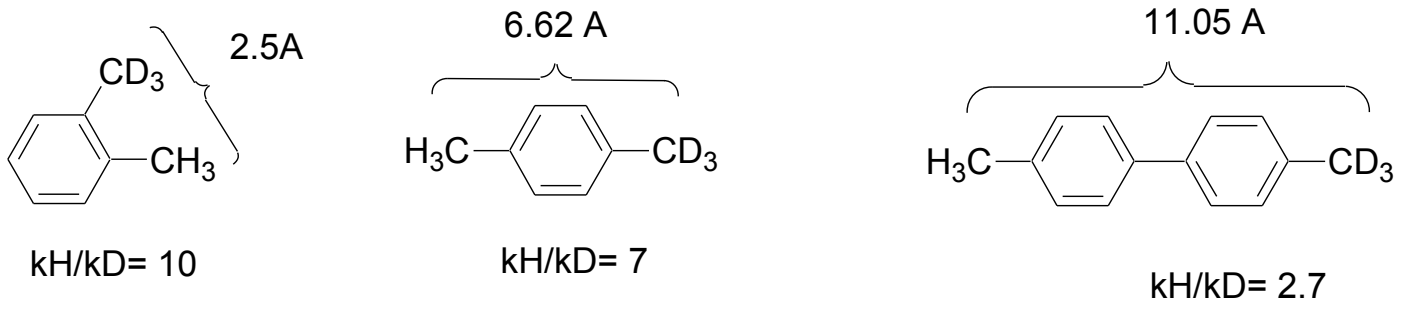
k_H/k_D intra = 6.7

High deuterium isotope effects (kH/kD > 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 [C•, •H], [C+, H-] or [C-, 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. Biochemisty 1989, 28: 9012-9018, Hanzlik et al. JACS 1993, 115: 9363-9370 and JACS 1999 121: 41-47.



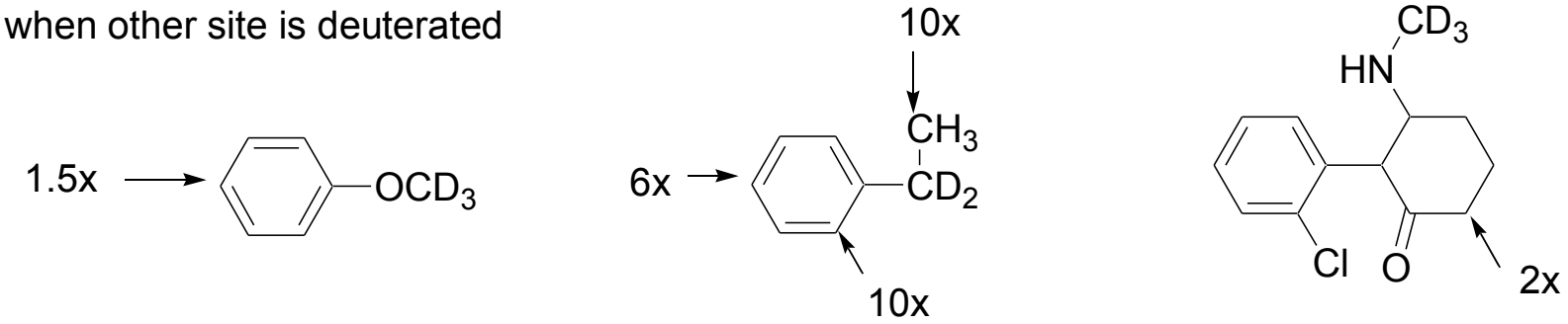
As the distance between chemically identical sites increases, the ratio of metabolites due to hydrogen vs. deuterium abstraction decreases. High switching means that the lifetime of the perferryl intermediate is much longer than the rate of reorientation in the active site. Low metabolic switching means 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.

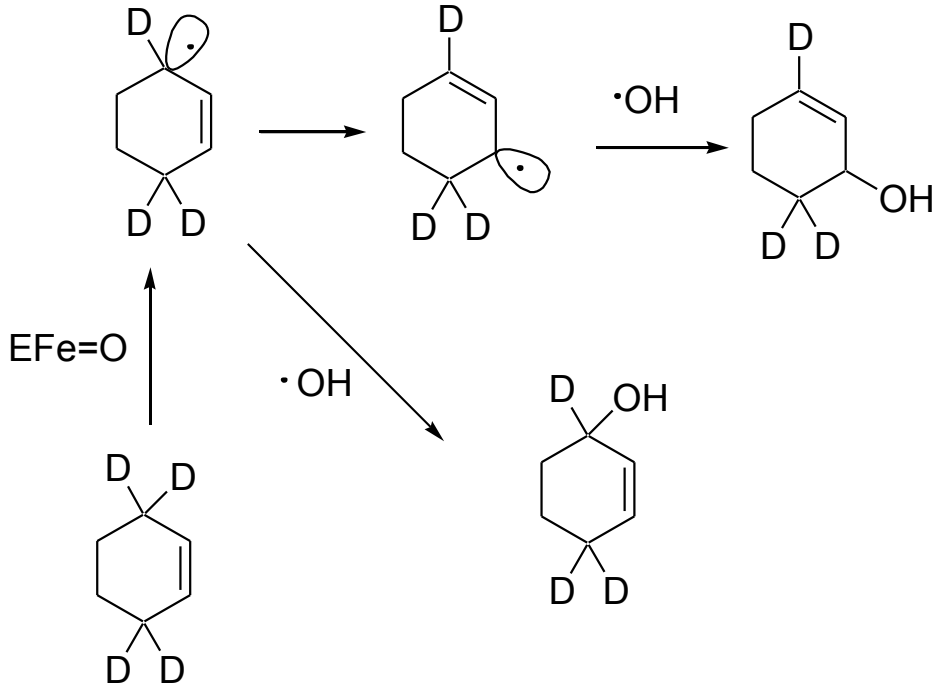
Rate of oxidation increase when other site is deuterated

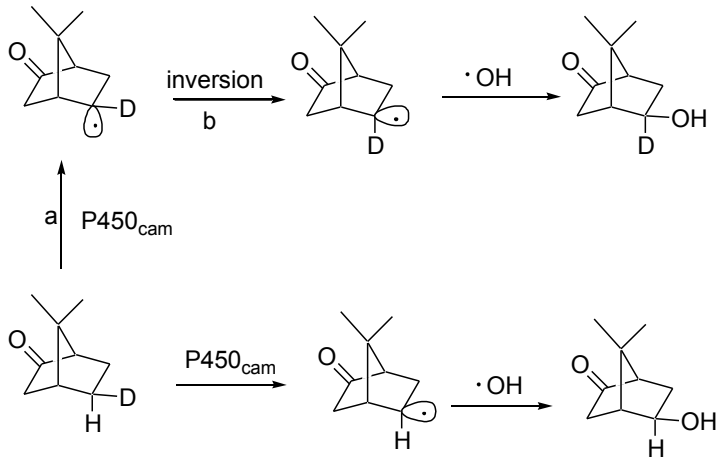


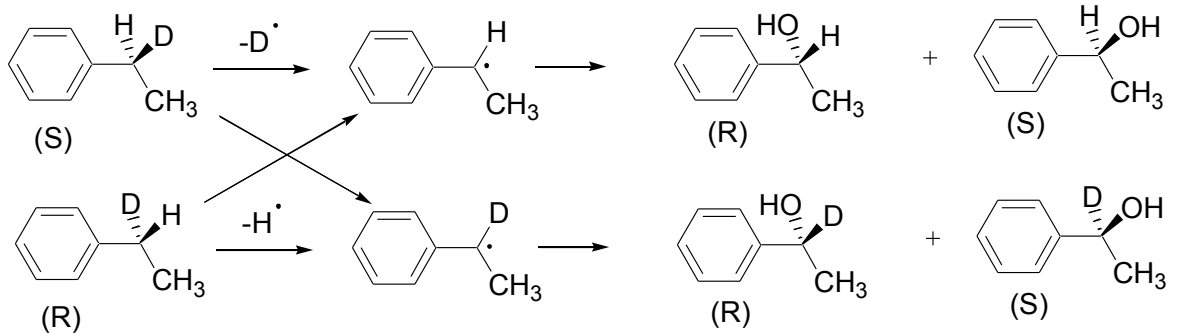
What is the nature of the intermediate formed in aliphatic hydroxylations?

- 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.*







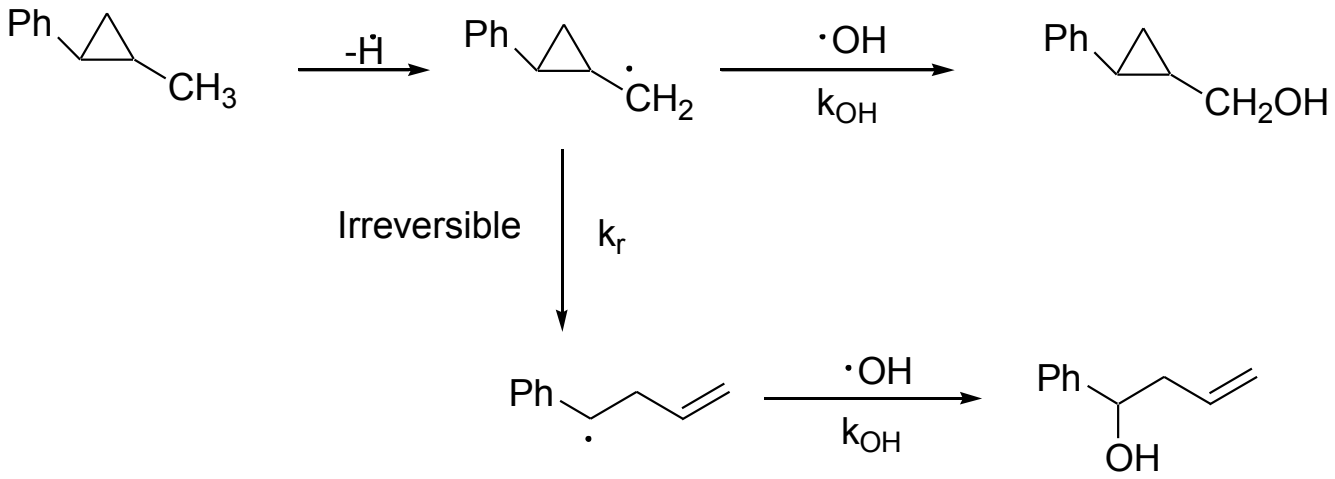
These results, along with the large intrinsic kinetic isotope effects, strongly suggest that a discrete radical is formed during aliphatic hydroxylation reactions and that it has a finite lifetime, which allows it to rearrange or invert prior to the C-O bond-formation step.

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

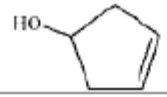
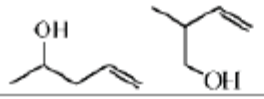
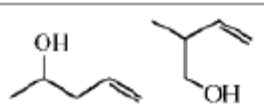
Radical clocks contain a highly sensitive functionality (e.g. a cyclopropane ring) that will rapidly rearrange if a radical is formed during the reaction.

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.

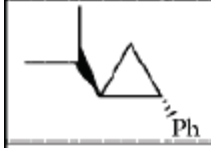
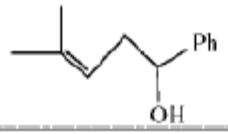
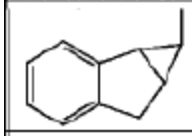
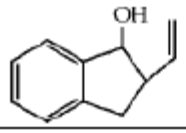
The unimolecular Cyclopropylcarbinyl radical ring opening rate constant provides a clock for oxygen rebound along with the ratio of rearranged vs. unrearranged products. The rate of oxygen rebound for radical clock substrates have been estimated from 1010 to 10^{11} s-1.



"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} 1.8×10^{8}	Rat liver microsomes	1.5×10^{10} 1.6×10^{10}
		8.0×10^{8} 2.3×10^{8}	Rat liver microsomes	1.9×10^{10} 1.8×10^{10}

"Fast" radical clocks

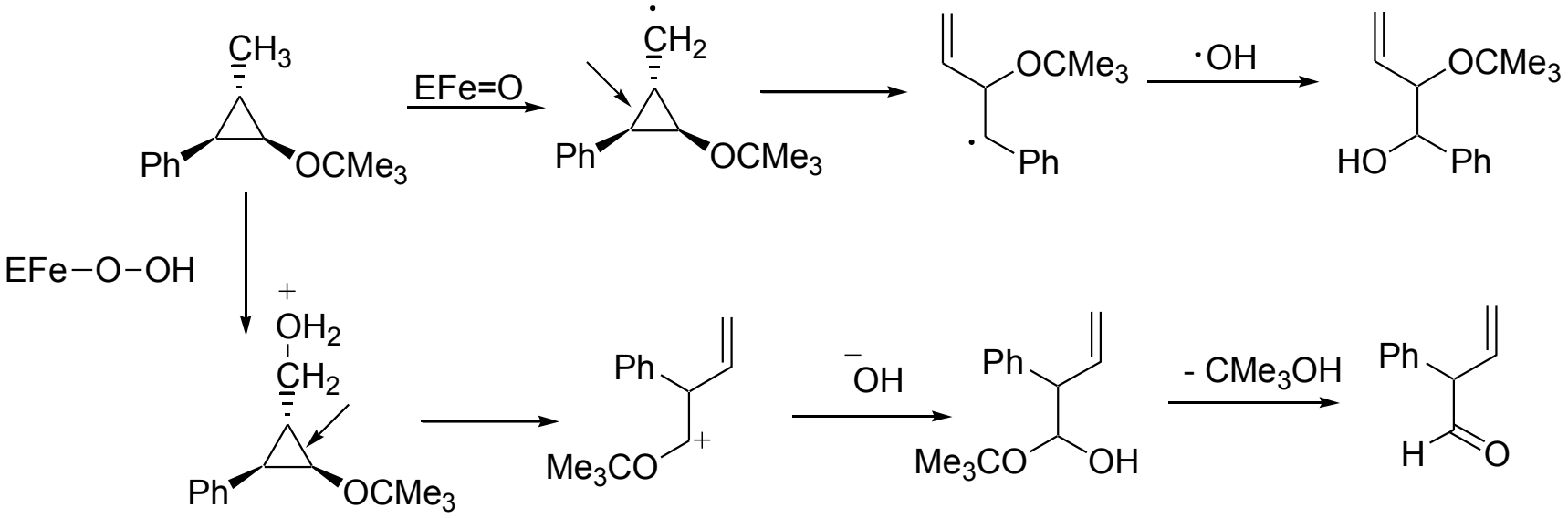
		1.2×10^{11}	CYP2B1	$>1,2 \times 10^{13}$
		3×10^{11}	Rat liver microsomes, CYP2B1	1.4×10^{13}

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 (kOH) 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.

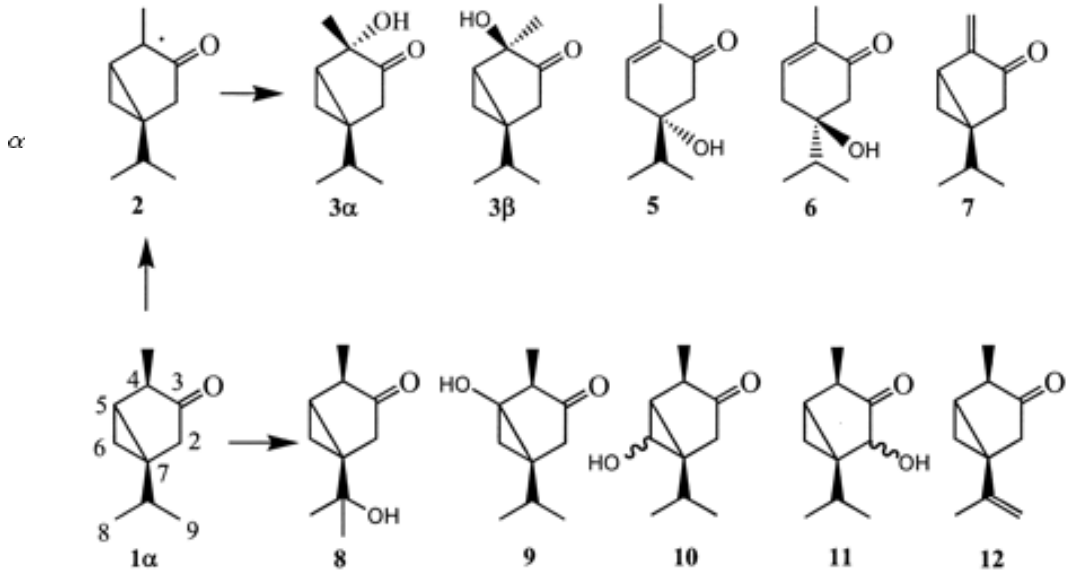
Newcomb *et al*. observed rearrangement products resulting form cationic intermediates as well as products from radical rearrangement. The Measured rate of oxygen rebound measured at 10^{13} s^{-1} (too fast for a true intermediate). Newcomb has interpreted these results to suggest that a discrete carbon 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



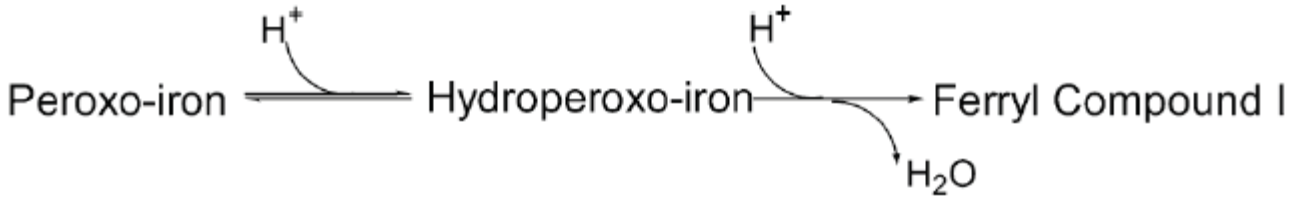
Auclair K, Hu, Z., Little, D., Ortiz de Montellano, P., and Groves, J. 2002 JACS, 124 2806-2817. Results agree with earlier studies implicating a radical mechanism rather than an OH+ mechanism and suggests radical lifetimes from 16 to 50 ps. Only trace amounts of cationic rearrangement products were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

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}.

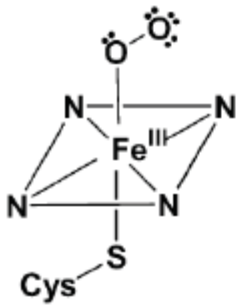


Products resulting from formation of a radical intermediate upon P-450-catalyzed oxidation of α-thujone

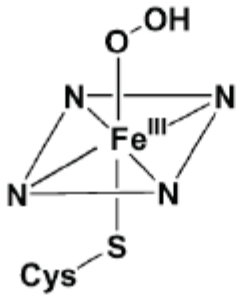
P450 – Multiple Oxidants? – "Two-state" Mechanism



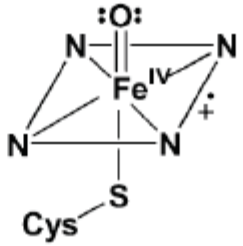
Nucleophilic	Electrophilic	Electrophilic	Electrophilic
Deformylation	Epoxidation Sulfoxidation? Hydroxylation (inserts OH^+)?	Epoxidation Sulfoxidation Hydroxylation (inserts O)?	Hydroxylation (abstracts H)



Ferric peroxo anion

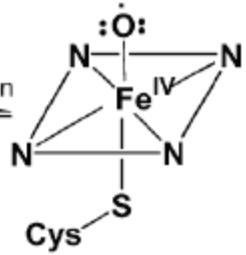


Ferric hydroperoxo



Low Spin

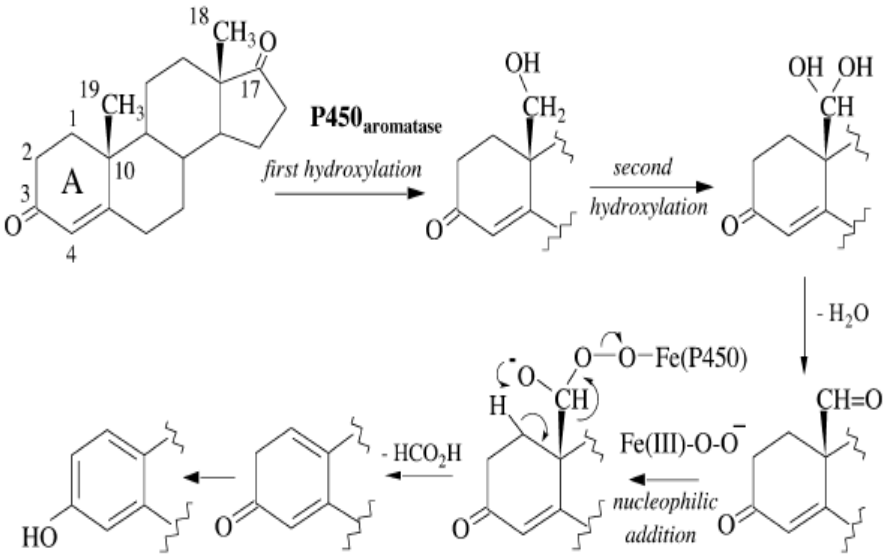
Spin Inversion



High Spin

Evidence for reactions involving alternative oxidant species

Peroxo-iron acting as a nucleophilic oxidant in CYPC19 (aromatase) catalyzed biotransformation of androgens to estrogens via oxidative deformylation of the 19-aldehyde to release formic acid and estrone.

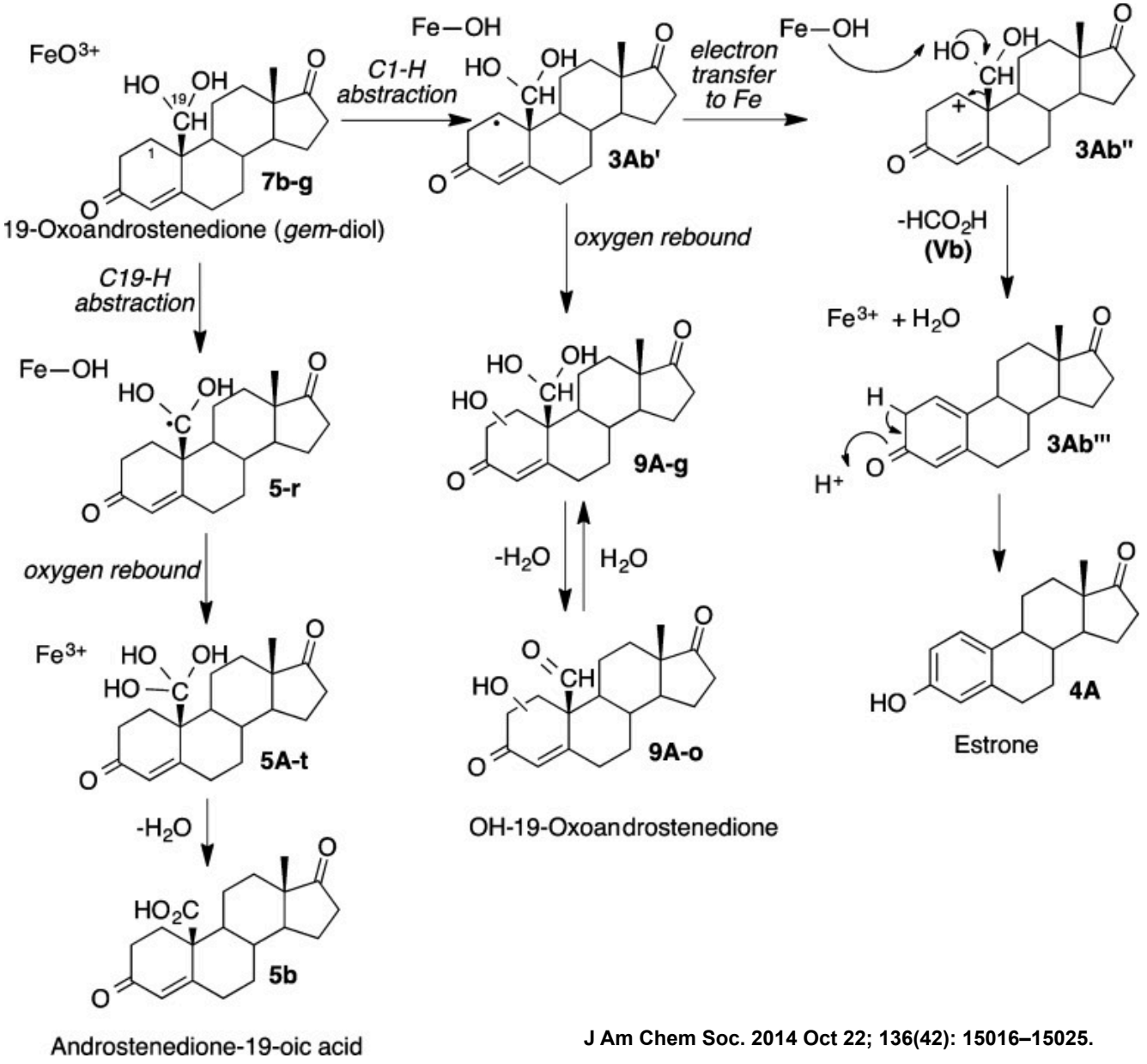


The peroxy-hemiacetal like complex was shown to be a strong contender to explain the C-C bond scission in experiments using deuterated starting material and $^{18}O2$. Formic acid also enriched with ^{18}O.

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.

However even this reaction can be catalyzed by Cmp I



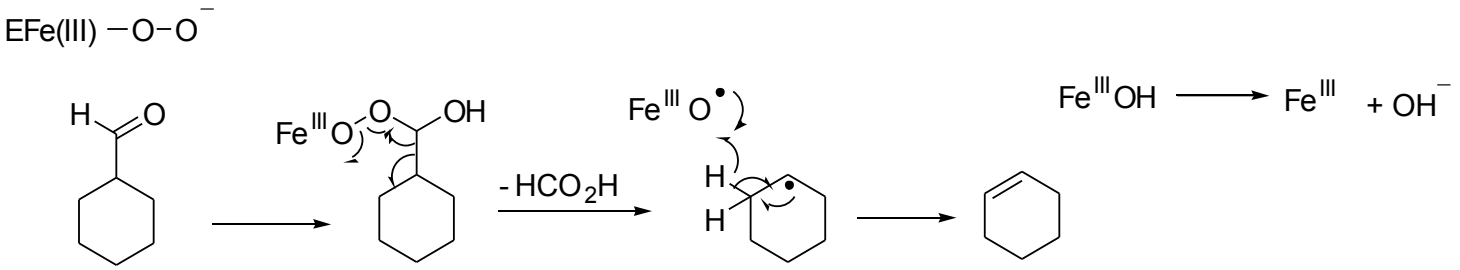
J Am Chem Soc. 2014 Oct 22; 136(42): 15016–15025.

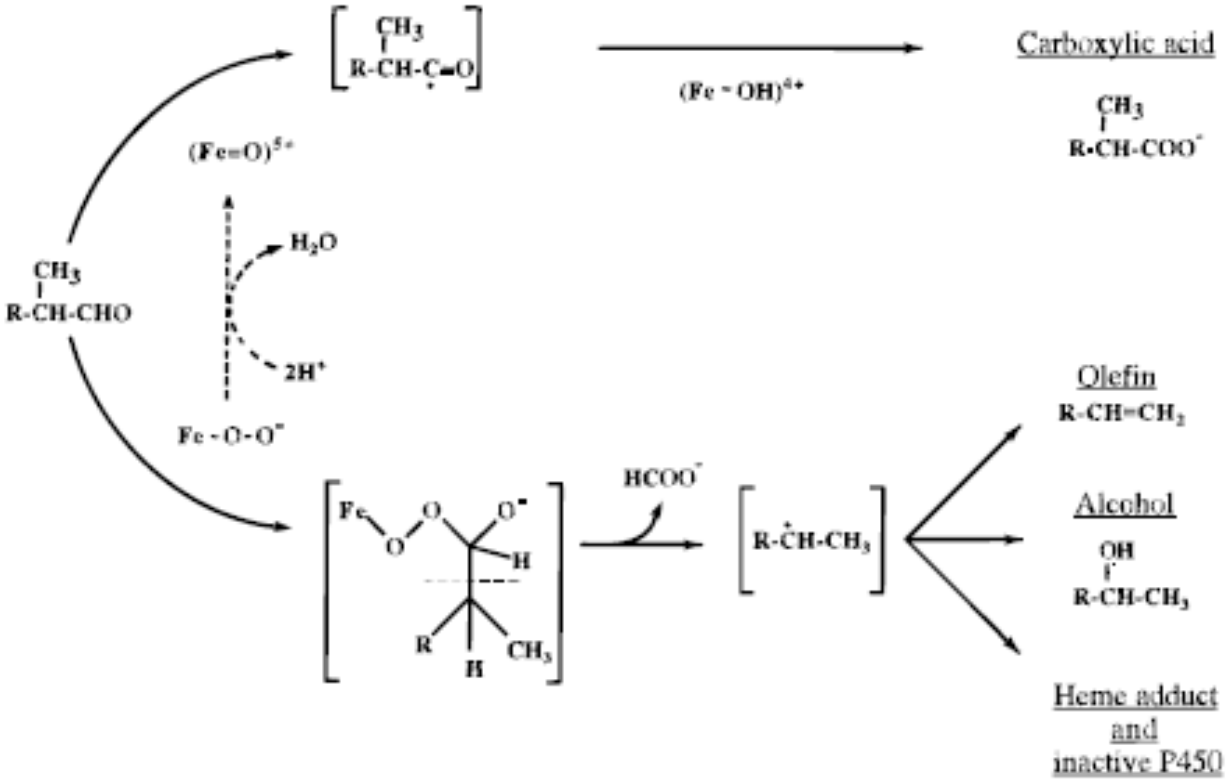
Historic evidence for alternative oxidants in drug metabolizing P450 enzymes

- Vaz and Coon invoked the iron-peroxo intermediate to explain the deformylation of aldehyldes 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

	Ratio of initial rates, epoxidation/hydroxylation			
Substrate	Δ2B4	Δ2B4 T302A	Δ 2E1	Δ 2E1 T303A
Cyclohexene	1.7	1.7	1.0	2.4
cis-butene	43	ND*	90	460
trans-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 O_2 to H_2O_2 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 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.