Cytochrome P450 Catalytic Mechanisms I

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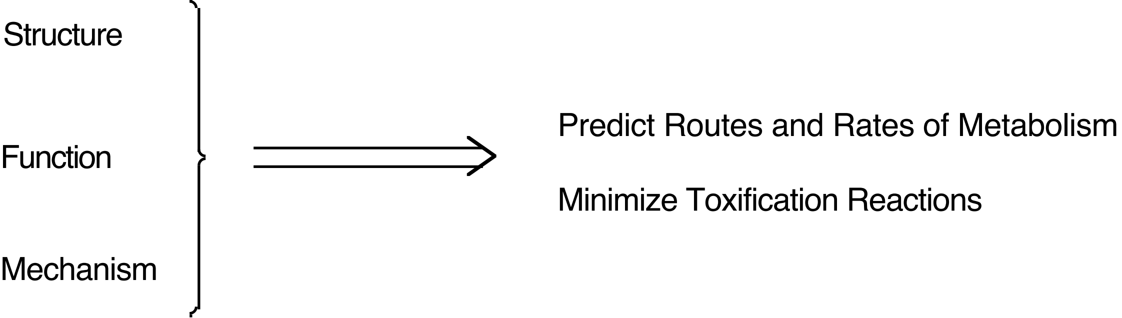
We don't have a crystal ball here, and progress is as much as anything a function of knowing when we are in one of those periods where what we know isn't worth much, and what we don't know is really important. Roger MacNamee (Silver Lake Partners)



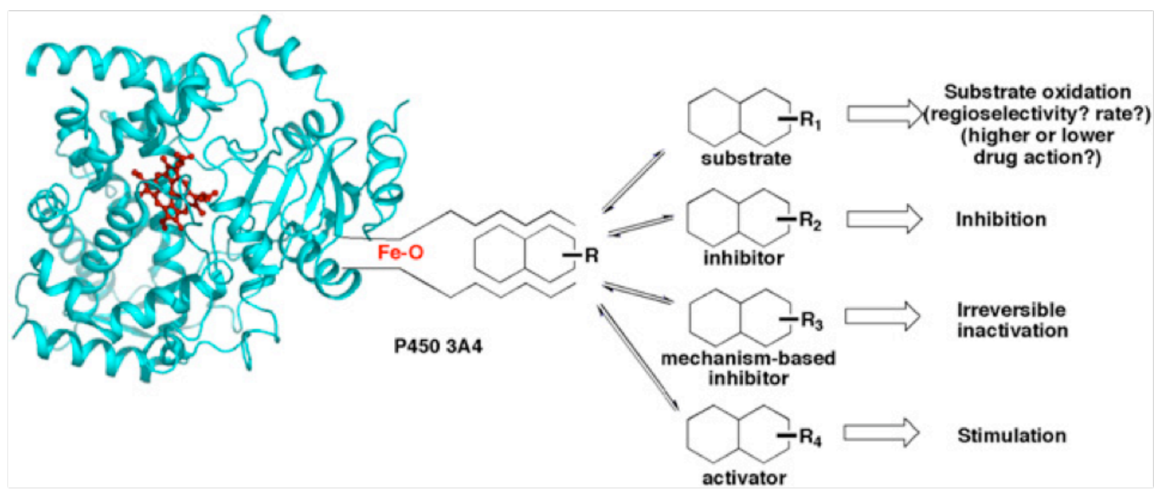


Figure 1. Outcomes of Interaction of a Ligand with a P450
The interaction of a compound with a P450 (P450 3A4 structure is shown, pdb code 1TQN) can be related to the chemical being a substrate, inhibitor, irreversible inhibitor, or stimulator—or combinations thereof. Also note that many P450 ligands can interact via multiple binding modes, leading to multiple products in the case of substrates (Guengerich, 2005).

General References:

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

2. Sono, M. Roach, M., Coulter, E, and Dawson, J. *Heme Containing Oxygenases*. Chem. Rev. **96**: 2841-2887 (1996).

3. Ortiz de Montellano, P. and De Voss, J. *Oxidizing Species in the Mechanism of Cytochrome P45*0 Nat. Prod Rep **19**: 477-493 (2002)

4. Ortiz de Montellano, P (ed) *Cytochrome P450; Structure, Mechanism and Biochemistry 3rd Ed*. Plenum Press (2005).

5. Meunier, B., De Visser, S. and Shaik, S. *Mechanism of Oxidation Reactions Catalyzed by Cytochrome P450 Enzymes* Chem. Revs. **104**, 3497-3980 (2004).

6. Rittle and Green *Cytochrome P450 Compound I: Capture, Characterization and C-H Bond Activation Kinetics* Science 330: 933-937 (2010) also supplement

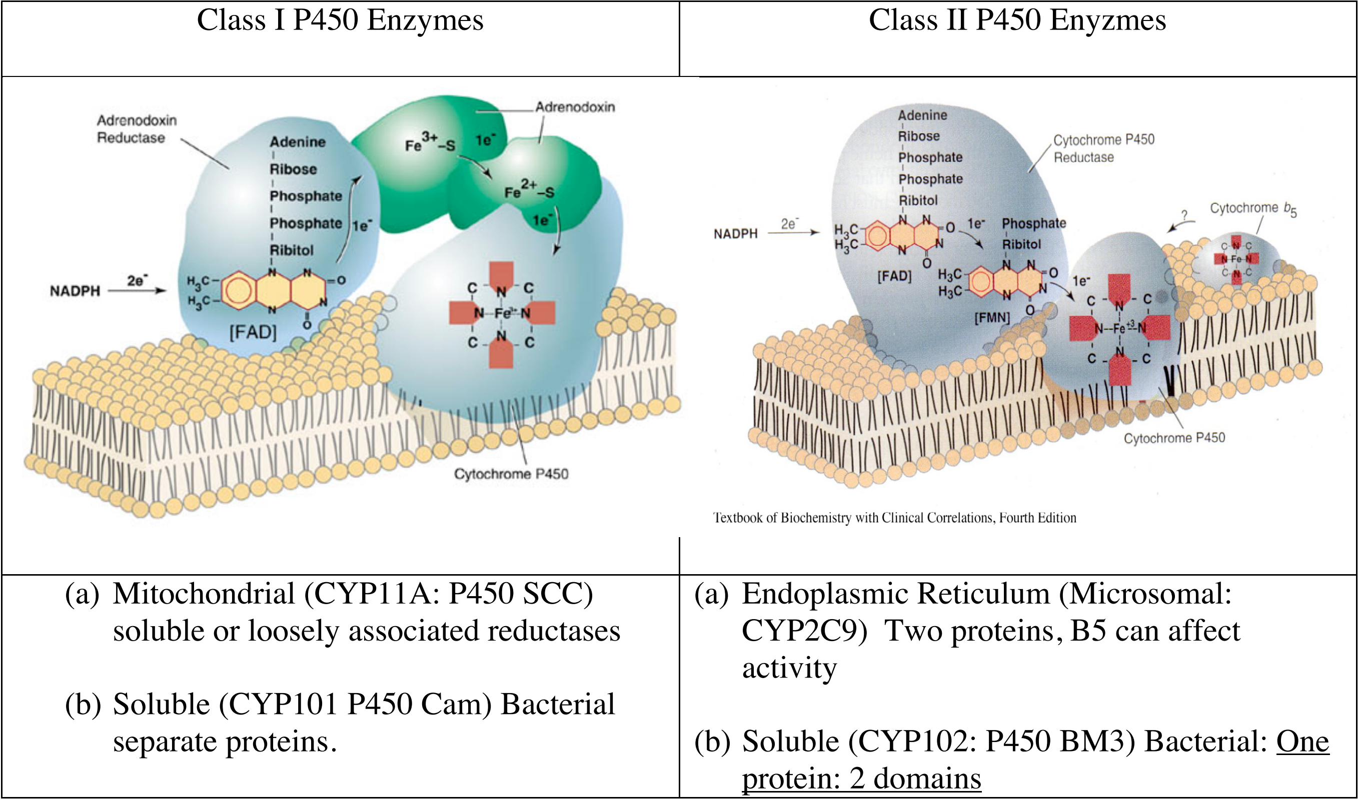
7. Isin and Guengerich *Substrate Binding to Cytochromes P450* Anal Bioanal Chem 392: 1019-1030 (2008)

8. Im and Waskell *The interaction of microsomal cytochrome P450 2B4 with it's redox partners* Arch. Biochem. Biophys 2010 in press.

<u>Basic Stoichiometry of P450 Enzymes: Mixed Function Oxidases</u>

$$RH + NAD(P)H + O_2 + H^+ \rightarrow ROH + NAD(P)^+ + H_2O$$

<u>Major P450 Classes Require Multiple Proteins/Domains for Activity</u>



Class I P450 Enzymes	Class II P450 Enyzmes
(a) Mitochondrial (CYP11A: P450 SCC) soluble or loosely associated reductases (b) Soluble (CYP101 P450 Cam) Bacterial separate proteins.	(a) Endoplasmic Reticulum (Microsomal: CYP2C9) Two proteins, B5 can affect activity (b) Soluble (CYP102: P450 BM3) Bacterial: <u>One protein: 2 domains</u>

<u>Reductase enzymes are required to supply reducing equivalents to the P450 enzymes from NADPH.</u>

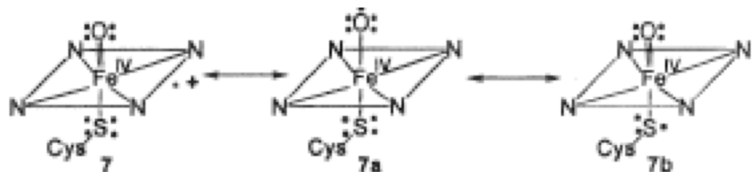
1. NADPH (source of electrons) is an obligate two electron donor (must transfer hydride).

2. Flavins (FAD and FMN) each have 2 accessible oxidation states.

FAD or FMN (oxidized) $\overset{e^- + H^+}{\rightleftharpoons}$ FAD· or FMN· (semiquinone) $\overset{e^- + H^+}{\rightleftharpoons}$ $FADH_2$ or $FMNH_2$ (reduced)

3. Electrons must be delivered one at a time to the enzyme during the cycle so the odd-electron semiquinone form of FMN is important in the Class II enzymes. FAD accepts hydride from NADPH which is immediately split amongst FAD and FMN.
(See Biochem Biophys Acta **1698** 1-24 (2004) for a recent review)

P450 enzymes catalyze a broad range of oxidative reactions. The oxidation chemistry is usually driven by the reaction of a hypervalent iron-oxo species, called Compound I created by the P450 cycle, with the substrate.



(a) Hydrocarbon hydroxylation

$-CH \rightarrow -C-OH$

(b) Alkene epoxidation / Alkyne oxygenation

(i) $C=C \rightarrow C(O)C$ (epoxide)

(ii) $R-C\equiv C-H \rightarrow [R(H)C=C=O] \xrightarrow{H_2O} RH_2C-C(=O)OH$

(c) Arene epoxidation, aromatic hydroxylation, NIH shift

R–C₆H₄–X → arene oxide → R–C₆H₄–OH + (X, OH)-substituted arene

(d) *N*-Dealkylation

$R-NH-Me \rightarrow [R-NH-CH_2OH] \rightarrow R-NH_2 + HCHO$

(e) *S*-Dealkylation

$R-S-Me \rightarrow [R-S-CH_2OH] \rightarrow R-SH + HCHO$

(f) *O*-Dealkylation

$R-O-Me \rightarrow [R-O-CH_2OH] \rightarrow R-OH + HCHO$

(g) *N*-Hydroxylation

$-C-NH_2 \rightarrow -C-NHOH$

(h) *N*-Oxidation

$=N \rightarrow =N^{+}-O^{-}$

(i) *S*-Oxidation

$R-S-Me \rightarrow R-S^{+}(O^{-})-Me$

(j) Oxidative deamination

$R-CH(NH_2)-Me \rightarrow [R-C(NH_2)(OH)-Me] \rightarrow R-C(=O)-Me + NH_3$

(k) Oxidative dehalogenation

$R_1-CH(R_2)-X \rightarrow [R_1-C(R_2)(OH)-X] \rightarrow R_1-C(R_2)=O + HX$

(l) Alcohol and Aldehyde oxidations

(i) $R-CH(R'(H))-OH \rightarrow [R-C(R'(H))(OH)-OH] \rightarrow R-C(R'(H))=O + H_2O$

(ii) $R-C(=O)H \rightarrow R-C(=O)OH$

(m) Dehydrogenation

(i) $-CH_2-CH_2- \xrightarrow{-H\cdot} [-CH^{\cdot}-CH_2-] \xrightarrow{-e^-, -H^+} -CH=CH-$; (Hydroxylation) $\rightarrow -CH(OH)-CH_2-$

(ii) HO–C₆H₄–NHAc $\xrightarrow{-2e^-, -2H^+}$ O=C₆H₄=NAc

(n) Dehydrations

(i) $R(H)C=N-OH \rightarrow R-C\equiv N + H_2O$

(ii) $R-CH=CH-CH(OOH)-R' \rightarrow$ R-epoxide-R' $+ H_2O$

(o) Reductive dehalogenation

$R_1-C(R_2)(R_3)-X \xrightarrow{+e^-} R_1-C^{\cdot}(R_2)(R_3) + X^-$

(p) *N*-Oxide reduction

$\geq N^{+}-O^{-} \xrightarrow{+2e^-\ (+2H^+)} \geq N\ (+H_2O)$

(q) Epoxide reduction

arene oxide $\xrightarrow{+2e^-, +2H^+}$ benzene $+ H_2O$

(r) Reductive β-scission of alkyl peroxides

$X-C(R)(R')-OOH \xrightarrow{+2e^-, +2H^+} X-C(R)=O + R'H + H_2O$

(s) NO reduction

$2NO \xrightarrow{+2e^-, +2H^+} N_2O + H_2O$

(t) Isomerizations

Prostaglandin H_2 (PGH_2) → Prostaglandin I_2 (PGI_2) / Thromboxane A_2 (TxA_2)

(u) Oxidative C-C bond cleavage

(i) $R-C(OH)-CH(OH)-R' \xrightarrow{+O_2,\ +2e^-,\ -H_2O} R-C(=O)- + R'-CHO$

(ii) cyclic $-C(R)-C(=O)R' \xrightarrow{+O_2,\ +2e^-,\ +2H^+,\ -H_2O}$ cyclic alkene $-R$ + $R'-COOH$

Below see a proposed sequence for oxidation of a carbon hydrogen bond to an alcohol. Notice the degnerate states of Compound I lying close in energy as well as the intermediates of the reaction. Rheem will talk more about this.

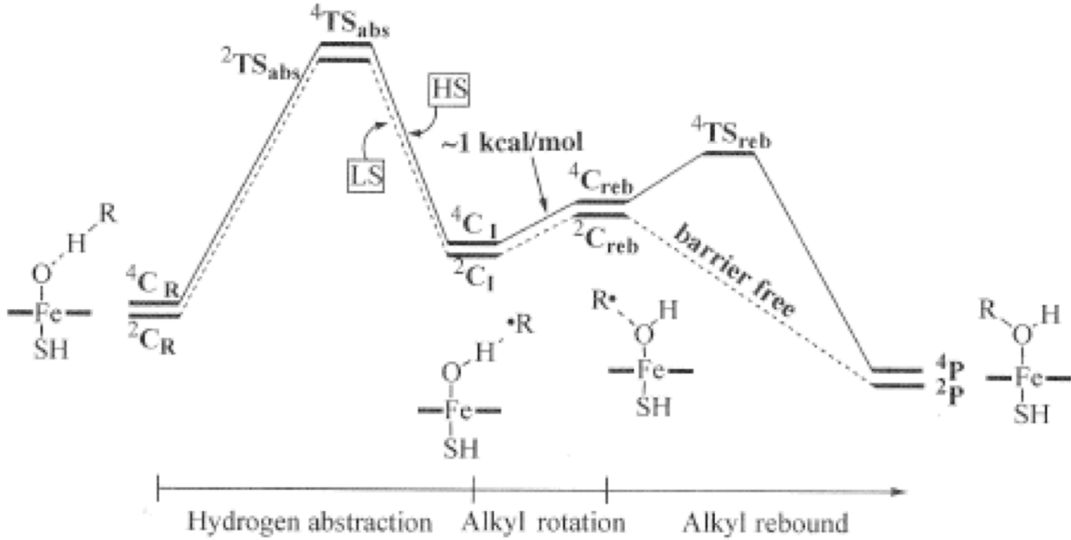


Figure 2.17. A two-state reactivity potential energy surface for alkane (R–H) hydroxylation by Cpd I.

P450 enzymes have broad and overlapping substrate specificities.

1. Multiple binding orientations: Single enzyme-single substrate: multiple products

2. Large active sites: Single substrate-different enzymes: same products or products formed at highly different rates (product ratios and rates different: why?).

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TABLE 1.3 *Substrate specificity and product regiospecificity of purified rat hepatic cytochromes P450* (Levin, 1990)

Isozyme*	Substrate and reaction (nmol min^{-1} [nmol P450]$^{-1}$)†												
	A	B	C	D	E	F	G	H	I	J	K	L	M
P450a	2.3	0.5	0.2	0.9	—	0.6	—	—	—	—	20.9	—	—
P450b	132.5	42.7	0.4	3.3	—	9.6	1.8	1.8	—	—	—	9.1	—
P450c	6.7	0.9	23.4	60.8	1.7	97.1	21.6	1.0	—	—	—	—	1.9
P450d	3.9	—	0.3	21.1	13.8	0.6	0.7	9.6	—	0.5	—	—	0.7
P450e	19.8	8.2	0.1	1.1	0.9	2.0	—	—	—	—	—	0.8	—
P450f	1.3	—	—	0.9	0.5	—	—	—	—	0.4	—	0.8	—
P450g	4.9	1.1	—	—	0.6	1.1	—	—	—	—	—	0.3	3.8
P450h	52.1	22.6	1.8	7.4	8.0	0.9	1.5	1.5	—	1.1	—	7.9	0.2
P450i	2.8	1.0	—	0.7	1.3	0.7	—	—	4.8	—	—	—	—
P450j	5.5	—	—	3.4	0.8	1.2	1.6	12.7	—	15.9	—	—	—
P450k	14.1	4.5	0.4	3.4	2.5	0.5	1.3	1.0	—	0.5	—	—	—

*See Table 3.2 in Chapter 3 for current nomenclature.
†Empty boxes indicate negligible values.

A: Benzphetamine N-demethylation
B: Hexobarbital 3-hydroxylation
C: Benzo[*a*]pyrene 3/9-hydroxylation
D: Zoxazolamine 6-hydroxylation
E: Estradiol-17β 2-hydroxylation
F: 7-Ethoxycoumarin O-dealkylation
G: *p*-Nitroanisole O-demethylation
H: Aniline *p*-hydroxylation
I: Androstane disulfate 15β-hydroxylation
J: N-Nitrosodimethylamine N-demethylation
K: Testosterone 7α-hydroxylation
L: Testosterone 16α-hydroxylation
M: Testosterone 6β-hydroxylation

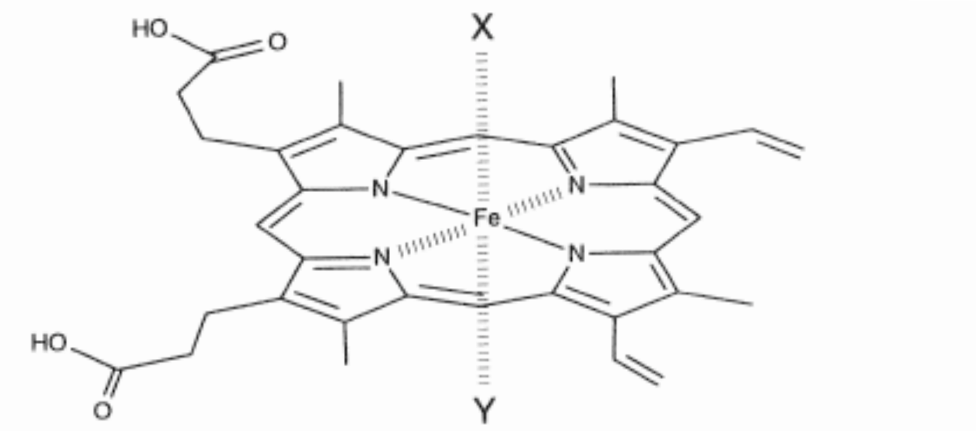
Testosterone (L) (K) (M)
7-ethoxycoumarin (F)
aniline (H)

CYP3A4
Multiple binding modes
Single Enzyme-Multiple Products

Heme iron is the catalytic center of the P450 enzymes (Fe + Protoporhyrin IX = Heme).

a. The heme iron has two stable oxidation states: Ferric (Fe^{+3}) and Ferrous (Fe^{+2}).

b. The iron itself can be either high spin (more unpaired electrons in the iron d orbitals) or low spin (fewer unpaired electrons in the iron d orbitals).



c. The iron atom of heme is bonded to the 4 pyrrole nitrogens of protoporphyrin IX. These nitrogen atoms are referred to as equatorial ligands. The 4 nitrogens have a net negative charge of -2. Thus ferrous heme is electronically neutral and ferric heme has a charge of +1.

d. Mixing between orbitals of porphyrin and iron occur. The electronics of the porphyrin macrocycle are important in stabilizing hypervalent states of the heme iron that occur during the P450 cycle.

e. The heme iron has two additional sites for interaction with ligands. These positions are referred to as the axial ligand positions (X and Y above).

f. The 5th ligand position of all P450 enzymes is occupied by a cysteinate sulphur anion (thiolate). This cysteine is located on the proximal face of the heme and is critical to the generation Compound I. Generally, high spin (iron out of plane towards cysteinate) and low spin (iron in plane) states are often in equilibrium and their distribution is ligand dependent.

low spin

high spin

UV-VIS Spectra

The heme electronic absorption spectrum (UV-VIS) is dominated by a $\pi \rightarrow \pi^*$ transition of the aromatic 18 π electron porphyrin macrocycle which gives rise to the Soret absorbance. The λ_{max} of the Soret peak occurs in the 380 to 460 nm region. The wavelength of the maximum absorption is highly dependent on the identity of the sixth ligand, the iron spin state and oxidation state as well as other factors. There are other low intensity bands at higher wavelengths. We will only look at the Soret peak.

UV-VIS spectroscopy is a sensitive tool in P450 structure function research.

1. Characterize changes that occur in the distal substrate binding pocket upon binding of substrates and inhibitors via changes in spin state.
2. Determine ligand affinities and mode(s) of ligand binding.
3. Characterize mutant proteins and detect protein-protein interactions with reductase partners.
4. Assess P450 content using the extinction coefficient for the ferrous CO complex.
5. Diagnose mechanisms of enzyme inactivation.

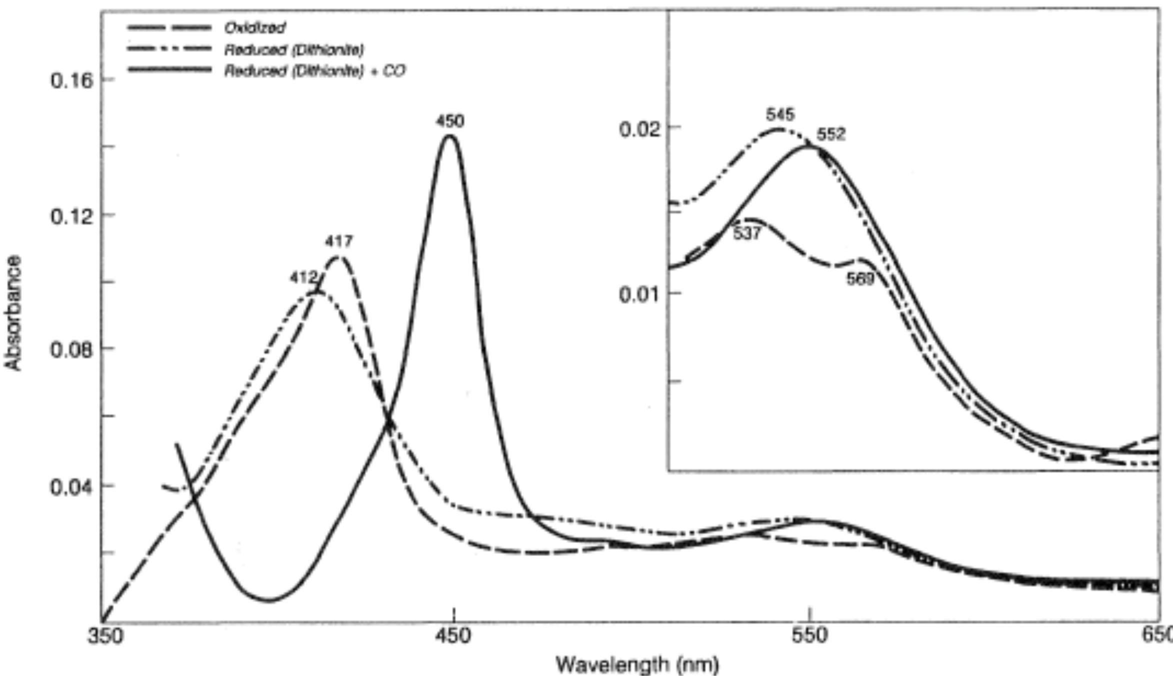


FIGURE 3.6 Absolute spectra of oxidized (low-spin), reduced, and reduced CO-complexed cytochrome P450 in microsomes from untreated male rats; the reference cuvette contained the same microsomal suspension in which cytochrome P450 (but not cytochrome b_5) had been destroyed by linoleic acid hydroperoxide. Reproduced from Nerland *et al.* (1981) with the permission of the copyright holder.

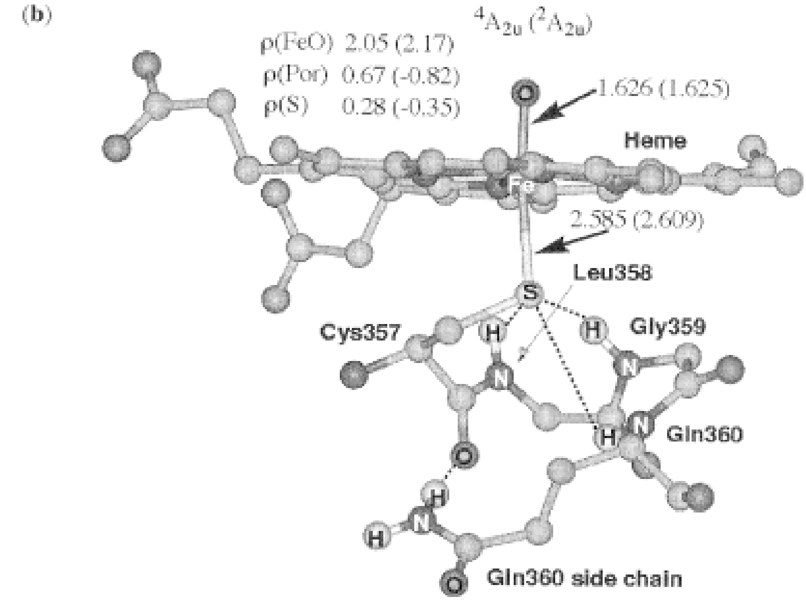
Cytochrome P450 Catalytic Cycle

1. The point of the P450 cycle is to generate Compound I shown below as calculated by Shaik. This is the species that usually causes oxidation of substrates. The oxygen atom itself is electrophilic and reacts with substrates in 3 major ways:

a. Oxygen attack on a pi bond which starts with substrate pi electrons forming a bond with the Fe-O.

b. Hydrogen atom abstraction from a C-H bond

c. Electron abstraction from a heteroatom such as nitrogen.

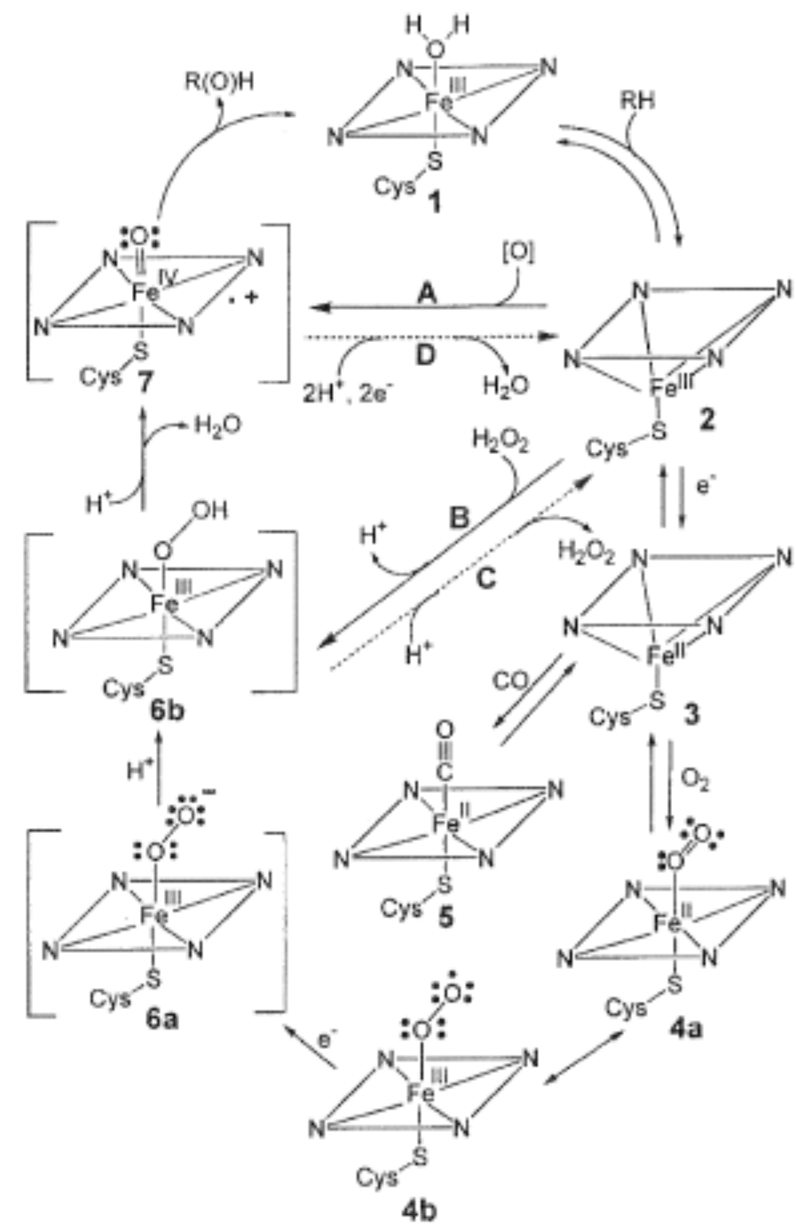


2. The heme iron is found in different oxidation and spin states during the progression through the catalytic cycle. The heme iron is highly sensitive to environment. Thus the iron moves in and out of the plane of the porphyrin ring in response to the presence of 6th ligands. Generally high spin states (more unpaired electrons in the heme) find the iron displaced further out the plane of the porphyrin ring relative to complementary low spin states.

Molecular orbital accounting for unpaired electrons, oxidation states and spin states is complex as many of what might be considered to be single species are actually degenerate (See Chapters 1 and 2 in Ref 5).

The nomenclature for the different P450 species involved in the catalytic cycle focuses on the oxidation state of the iron and the presence of ligands attached to the heme iron or somewhere in the active site. Generally any electrons shared via bonding interactions with the pyrrole nitrogens and macrocyle, thiolate or other ligands are usually viewed as belonging the ligand rather than the iron.

3. The Classical P450 Cycle (Reference 2)



1. Resting State of the Enzyme (ferric iron- no substrate):

The resting state (no substrate) of P450 is variably comprised of two states low spin(mostly) and high spin. The ratio of the spin states of the membrane bound forms may be sensitive to methods of isolation, phospholipid content in the membrane, the presence of other proteins such as the P450 redox partners (b5 and reductase) and aggregation state. Note that in the cycle diagram only the low spin state is shown with water as the sixth ligand. Multiple water molecules are normally present in the active site. These two spin states as in rapid equilibrium, particularly for the membrane-bound P450 enzymes.

a. Low Spin State (S=1/2) Water is the 6th ligand; difficult to reduce

Conventionally viewed as the resting state of the enzyme and the dominant state. H_2O occupies the ferric heme sixth ligand position in the distal (substrate binding) cavity of the enzyme. The heme exhibits a Soret λ_{max} = 418 nm, the heme iron is low spin (S=1/2), more in the plane of the porphyrin ring and difficult to reduce to the ferrous state (-300 mV). This is the dominant resting state of P450 cam and most other P450s. For P450 cam this state cannot be naturally reduced by redox partners. That may not be universally true for all enzymes and redox partners. All ferric P450's can be reduced by the artificial reductant sodium dithionite to the ferrous state.

b. High Spin State (S=5/2) like **(2)** No 6th ligand: easier to reduce

The heme sixth ligand position is not occupied in the high spin state. The heme exhibits a Soret λ_{max} = 390 nm, the heme iron is high spin (S=5/2), displaced further out of the plane of the porphyrin ring towards the thiolate 5th ligand and is much easier to reduce (-170 mV) to the ferrous state. The binding of certain types of non-polar substrates (Type I substrates, see below) 'displaces' H_2O and promotes the high spin state. In fact the binding of the natural substrate, camphor, to CYP101 releases 5-6 water molecules from the enzyme active site cavity. CYP3A4 is approximately 20% high spin. Binding of a ligand substrate, testosterone, changes it to 50% high spin.

2. Substrate (Ligand) Binding Spectra

Binding of substrates and other ligands to the ferric enzyme causes changes in the UV-VIS spectra due to changes in spin state populations and the effect of direct ligand interaction with the iron (think of nitrogens and oxygens). Let's remember that the resting state may be made up of high and low spin states to begin with. Generally the types of substrate binding spectra reflect the effects of ligand on the high spin/low spin equilibrium, displacement of the water sixth ligand and bonding interactions between ligand functional groups and the heme iron.

Absolute vs Difference Spectra: P450 enzymes have absolute spectra which are measured with P450 in one cuvette and buffer in the other. However most spectral work is carried out using difference spectroscopy where the P450 is placed in both cuvettes the instrument zeroed (flat line absorbance). Substrate/ligand is added to one cuvette only with an equivalent amount of buffer or solvent to the other cuvette. Perturbations in the Soret absorbance are given by negative and positive deflections in the absorbance spectra. Difference spectroscopy is the "only way to go" in turbid microsomal systems where other enzymes and chromopores are present. Think of a couple of reasons why.

For example, note then that in difference spectra shown on the right we have "negative " or minimum and "positive" or maximum absorbance values. The absolute spectra for ligand free (418 nm) and ligand bound (392 nm) enzyme are shown on the left. Note that the max and min absorbances in the difference spectra are different than the absolute spectra. What type of ligand is this substrate and what is the spin state of the iron in the ligand bound and ligand free states?

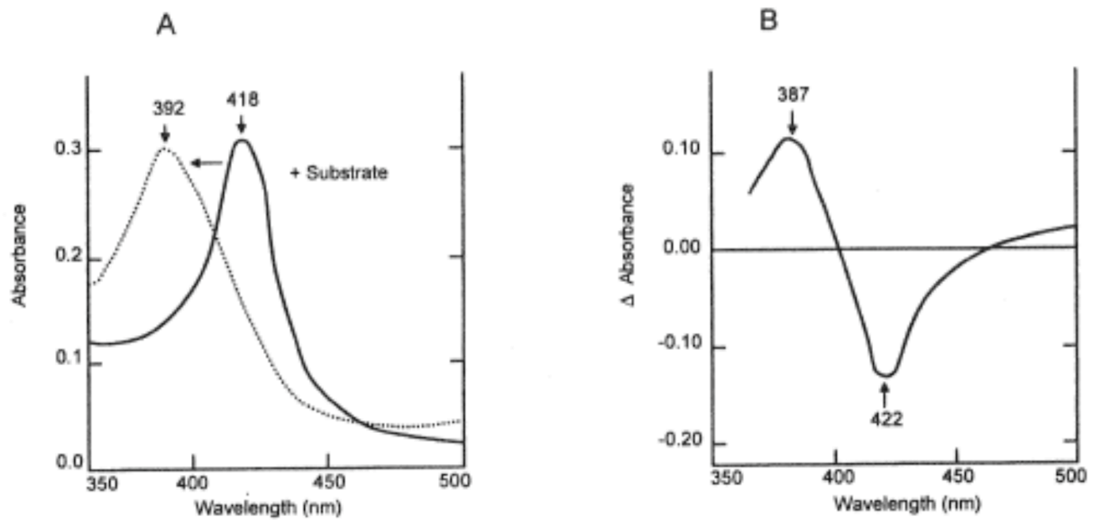
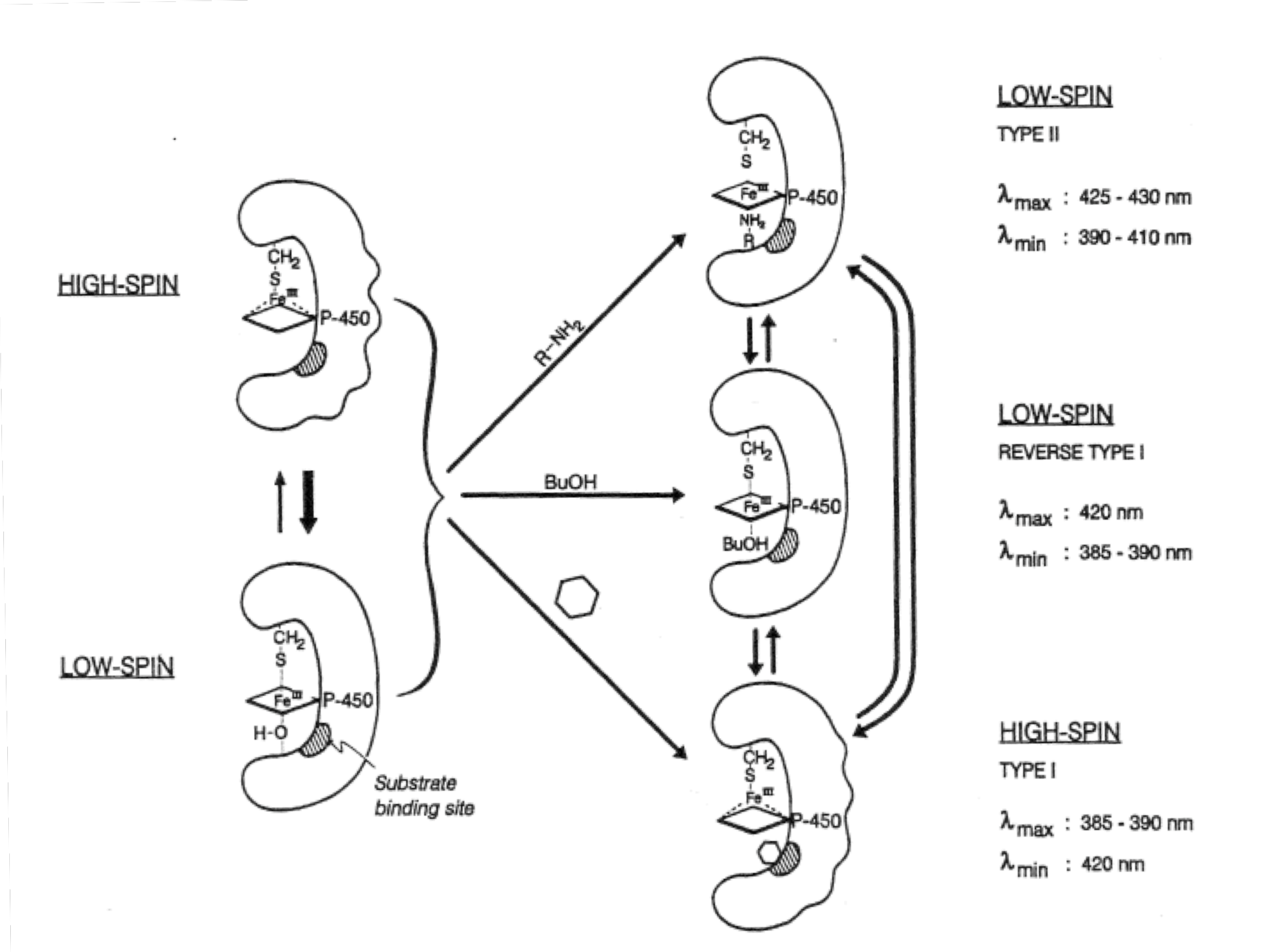


FIGURE 3.7 Absolute spectrum (A) and difference spectrum (B) of cytochrome P450 with bound substrate. Reproduced from Hall (1986) with the permission of the copyright holder.

<u>Type I substrates</u> [difference spectra; λ_{max} = 390 nm : λ_{min} = 418 nm:] 'displace' the water ligand from the low spin state (Soret λ_{max} = 418 nm) and convert the spectrum to that of the high spin form of the enzyme (Soret λ_{max} = 390 nm). Absolute and difference spectra shown below. The key feature here is that substrate binding typically drives water molecules out of the active site. In this figure benzene is binding to the active site. Type I substrates and ligands generally do not occupy the distal (sixth ligand) binding site. Thus the net effect of binding of Type I substrates increases the population of the high spin state. Remember that the high spin state is the state that is most easily reduced (the next step in the cycle). <u>This type of binding "makes sense" as substrate is required to be bound in the active site before the cycle can start.</u> It is not always the case however.



Reverse Type I substrates [difference spectra; _λ_{max} = 420 nm : λ_{min} = 390 nm:] provide an alternate ligand functional group to the heme iron (typically ROH) and promote the low spin state. Thus they supply a functional group in the the sixth ligand position that forms a non-covelent bond to the heme iron. Note that this type of ligand will not only displace water (nominally promoting high spin state) but also provides an alternate sixth ligand to the heme iron (alcohols such as butanol). The ligand-iron bond promotes the low spin state of the enzyme similar to that found with water so the shift is often more subtle. Despite the overall shift to the low spin state many Reverse Type 1 ligands are oxidized by the enzyme.

Note that the intensity of the spectral change is dependent on the identity of the reverse type 1 ligand in this simple series of alcohols. <u>Reverse Type 1 ligands are weak field ligands for P450 heme iron and favor the low spin state.</u>

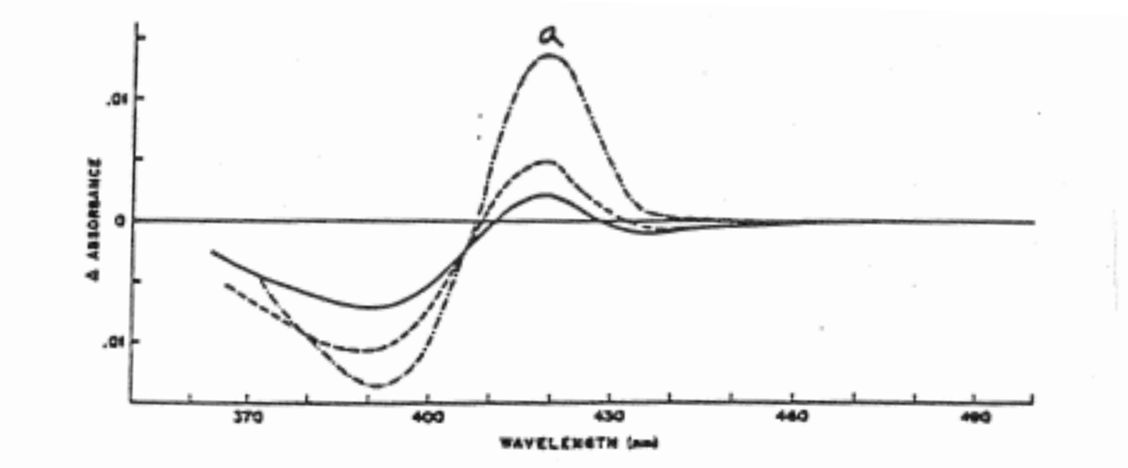


FIG. 3B. Reverse Type I spectral changes evoked by addition of different alcohols (20 μl/ml) to liver microsomes. a = n-butanol, b = ethanol, c = methanol. With permission, from Schenkman *et al.*, 1972.

Type I and Reverse Type 1 spectra were identified as such early in the P450 literature due to the fact that they engendered mirror image changes in difference spectra.

Note the "mirror image" appearance of the two types of difference spectra. What kind of ligand is phenacetin in this prep?

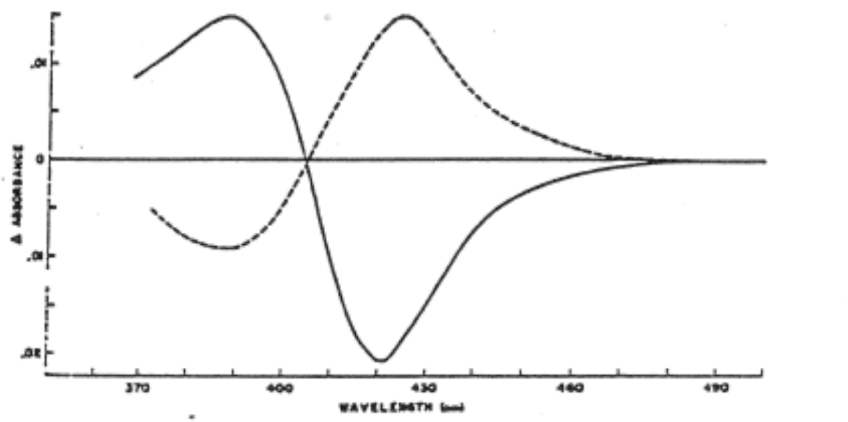
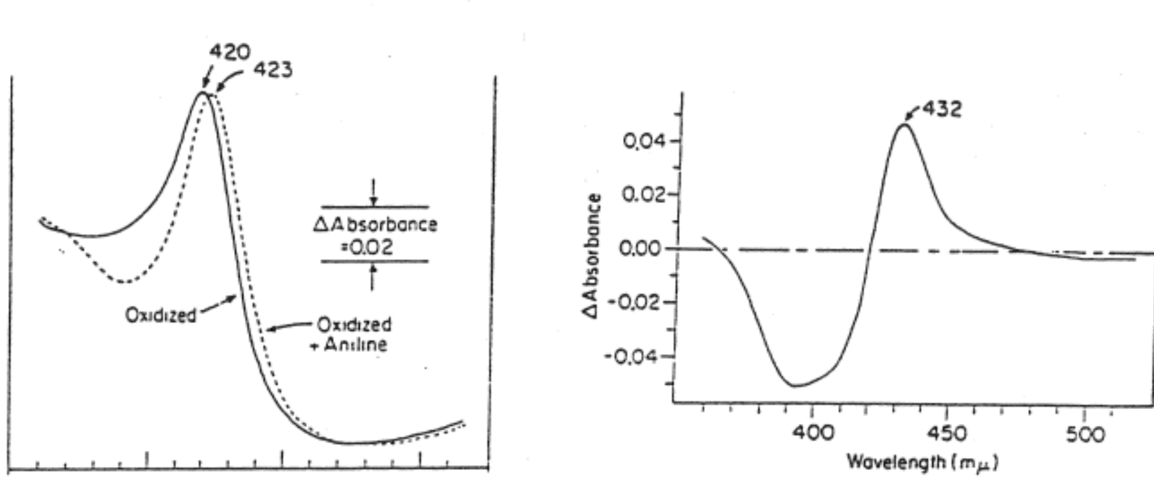


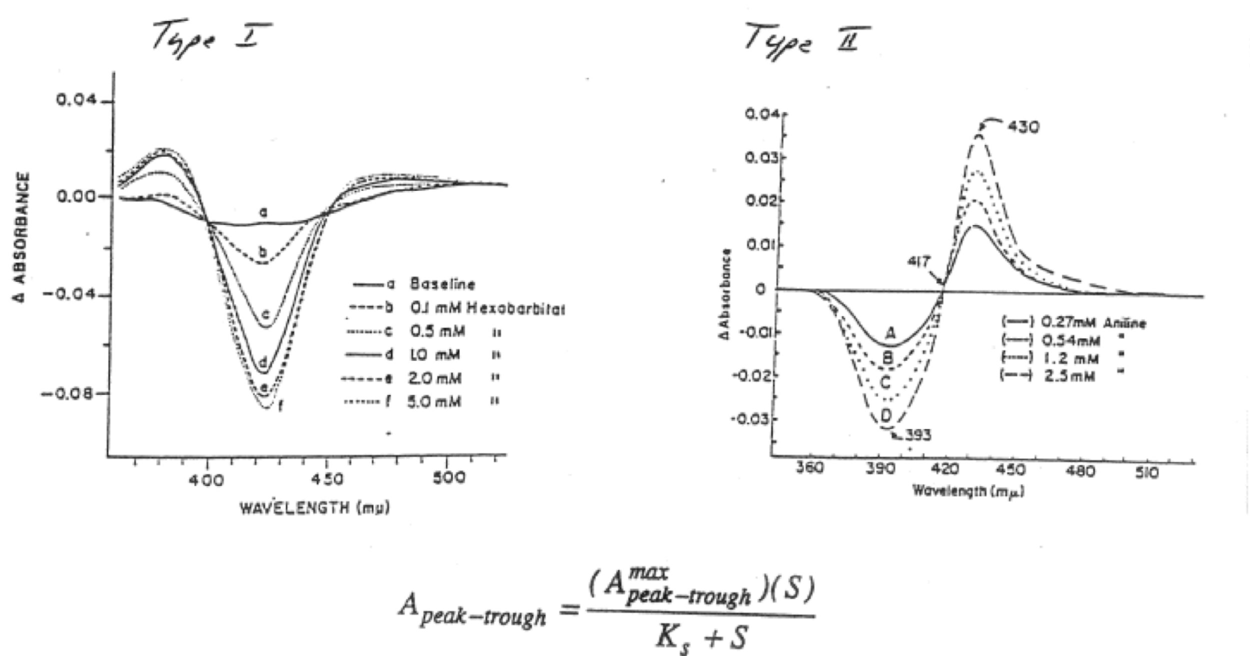
FIG. 3A. Comparison of the Type I and Reverse Type I spectral changes with rat liver microsomes. Solid line shows the difference spectrum obtained on addition of hexobarbital to microsomes and the dashed line shows the effect of phenacetin addition to the microsomal preparation.

Type II substrates [difference spectra; λ_{max} = 430 nm : λ_{min} = 390 nm:] provide an alternate ligand functional group to the heme iron (typically nitrogen) and promote the low spin state. Note that Type II substrates are similar to Reverse Type I substrates in that they also provide an alternate sixth ligand for the heme iron and promote the low spin state. However the functional group that binds to the iron is a "strong field ligand". Classic Type II substrate are nitrogen-containing ligands such as anilines, amines, imidazoles and azoles.

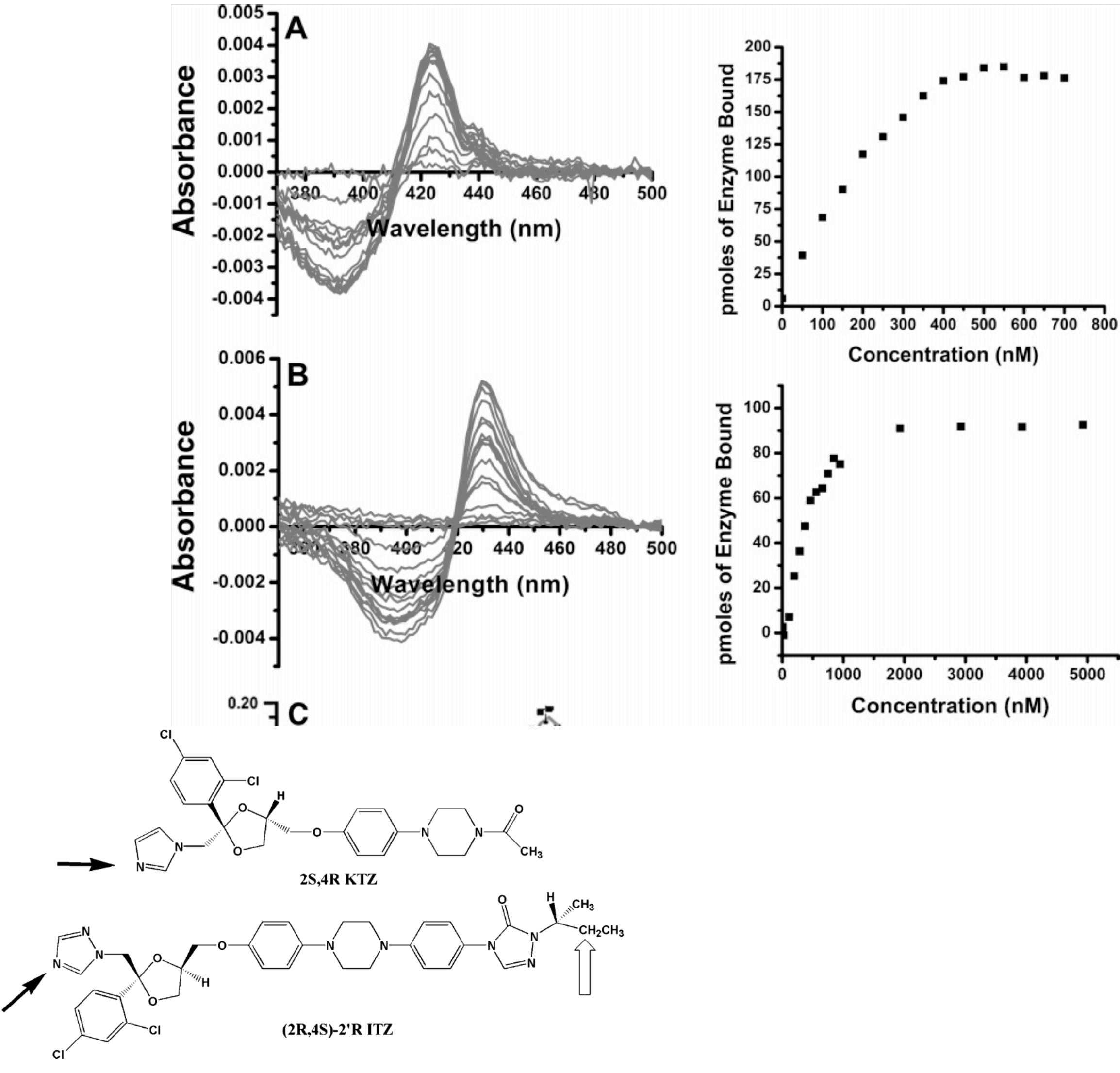
Type II Binding Spectra of Aniline



Ligand and Protein Affinities: Determination of ligand (substrates and inhibitors) affinity for the enzyme active site can be carried out by monitoring spectral changes in the difference spectra as the enzyme is titrated with ligand. These values are referred to as spectral binding constants (K_S).



$$A_{peak-trough} = \frac{(A_{peak-trough}^{max})(S)}{K_s + S}$$



Binding of redox protein partners (b5 and reductase) can also cause spin shifts. Thus Ks values for binding of accessory proteins to the surface of P450 enzymes can be determined in a similar manner.

Other methods to study P450 ligand interactions besides UV-Vis.

1. Fluorescense Spectroscopy
2. NMR
3. Electron Paramagnetic Resonance
4. Raman Spectroscopy
5. Surface Plasmon Resonance
6. Isothermal Titration Calorimetry
7. Circular Dichroism
8. X-ray Crystallography.

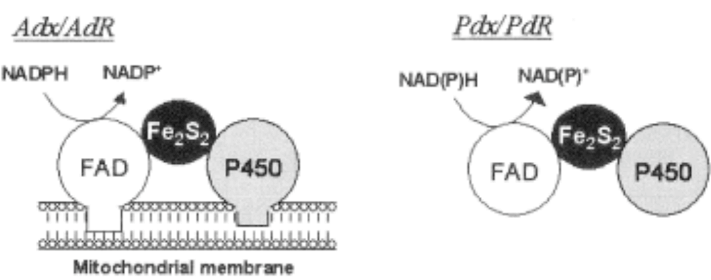
See review in Anal. Bioanal. Chem 392: 1019-1030 (2008)

All of these methods provide information about the structure of the ligand bound enzyme. For the most part, these are static representations. It is important to keep in mind that P450 substrates and ligands adopt multiple conformations in a flexible active site. How do we know this? Multiple products from a single substrate is commonly observed.

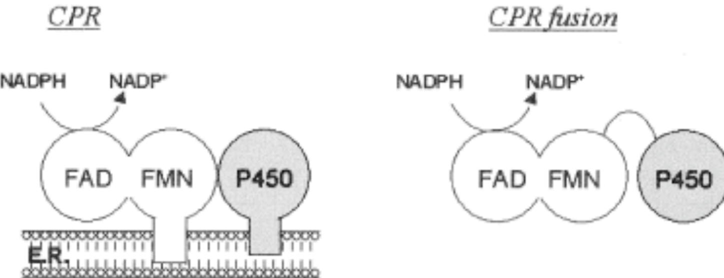
Step 3. Introduction of the first electron from the redox partners reduces the heme iron to the ferrous state.

There are different types of electron transfer partners in nature. Microsomal (endoplasmic reticulum) P450's use CPR (cytochrome P450 reductase: Class II below left). Mitochondrial P450's use irons sulphur proteins (AdX/AdR: Class I below left).

Class I: Iron–sulfur partners



Class II: Diflavin reductase partners



Addition of the first electron occurs via the action of redox active electron transfer proteins. Cofactors NADPH and NADH provide the reducing equivalents to the electron transport proteins which in turn supply electrons to the heme one at a time. The electron transport process requires the presence of two redox-active prosthetic groups (eg., FAD, FMN: microsomal P450 reductase) located on the same or different proteins. NADPH supports enzyme turnover in mammalian systems (much lower Km for the reductase (1 μM vs 1 mM).

The factors that control the physical association of the P450 enzymes with their partner electron transport proteins as well as the subsequent rates of electron transfer have been heavily studied. An outstanding exception to this protein complex paradigm is the bacterial enzyme BM3 where the heme prosthetic group as well as the electron transport prosthetic groups (FMN, FAD) are located within a single enzyme.

Transfer of the first electron to P450 is considered to be a fast step in the cycle. Generally the heme iron must be in the high spin state for reduction to occur. A central tenet of the P450 cycle built on the concrete observations with CYP101 (P450 cam) is that substrate binding is universally obligatory to

electron transfer as substrate binding in this enzyme converts low spin to high spin iron. This does not appear to be true for mammalian membrane associated P450 enzymes.

"In many cases binding is not obligatory for reduction, contrary to common dogma…Overall, the kinetics of ferric P450 reduction cannot be generalized among different P450s in various systems, and concepts regarding influence of substrate, reaction sequence, and a rate-limiting step are not very universal. There is no reason that substrate binding cannot occur later in the reaction cycle" (Guengerich, F Biochem. 36: 14741-50 (1997).

The P450 CO Complex (A Sideshow in the Catalytic Cycle)

Carbon monoxide has a high affinity for the ferrous heme. The CO complex has the signature Soret absorbance λ_{max} = 450 nm. P450s can be quantitatively converted to the CO complex using the artificial reductant sodium dithionite in the presence of CO. This is the most common method to measure P450 content in tissue preps like microsomes. The extinction coefficients for most P450 enzymes are highly similar. Studies of the mechanisms and kinetics of P450 reduction by natural and artificial reductants are often carried out anaerobically in the presence of carbon monoxide.

Step 4: Binding of dioxygen

Regardless of the timing issues relative to enzyme reduction, the P450 heme iron must be in the ferrous state before oxygen will bind to the iron. The ferrous form of P450 has a high affinity for molecular oxygen (30 nM) which is much lower than the natural dissolved oxygen concentration. Oxygen binding is rapid and reversible. The Soret absorbance of the ferrous dioxygen complex is λ_{max} = 418 nm.

The high affinity of ferrous hemoproteins with an open 6th ligand position for dioxygen is well known (hemoglobin, myoglobin). As for most other hemoproteins , O_2 may occasionally dissociate from the ferrous iron as O_2^- (superoxide), a process that leaves the iron in the ferric state and superoxide loose in the system. Toxic superoxide is decomposed by superoxide distmutase and glutathinone peroxidase.

Step 5: Introduction of the second electron

Introduction of the second electron to produces the ferric peroxyanion species which has been observed by ENDOR, EPR and Xray upon cryoreduction of the precursor in P450cam. Science 287: 1615 (2000), J. Amer. Chem. Soc. 121 10654 (1999).

This step is generally believed to be the rate limiting step in the cycle.

Reduced cytochrome b5 present in microsomal membranes may be involved in transfer of the second electron but it is not obligatory. We will return to the role of b5 in the second lecture. As a preview, the effects of b5 are paradoxical. P450 reductase and reduced forms of P450 can reduce oxidized b5. B5 can stimulate the turnover of some substrates and enzymes but not uniformly.

The ferric peroxyanion species has been identified as the active component in deformylation reactions including the last step in the aromatase reaction sequence. In a sense the peroxoanion can be intercepted by properly oriented aldehyde.

Figure 6.39. The currently accepted mechanism for the final step in the aromatase catalyzed reaction. The timing of enolization of the carbonyl with respect to the addition of the ferric peroxide to the aldehyde and to C–C and O–O bond fission is still uncertain.

Protonation to give the perferryl oxygen (good) or hydrogen peroxide (bad)

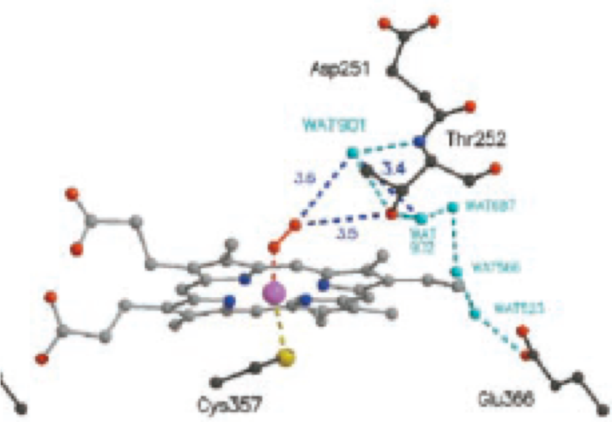
The addition of 2 protons to the terminal oxygen of the ferric peroxyanion (middle below going right) leads to heterolytic cleavage of the oxygen-oxygen bond producing water and Compound I.

a. The "push" from the 5th ligand thiolate is important in this O-O cleavage reaction.

b. Proton relay systems are important in the protonation steps.

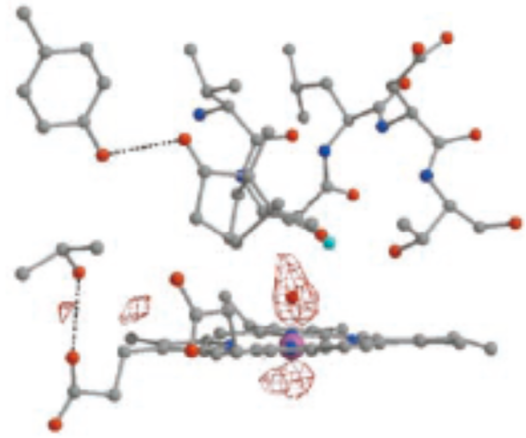
A very important branching reaction also occurs at this point although we don't know the details. The cycle aborts (decouples) with the release oxygen as hydrogen peroxide. One might imagine that this is a result of protonating the proximal oxygen with one of the two protons (middle going left).

The mechanism of productive dioxygen cleavage is being extensively studied by deuterium solvent isotope effects as well as point mutants of P450 enzymes. It appears that the first protonation step on the distal oxygen atom is slower than the second and that a highly, though not totally, conserved distal threonine and a chain of water molecules are important in oxygen scission. Oxygen cleavage is heterolytic!...both electrons in the O-O bond leave with the water oxygen. This generates an electrophillic oxygen bound to iron. It is also thought that the effectiveness of the proton delivery machinery is substrate dependent and highly sensitive to mutations in the enzyme active site. As we will see later these mutants have been used to assess the ability of the peroxyanion and hydroperoxide (largely discounted now) to carry out substrate oxidations.



The cysteinate sulphur is believed to play an important role in promoting heterolytic (observed) over homolytic (rarely observed) cleavage of the dioxygen bond (push effect: J. Inorganic Biochem 91: 554 (2002)) as well as controlling the chemical behavior of the active oxidant.

<u>The structure function of the active oxidant has been the long sought after holy grail of P450 research: Picture P450cam (2.3 angs) Science 287: 1615 (2000) Is that really it?</u>



<u>UV-Vis Spectrum CYP119 Compound I   JBC 277: 9641 (2002)</u>

1. (Blue Shifted Soret: λ_{max}=370 nm) when CYP119 was treated with MCPBA however spectra not all that good.
2. Hydroxylation of a substrate observed.
3. First estimate of the lifetime of compound I.

CYP119
+3 S Cys
N—Fe—N
4 °C
+4 S Cys
N—Fe—N ·+
O
e (Protein?)
$t_{1/2}$=30 msec
+4 S Cys
N—Fe—N
O
Compound II?
Laurate
Hydroxy Laurate
+3 S Cys
N—Fe—N
OH
O
Cl

Compound I "decomposed" with a t1/2 of 30 msec (barrier 18 kcal/mol). The most likely route of decomposition is oxidation of the protein to give compound II however this is not known.

Other studies indicate that Compound I can also be reduced in presence of exogenous reducing equivalents (like reductase). Decomposition of Compound I in the presence of reducing equivalents is of great interest because it is clear that the Compound I oxygen can be reduced to water during turnover of substrates. Thus two major banching reactions of compound I are: (1) reaction with substrate (2) reduction to water. We will look at this again later on.

Rittle and Green *Cytochrome P450 Compound I: Capture, Characterization and C-H Bond Activation Kinetics* Science 330: 933-937 (2010)

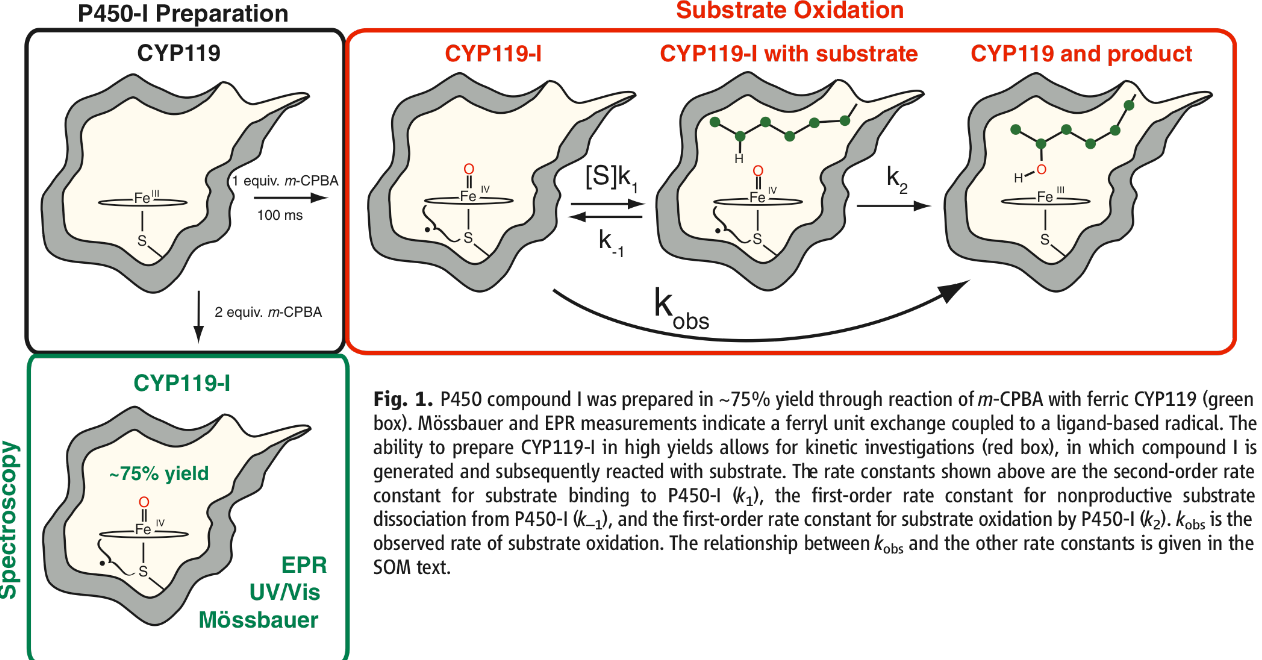
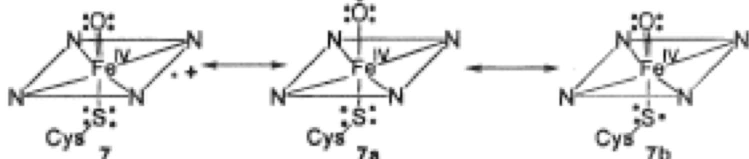


Fig. 1. P450 compound I was prepared in ~75% yield through reaction of *m*-CPBA with ferric CYP119 (green box). Mössbauer and EPR measurements indicate a ferryl unit exchange coupled to a ligand-based radical. The ability to prepare CYP119-I in high yields allows for kinetic investigations (red box), in which compound I is generated and subsequently reacted with substrate. The rate constants shown above are the second-order rate constant for substrate binding to P450-I (k_1), the first-order rate constant for nonproductive substrate dissociation from P450-I (k_{-1}), and the first-order rate constant for substrate oxidation by P450-I (k_2). k_{obs} is the observed rate of substrate oxidation. The relationship between k_{obs} and the other rate constants is given in the SOM text.

Highlights of what is sure to be a classic paper in P450 enzymology.

1. Compound I formation is observed at 4^0C producing much cleaner spectra including isosbestic points. Note the long wavelength absorbance at 690.
2. The complex is also studied at low temperature by EPR and Mossbauer spectroscopy.
3. It is concluded that Compound I is "best described as an S=1 iron (IV) oxo unit exchange-coupled with an S=1/2 ligand based radical. Sulfur, oxygen, porphyrin are all classified as ligands.



4. The Mossbauer spectra are very similar to the well characterized chloroperoxidase Compound 1. The EPR spectra are slightly different which may be important as chlorperoxidase Compound I is not able to hydroxylate C-H bonds.
5. Bimolecular rates of reaction of Compound I with substrates are very fast (>200 per sec at moderate concentrations of substrate).
6. Isotope effect studies demonstrate that the reaction with substrate is limited by the rate of binding of substrate to the pre-formed Compound I enzyme.

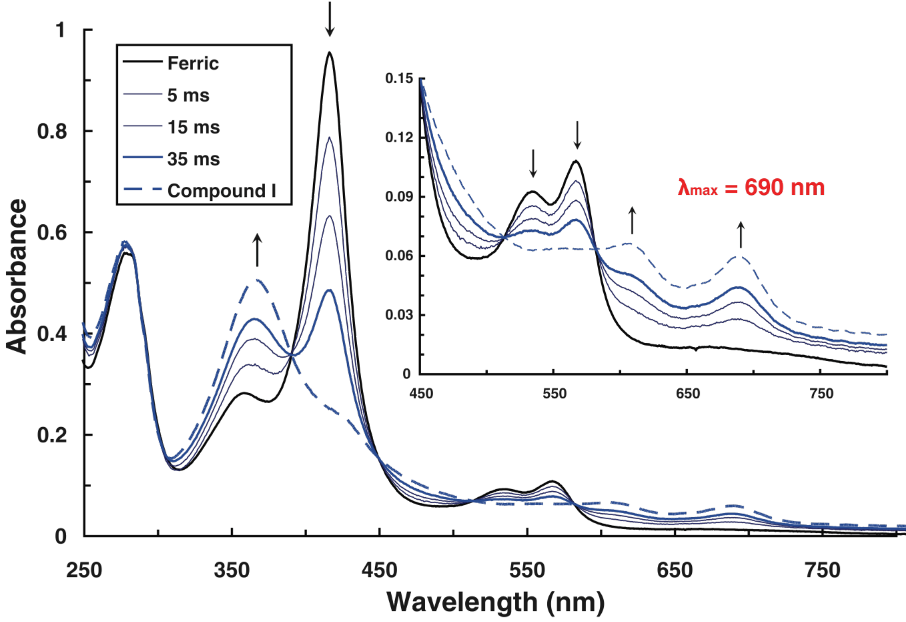


Fig. 2. UV/visible spectra obtained from the stopped-flow mixing (1:1) of 20μM ferric CYP119 with 40 μM *m*-CPBA at 4°C. The blue traces correspond to spectra taken 5, 15, and 35 ms after mixing. Maximum yield of P450-I was ~70% at 35 ms. At later times, isosbestic points were lost, and a portion of the protein was degraded from the use of excess *m*-CPBA as judged by the final Reinheitszahl (R_z = $Abs_{416\ nm}/Abs_{280\ nm}$) ($R_z$) of 1.31 (fig. S1). All spectral changes were completed within 2.5 s. Dashed line indicates the spectrum of CYP119 compound I obtained by difference techniques (*41*).