

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)

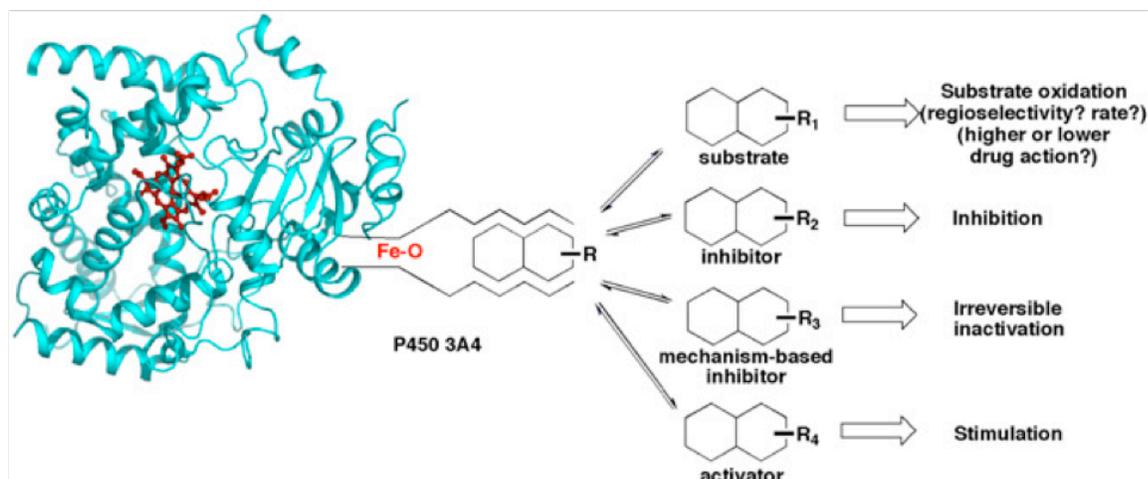
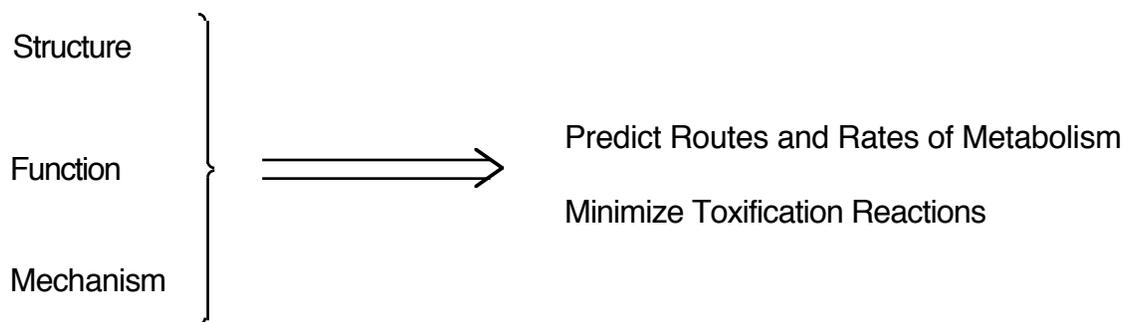


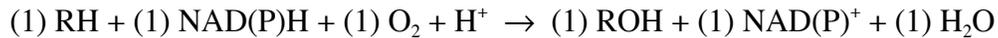
Figure 1. Outcomes of Interaction of a Ligand with a P450

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 P450* 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.

Basic Stoichiometry of P450 Enzymes: Mixed Function Oxidases

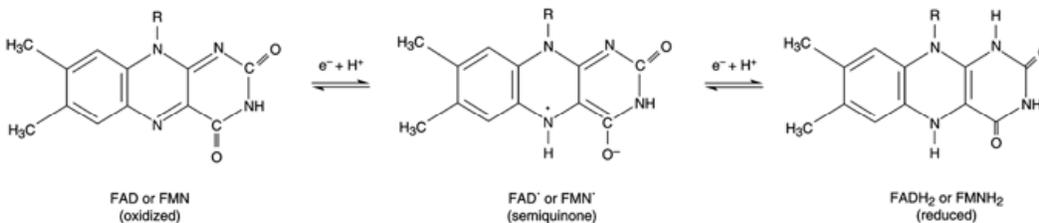


Major P450 Classes Require Multiple Proteins/Domains for Activity

Class I P450 Enzymes	Class II P450 Enzymes
	<p style="text-align: center; font-size: small;">Textbook of Biochemistry with Clinical Correlations, Fourth Edition</p>
<p>(a) <u>Mitochondrial</u> (CYP11A: P450 SCC) soluble or loosely associated reductases</p> <p>(b) <u>Soluble</u> (CYP101 P450 Cam) Bacterial separate proteins.</p>	<p>(a) <u>Endoplasmic Reticulum</u> (Microsomal: CYP2C9) Two proteins, B5 can affect activity</p> <p>(b) <u>Soluble</u> (CYP102: P450 BM3) Bacterial: <u>One protein: 2 domains</u></p>

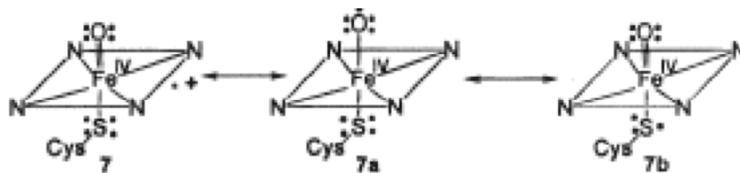
Reductase enzymes are required to supply reducing equivalents to the P450 enzymes from NAD(P)H.

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.

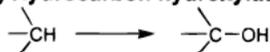


3. Electrons must be delivered one at a time to the enzyme during the cycle so the odd-electron semiquinone form of FMN is important in the Class II enzymes. FAD accepts hydride from NADPH which is immediately split amongst FAD and FMN.
(See Biochem Biophys Acta **1698** 1-24 (2004) for a recent review)

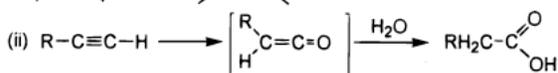
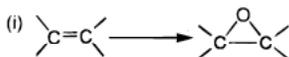
P450 enzymes catalyze a broad range of oxidative reactions. The oxidation chemistry is usually driven by the reaction of a hypervalent iron-oxo species, called Compound I created by the P450 cycle, with the substrate.



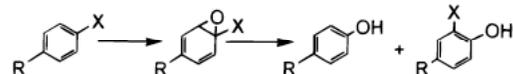
(a) Hydrocarbon hydroxylation



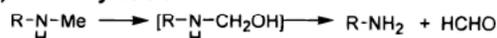
(b) Alkene epoxidation / Alkyne oxygenation



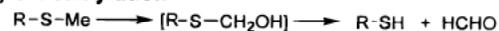
(c) Arene epoxidation, aromatic hydroxylation, NIH shift



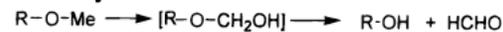
(d) N-Dealkylation



(e) S-Dealkylation



(f) O-Dealkylation



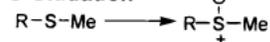
(g) N-Hydroxylation



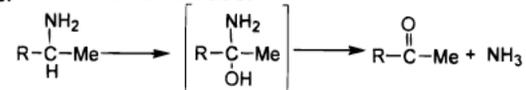
(h) N-Oxidation



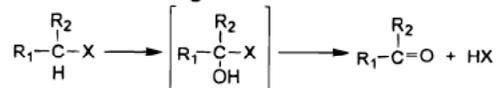
(i) S-Oxidation



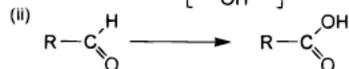
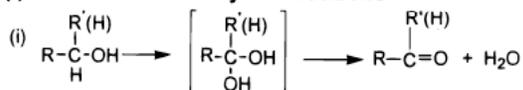
(j) Oxidative deamination



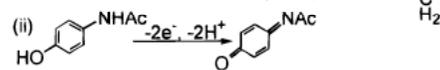
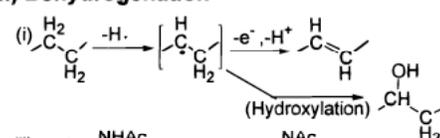
(k) Oxidative dehalogenation



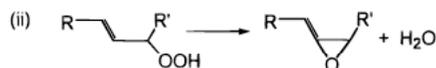
(l) Alcohol and Aldehyde oxidations



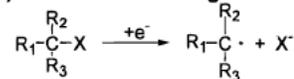
(m) Dehydrogenation



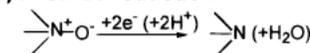
(n) Dehydrations



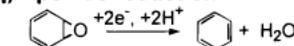
(o) Reductive dehalogenation



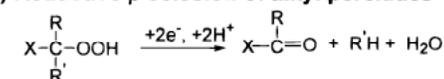
(p) N-Oxide reduction



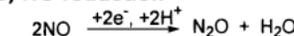
(q) Epoxide reduction



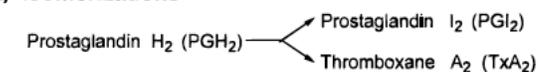
(r) Reductive β-scission of alkyl peroxides



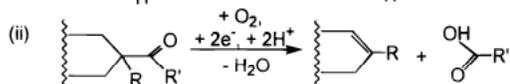
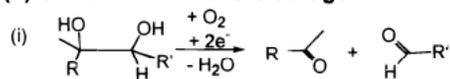
(s) NO reduction



(t) Isomerizations



(u) Oxidative C-C bond cleavage



Below see a proposed sequence for oxidation of a carbon hydrogen bond to an alcohol. Notice the proposal here, first postulated by Sason Shaik that degenerate states of Compound I exist. Thus Compound I is very likely a composite of two different species with similar energies and different energy profiles are calculated. One profile is called the high spin reaction coordinate (HS). The second coordinate is called low spin (LS). We have not yet devised a conclusive experiment to prove this postulate. At one level then it may not be all that relevant in drug metabolism. Rheem will talk more about this.

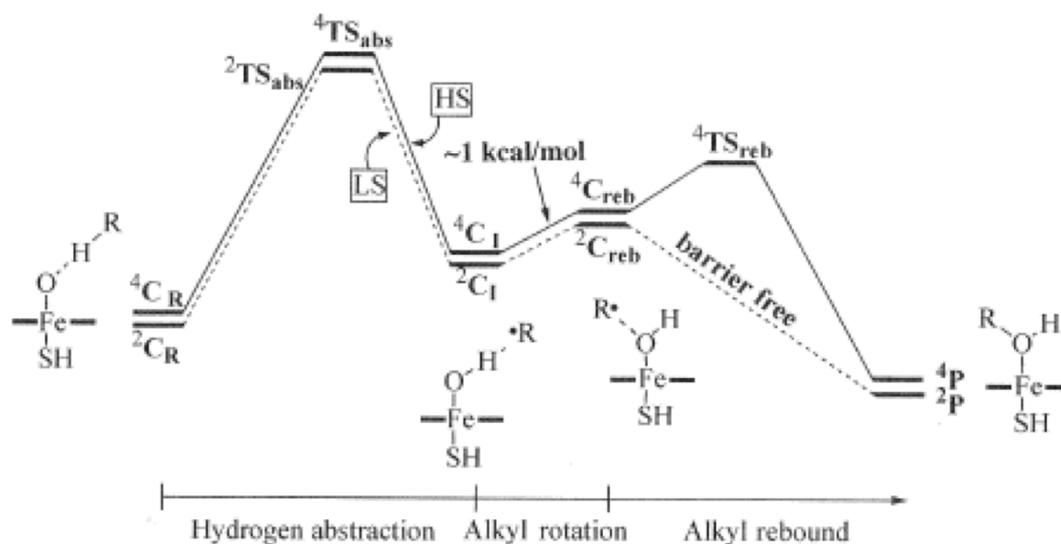
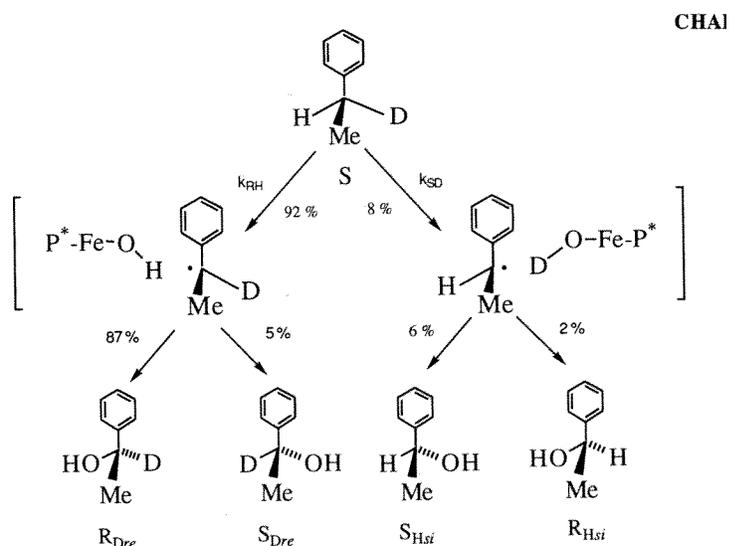


Figure 2.17. A two-state reactivity potential energy surface for alkane (R-H) hydroxylation by Cpd I.

For our purposes the profile above does show that the conversion of a carbon-hydrogen bond to an alcohol will proceed by hydrogen atom abstraction. There is overwhelming data in the literature using stereospecifically deuterated substrates that inversion of configuration does occur. Abstraction and recombination are not stereospecific.



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?).

Below we see a series of P450 substrates for the rat family of enzymes and the substrate selectivity observed.

1. By inspection you should be able to see that the product profiles for the different enzymes are different. Note in the case of testosterone (K,L,M) that 3 metabolites are formed.
2. Also note that the rate of metabolism of a given compound varies dramatically from enzyme to enzyme.

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

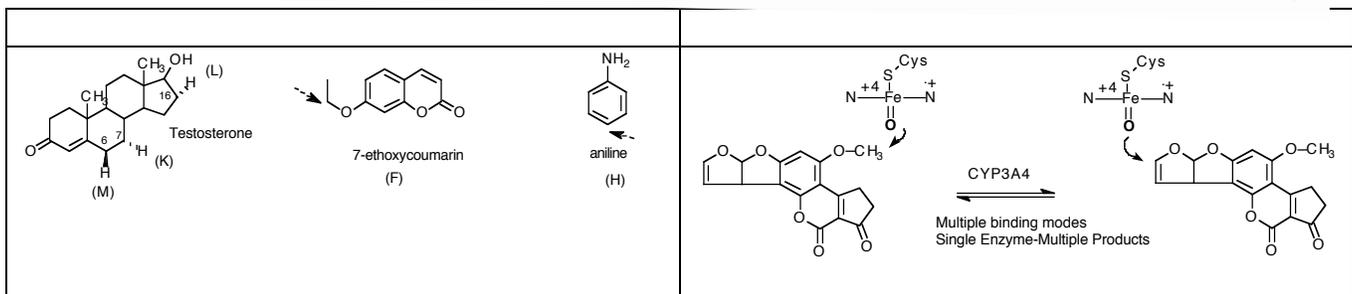
Isozyme*	Substrate and reaction (nmol min ⁻¹ [nmol P450] ⁻¹)†												
	A	B	C	D	E	F	G	H	I	J	K	L	M
P450a	2.3	0.5	0.2	0.9	—	0.6	—	—	—	—	20.9	—	—
P450b	132.5	42.7	0.4	3.3	—	9.6	1.8	1.8	—	—	—	9.1	—
P450c	6.7	0.9	23.4	60.8	1.7	97.1	21.6	1.0	—	—	—	—	1.9
P450d	3.9	—	0.3	21.1	13.8	0.6	0.7	9.6	—	0.5	—	—	0.7
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.

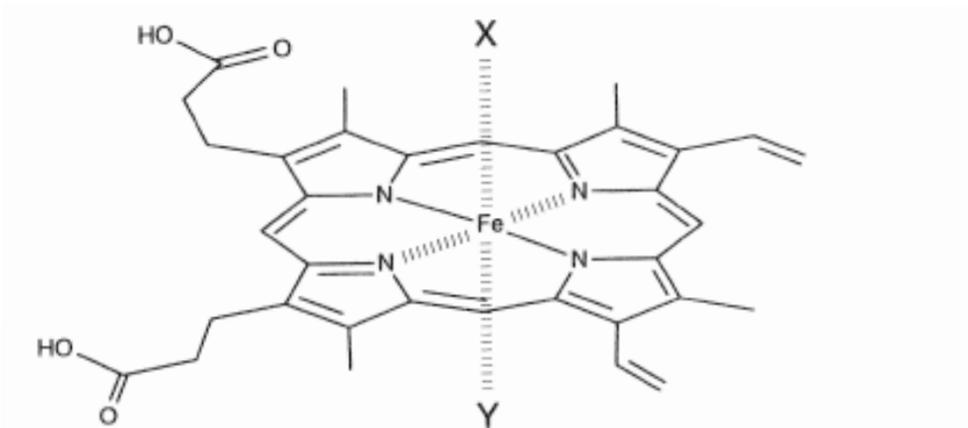
A: Benzphetamine N-demethylation
 B: Hexobarbital 3-hydroxylation
 C: Benzo[*a*]pyrene 3/9-hydroxylation
 D: Zoxazolamine 6-hydroxylation
 E: Estradiol-17 β 2-hydroxylation
 F: 7-Ethoxycoumarin O-dealkylation
 G: *p*-Nitroanisole O-demethylation

H: Aniline *p*-hydroxylation
 I: Androstane disulfate 15 β -hydroxylation
 J: N-Nitrosodimethylamine N-demethylation
 K: Testosterone 7 α -hydroxylation
 L: Testosterone 16 α -hydroxylation
 M: Testosterone 6 β -hydroxylation

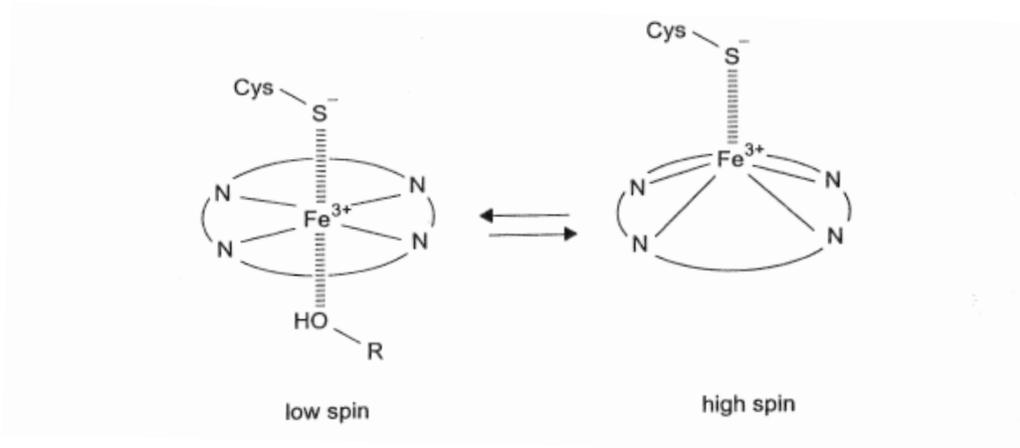


Heme iron is the catalytic center of the P450 enzymes (Fe + Protoporphyrin IX = Heme).

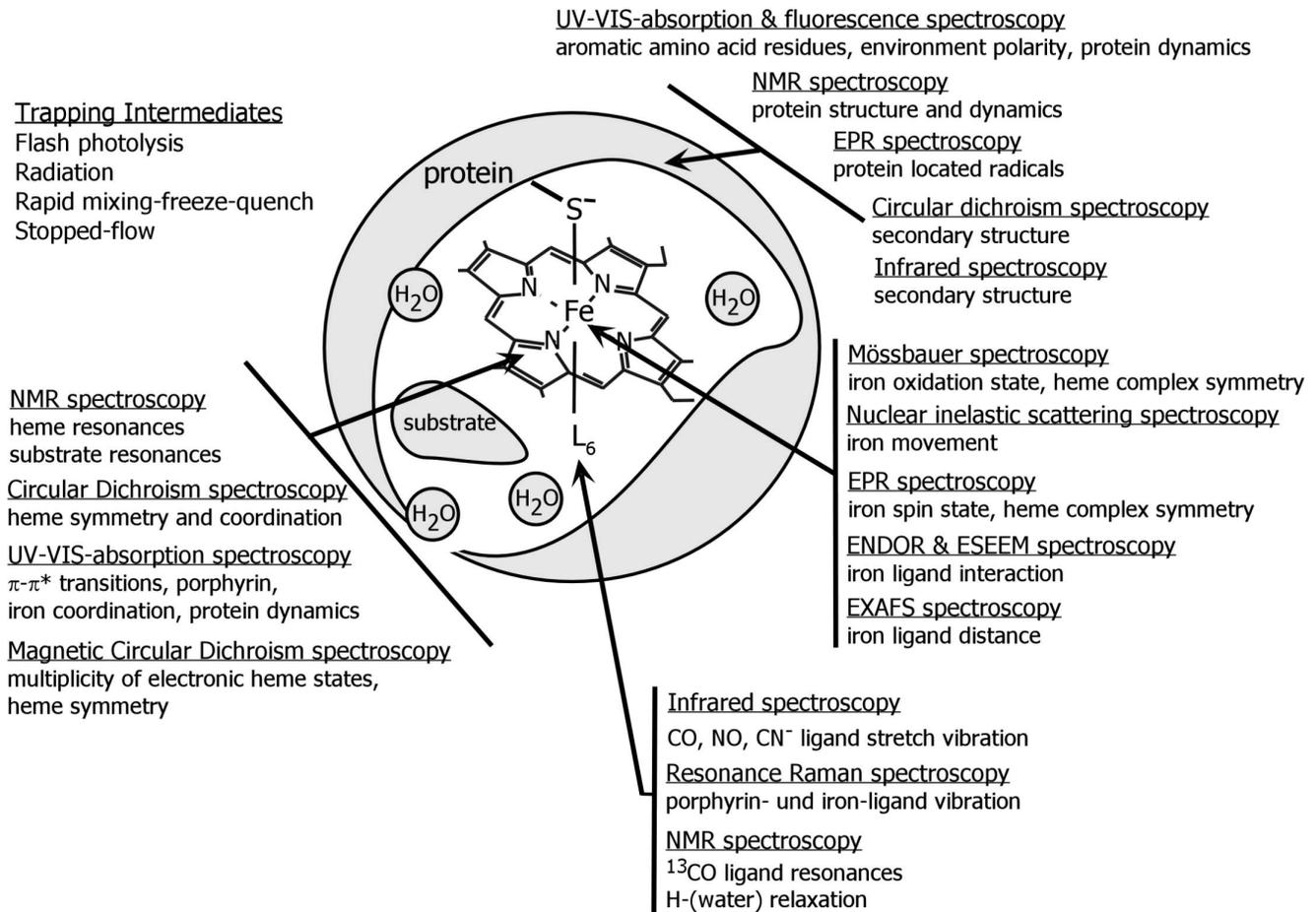
- a. The heme iron has two stable oxidation states: Ferric (Fe^{+3}) and Ferrous (Fe^{+2}) but can also exist in transient hypervalent states.
- b. The ferric iron itself can be either high spin (more unpaired electrons in the iron d orbitals) or low spin (fewer unpaired electrons in the iron d orbitals).



- 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 the porphyrin and iron occur so electrons can reside in different orbitals depending on the spin state. 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 cysteinyl sulphur anion (thiolate). This cysteine is located on the proximal face of the heme and is critical to the generation and reaction properties of Compound I. Generally, high spin (iron out of plane towards cysteinyl) and low spin (iron in plane) states are often in equilibrium and their distribution is ligand dependent.



There are a wide variety of spectroscopic techniques that are used to probe the electronic and structural features of the P450 enzymes with and without enzyme present.



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. This transition gives what is called the Soret absorbance. The λ_{max} of the Soret peak for heme containing enzymes occurs in the 380 to 460 nm region. The wavelength of the maximum absorption is highly dependent on the identity of the fifth and sixth ligands, 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 of the P450 enzymes for the time being.

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.

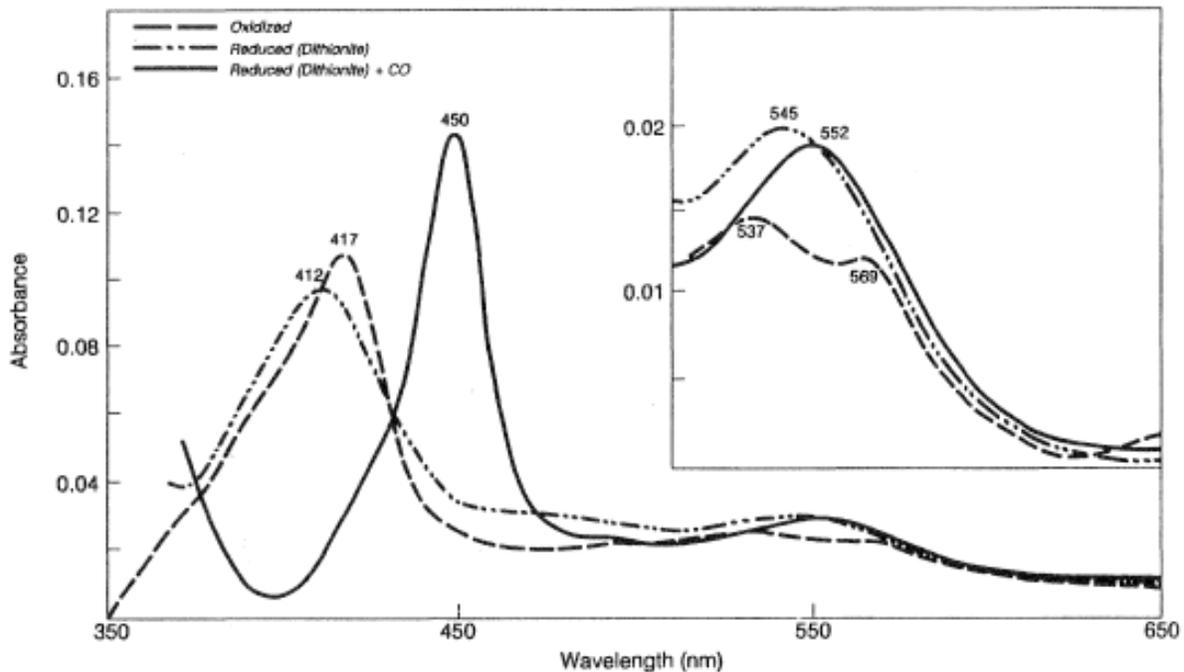
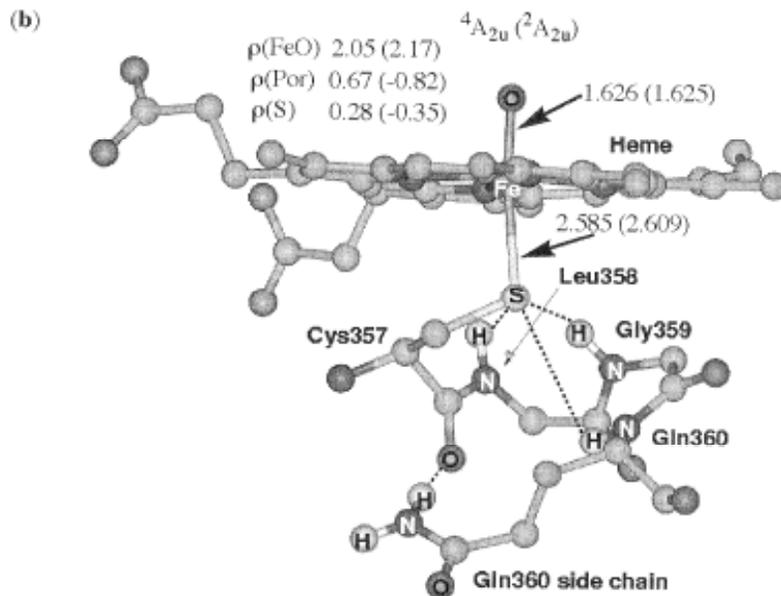


FIGURE 3.6 Absolute spectra of oxidized (low-spin), reduced, and reduced CO-complexed cytochrome P450 in microsomes from untreated male rats; the reference cuvette contained the same microsomal suspension in which cytochrome P450 (but not cytochrome b_5) had been destroyed by linoleic acid hydroperoxide. 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 normally but not always causes oxidation of substrates. Note the hydrogen bonds formed to the sulphur. Different P450 enzymes have different arrangements of hydrogen bond donors so the exact structure of Compound 1 is expected to be enzyme specific. The oxygen atom itself is electrophilic and reacts with substrates in 3 major ways:

- Oxygen attack on a pi bond which starts with substrate pi electrons forming a bond with the Fe-O.
- Hydrogen atom abstraction from a C-H bond
- Electron abstraction from a heteroatom such as nitrogen.

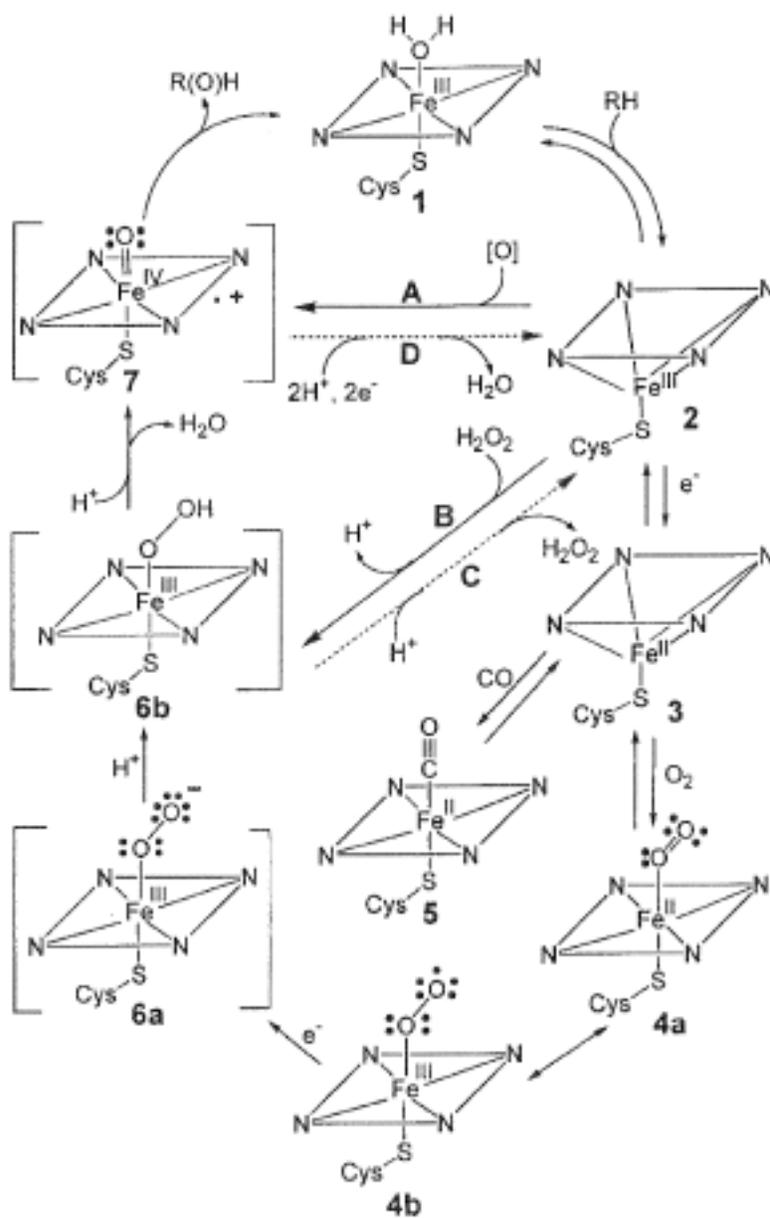


2. The heme iron is found in different oxidation and spin states during the progression through the catalytic cycle. The heme iron is highly sensitive to environment. Thus the iron moves in and out of the plane of the porphyrin ring in response to the presence of 6th ligands. Generally high spin states (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 macrocycle, thiolate or other ligands are usually viewed as belonging the ligand rather than the iron.

3. Here we see the Classical P450 Cycle (Reference 2) along with some short circuits (ABCD) we will return to later. Note that our knowledge of the cycle was largely built on studies of CYP101 (P450cam) which is an extraordinarily well behaved soluble non-mammalian P450 enzyme.



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

The resting state (no substrate) of the P450 enzymes is variably comprised of two different spin states, low spin(mostly) and high spin and these states are in equilibrium. The ratio of the two spin states of the membrane bound forms are sensitive to a host of factors such as method of isolation, phospholipid content in the membrane, the presence of other proteins such as the P450 redox partners (b5 and reductase), the presence of ligands such as fatty acids as well as aggregation state. Note that in the cycle diagram only the low spin state is shown where water as the sixth ligand. This is the dominant state for CYP101. As you have learned there are multiple water molecules normally present in the active site. These two spin states are in rapid equilibrium, particularly for the membrane-bound P450 enzymes. The properties of these two spin states are reasonably well understood.

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. H₂O occupies the ferric heme sixth ligand position in the distal (substrate binding) cavity of the enzyme. The heme exhibits a Soret at $\lambda_{\max} = 418$ nm, the heme iron is low spin (S=1/2), lies in the plane of the porphyrin ring and is 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 the low spin state cannot be naturally reduced by the introduction of an electron by redox partners. That may not be universally true for all enzymes and redox partners which are more difficult to study. All ferric P450's can be reduced to the ferrous state by the artificial reductant sodium dithionite.

b. High Spin State (S=5/2) like (2) but not shown No 6th ligand: easier to reduce

The heme sixth ligand position is not formally occupied in the high spin state. The high spin heme exhibits a Soret $\lambda_{\max} = 390$ nm, the heme iron is high spin (S=5/2), displaced 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' H₂O from the active site and distal binding site and promotes the high spin state. By way of example 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 such as 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 forming a type of bond with the iron). Let's also 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. Absolute spectra are measured with the P450 only in one cuvette and buffer in the other (See A solid line below which shows a low spin ferric heme). However most spectral work is carried out using a technique called difference spectroscopy where the P450 is placed in both cuvettes and the instrument zeroed to achieve a flat line absorbance (B below). Substrate/ligand is then added to one cuvette only and an equivalent

amount of buffer or solvent to the other cuvette. Perturbations in the Soret absorbance caused by this addition are given by negative and positive deflections in the absorbance difference spectra. Difference spectroscopy is the “only way to go” in turbid microsomal systems where other enzymes and chromophores are also 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?

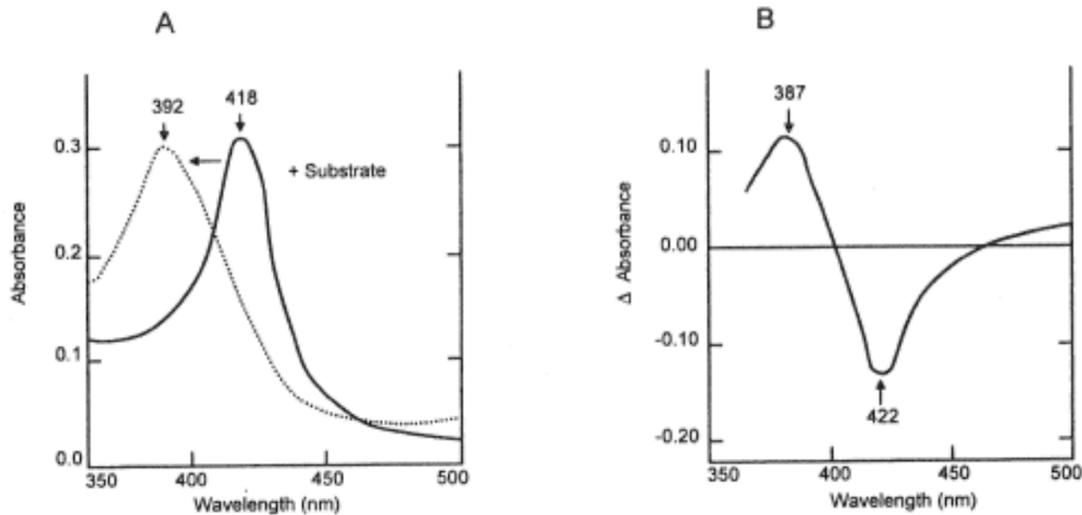
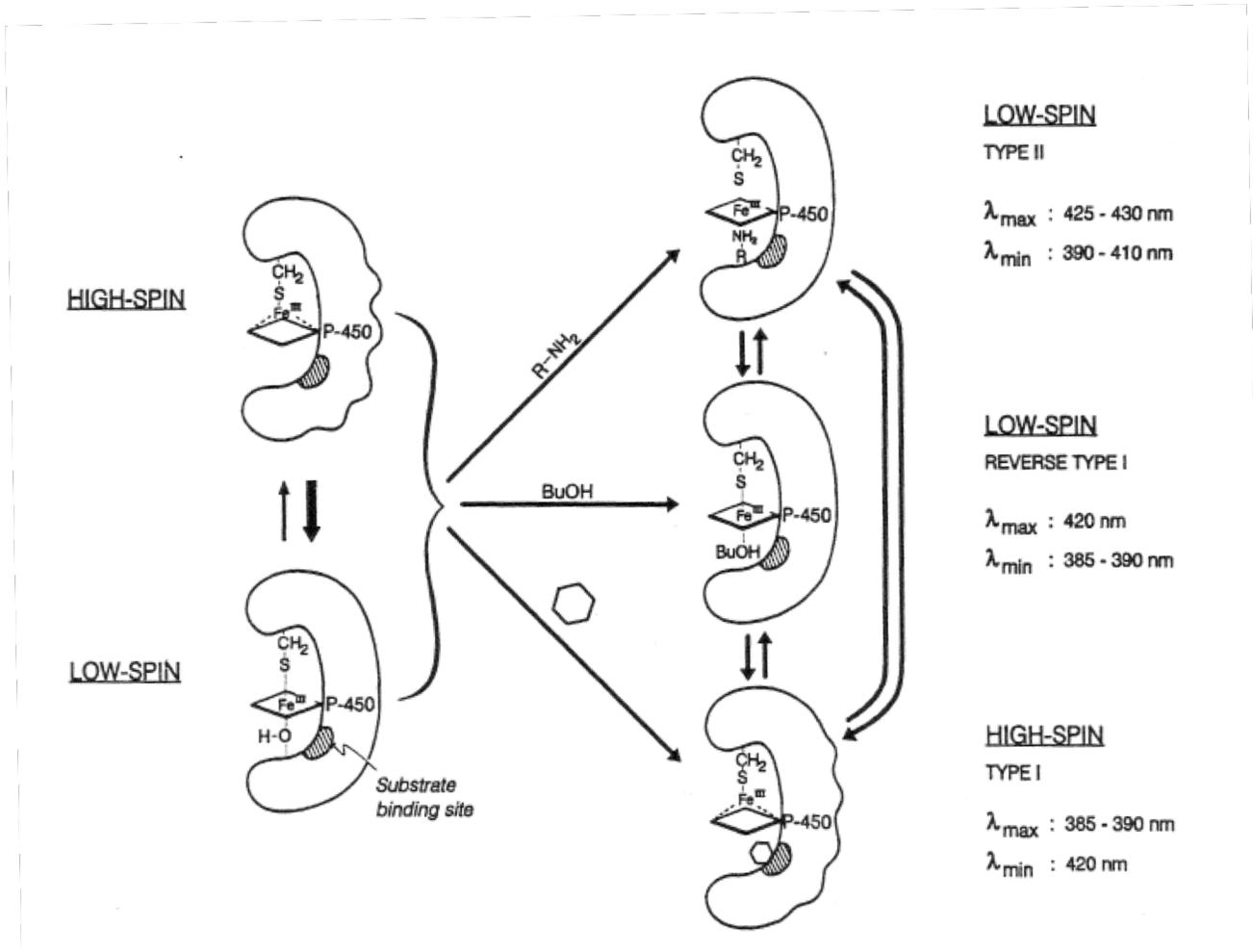


FIGURE 3.7 Absolute spectrum (A) and difference spectrum (B) of cytochrome P450 with bound substrate. Reproduced from Hall (1986) with the permission of the copyright holder.

How do we determine the spin state of a resting enzyme? Peak shape analysis, so it is not precise. Other methods can be used but are not routinely. We won't dwell on this.

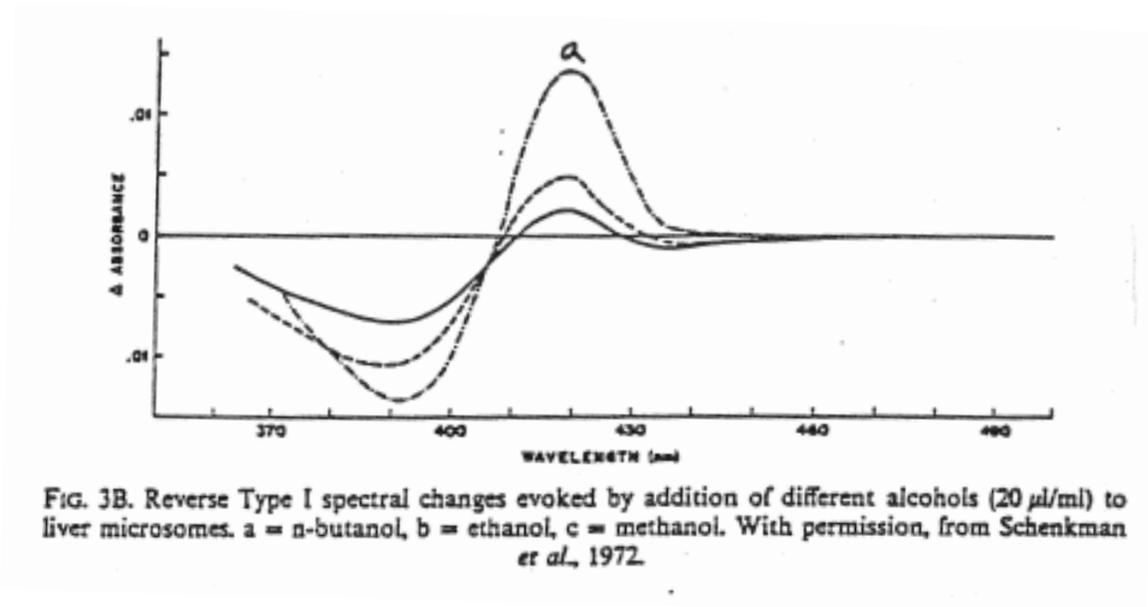
Substrate binding causes a perturbation in the Soret absorbance. In the above example the addition of a substrate changed the heme from low spin to high spin. This spectral conversion is referred to as a Type I spectra and substrates or ligands that produce this change are called Type I ligands or substrates. We can look at a cartoon to best understand the various spin state changes.

Type I substrates [difference spectra; $\lambda_{\max} = 390 \text{ nm}$: $\lambda_{\min} = 418 \text{ nm}$:] ‘displace’ the water ligand from the low spin state (Soret $\lambda_{\max} = 418 \text{ nm}$) and convert the spectrum to that of the high spin form of the enzyme (Soret $\lambda_{\max} = 390 \text{ nm}$). Absolute and difference spectra shown above. The key feature here is that the binding of substrate typically drives water molecules out of the active site. In the cartoon cyclohexane (non-polar, no heteroatoms to bind directly to the heme iron) occupies 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 by driving water (the sixth ligand in the resting low spin enzyme) out of the active site. Remember that the high spin state is the state that is most easily reduced (the next step in the cycle). This type of binding “makes sense” as substrate is required to be bound in the active site before the cycle can start.



Reverse Type I substrates/ligands [difference spectra; $\lambda_{\text{max}} = 420 \text{ nm}$: $\lambda_{\text{min}} = 390 \text{ nm}$:] provide a weakly bonding functional group to the heme iron (typically ROH). This binding then promotes the low spin state that is also stabilized by water in the resting state. Thus they supply a functional group in the sixth ligand position that forms a non-covalent 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. Reverse Type 1 ligands are weak field ligands for P450 heme iron and favor the low spin state.



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 of microsomes? Does the structure predict type of spectrum?

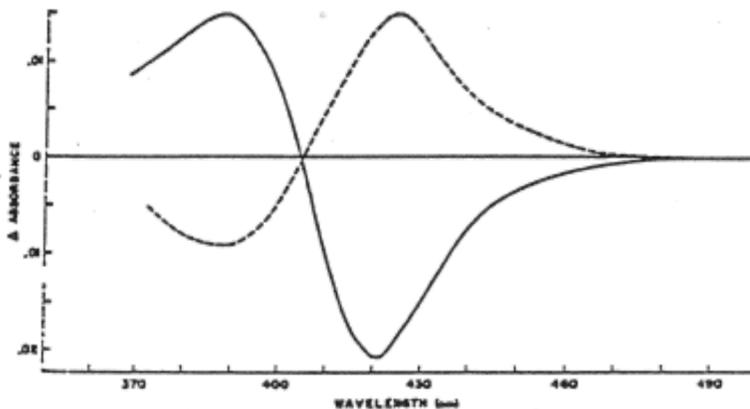
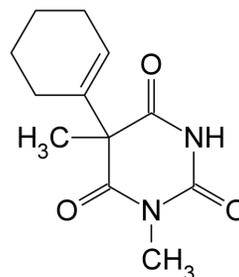
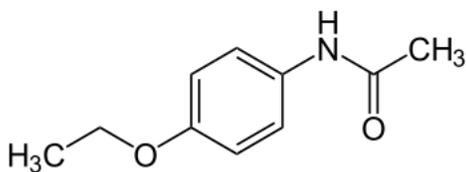
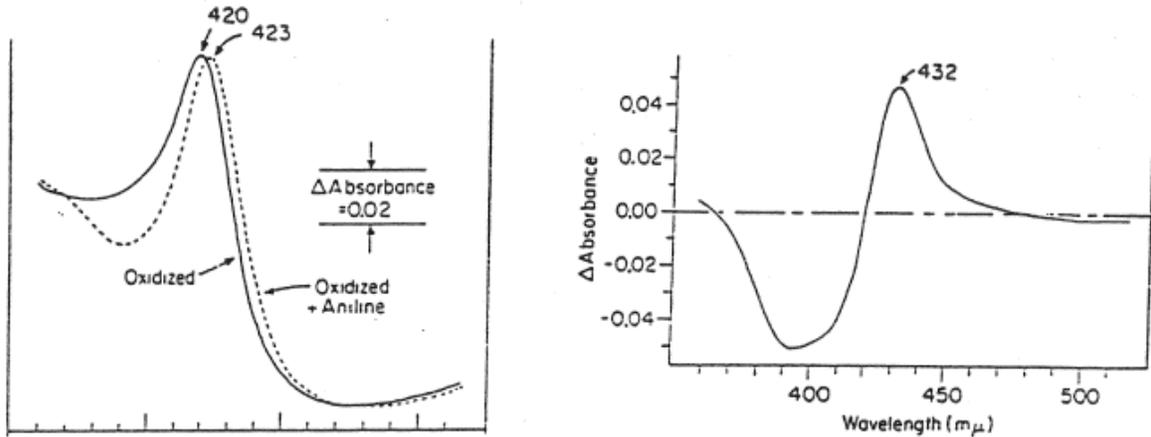


FIG. 3A. Comparison of the Type I and Reverse Type I spectral changes with rat liver microsomes. Solid line shows the difference spectrum obtained on addition of hexobarbital to microsomes and the dashed line shows the effect of phenacetin addition to the microsomal preparation.

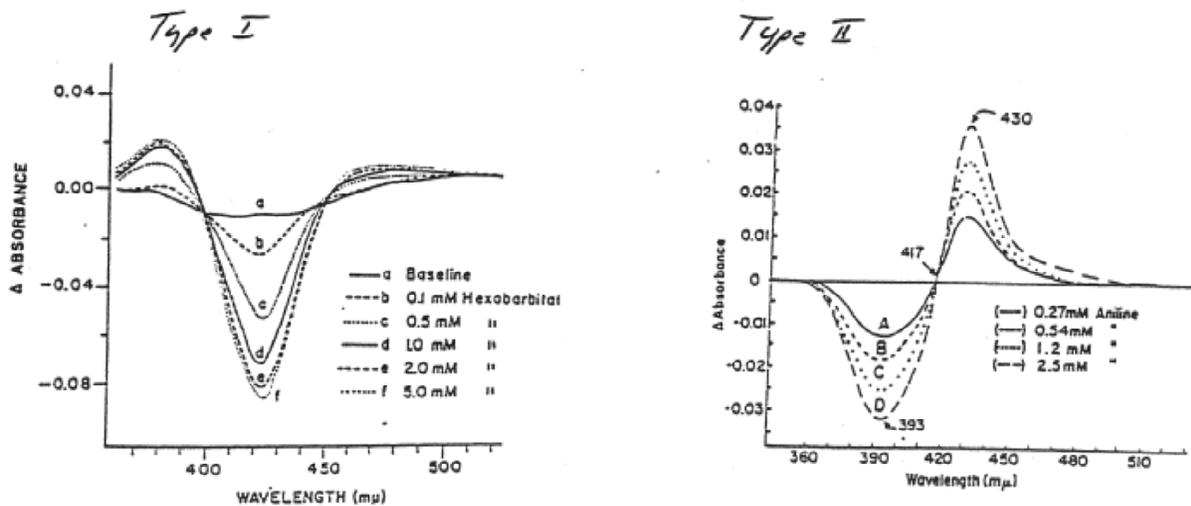


Type II substrates [difference spectra; $\lambda_{\max} = 430 \text{ nm}$: $\lambda_{\min} = 390 \text{ nm}$:] provide an alternate strong-field ligand functional group to the heme iron (typically nitrogen) and promote the low spin state. Note that Type II substrates are similar to Reverse Type I substrates in that they also provide an alternate sixth ligand for the heme iron promoting 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.

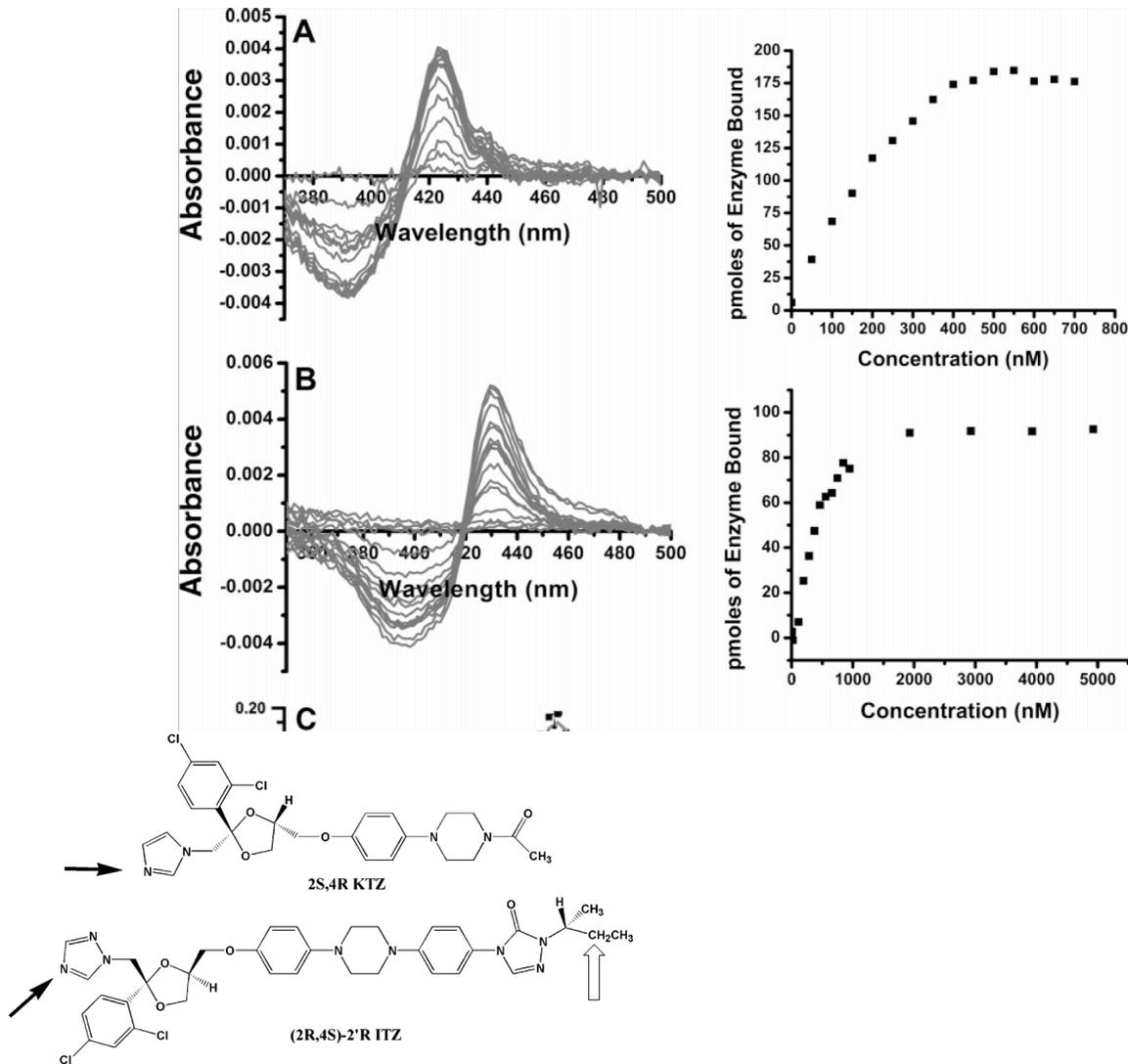
Type II Binding Spectra of Aniline



Determination of K_s values for ligands: Determination of ligand (substrates and inhibitors) affinity for the enzyme active site can be carried out by monitoring the magnitude of the spectral changes in the difference spectra as the enzyme upon addition of aliquots of the ligand. These values are referred to as spectral binding constants (K_s).



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



Note that binding of redox protein partners (b5 and reductase) can also cause subtle spin shifts in the Soret peak. Thus K_S 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. See reviews (1) *Anal. Bioanal. Chem* 392: 1019-1030 (2008) *Arch. Biochem Biophys* 507: 44-55 (2011)

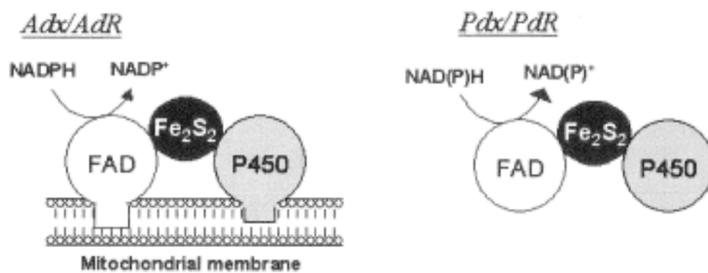
1. Fluorescence Spectroscopy
2. NMR
3. Electron Paramagnetic Resonance
4. Raman Spectroscopy
5. Surface Plasmon Resonance
6. Isothermal Titration Calorimetry
7. Circular Dichroism
8. X-ray Crystallography.

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 very flexible active site. How do we know this? Multiple products from a single substrate is commonly observed.

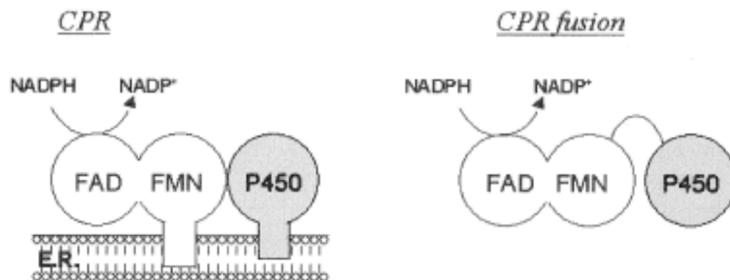
Step 3. Introduction of the first electron from the redox partners reduces the heme iron to the ferrous state.

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 iron 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 redox partner 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 although NADH will also drive turnover (much lower K_m for the reductase (1 μ M; NADPH vs 1 mM; NADH).

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 $\lambda_{\max} = 450 \text{ nm}$. 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 in the presence of carbon monoxide. Early on the demonstration that CO inhibited a NADPH dependent oxidation reaction in microsomes was required to prove the involvement of P450.

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 ($K_s = 30 \text{ nM}$) which is much lower than the natural dissolved oxygen concentration. Oxygen binding is both rapid and reversible. The Soret absorbance of the ferrous dioxygen complex is $\lambda_{\max} = 418 \text{ nm}$.

The high affinity of ferrous hemoproteins with an open 6th ligand position for dioxygen is well known and heavily studied (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 heme iron in the ferric state and releases superoxide in the system. As in red blood cells superoxide is decomposed by superoxide dismutase and glutathione peroxidase.

Step 5: Introduction of the second electron

Introduction of the second electron to produces the ferric peroxy anion 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 b_5 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 b_5 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 peroxy species has been identified as the active component in certain reactions catalyzed by P450. In particular certain deoxygenation reactions such as the last step in the aromatase reaction sequence shown below. Notice that the peroxy species is acting as a nucleophile and that the O-O bond is cleaved homolytically.

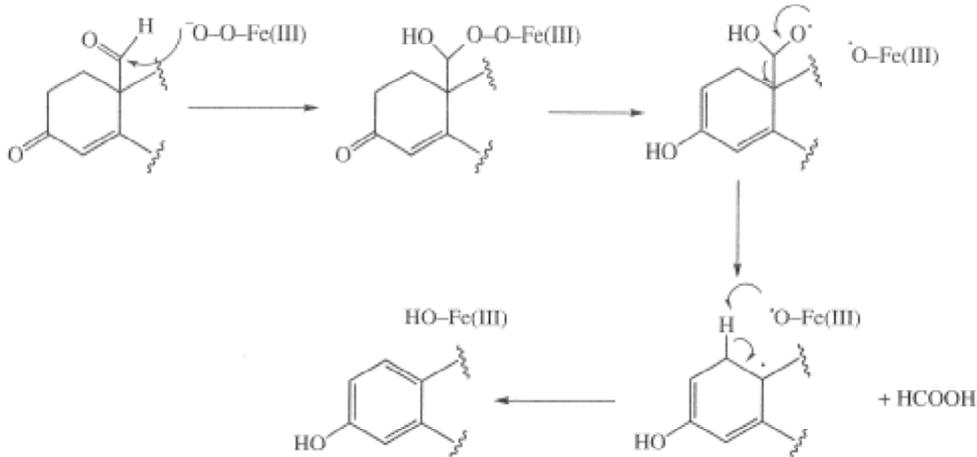


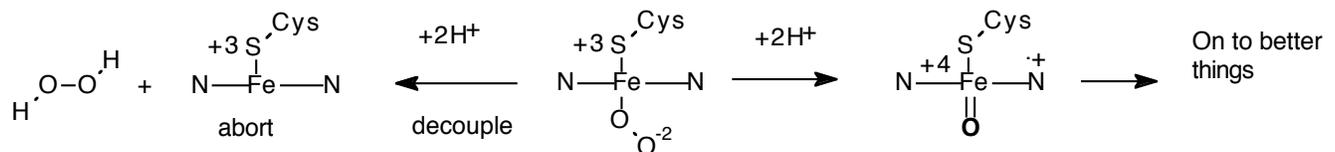
Figure 6.39. The currently accepted mechanism for the final step in the aromatase catalyzed reaction. The timing of enolization of the carbonyl with respect to the addition of the ferric peroxide to the aldehyde and to C-C and O-O bond fission is still uncertain.

6. Protonation to give the Compound I (good) or hydrogen peroxide (bad)

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

a. The “push” of electron density from the 5th ligand thiolate is important in this O-O cleavage reaction to assure that the electrons in the O-O bond leave with water. This is part of the push-pull effect.

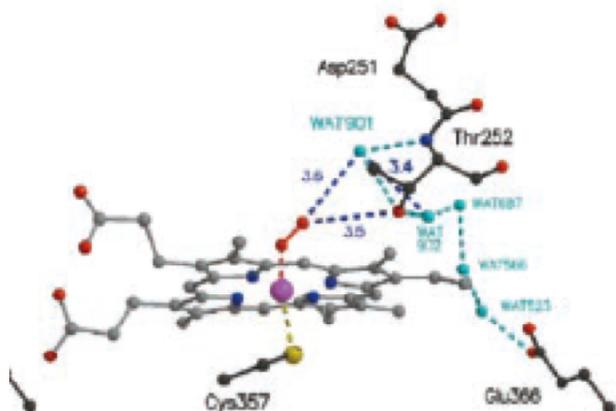
b. A proton relay system is important in the protonation steps.



c. 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 participation of a chain of water molecules are important in driving productive oxygen

scission. Oxygen cleavage is heterolytic!...both electrons in the O-O bond leave with the water oxygen. This generates an electrophilic oxygen bound to iron.



d. The remaining oxygen atom of Compound I is electron poor. Calculations show that the thiolate “pulls” electrons from the oxygen. Hence the name Push-Pull effect. 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.

The hydrogen bonds to the cysteinate are important and largely conserved. Recent QMM studies have focused on some interesting questions. JACS 133: 15464-15474 (2011).

1. Should we expect meaningful inter-CYP differences in the structure and reactivity of Compound I?
2. How important is conformational flexibility in the proximal peptides?
3. Compound I may be less stable in the presence of substrates which chase water out of the active site.

