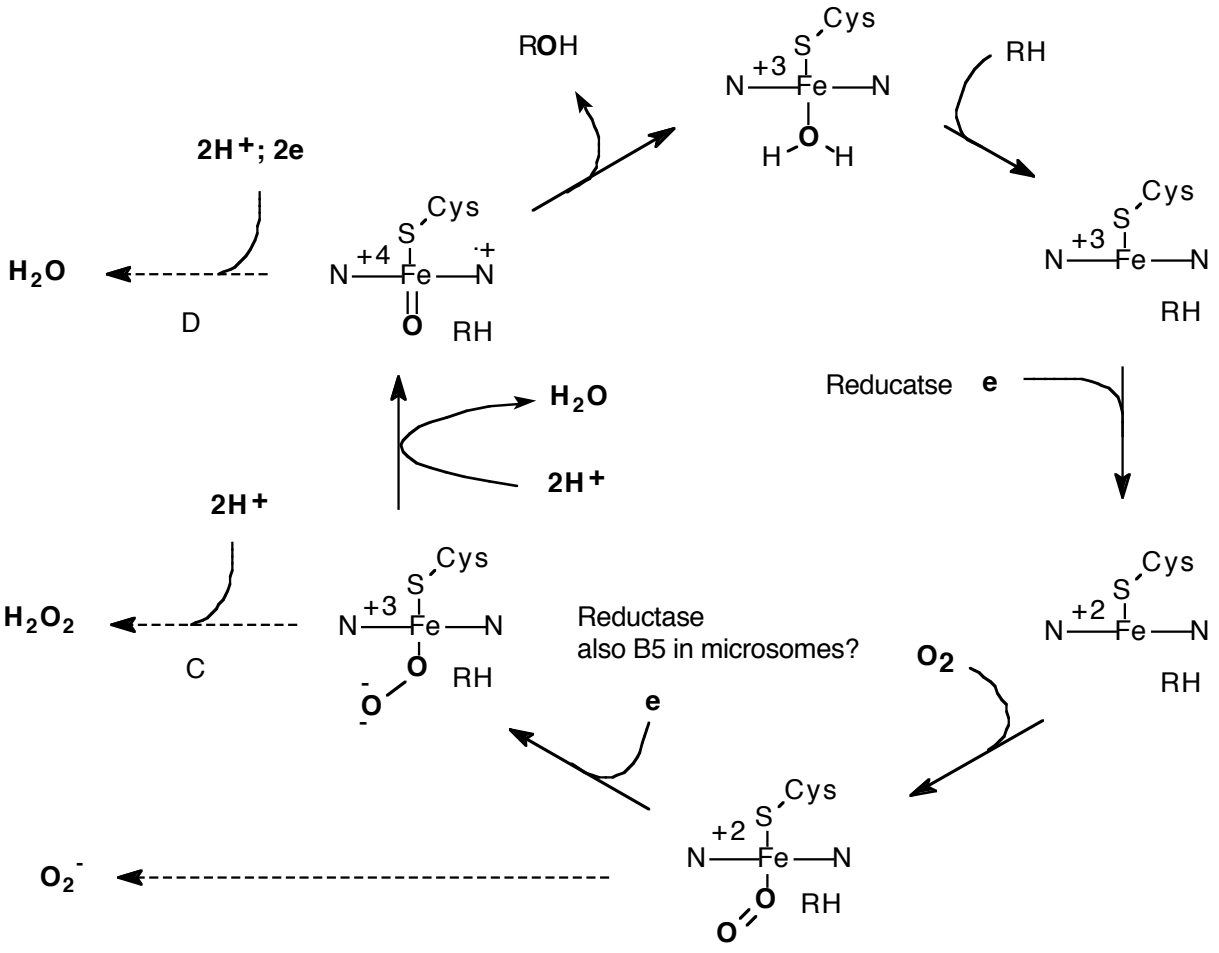
Cytochrome P450 Catalytic Mechanisms II

1. P450 Cycle Stoichiometry and Decoupling:

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

Actual vs theoretical overall stoichiometries for the P450 cycle have been estimated based on a consideration of the elementary reactions together with measurements of substrates and products:

1. "Substrates" consumed are: NADPH, O_2 and RH
2. "Products" formed during the catalytic cycle are (H_2O, O_2^-, ROH, NADP, H_2O_2) (note: only water cannot be measured).



Stoichiometries for P450 cam (exlcudes superoxide)

1 NADPH + 1 O_2 + 1 RH ⟶ 1 NADP + 1 H_2O + 1 ROH

1 NADPH + 1 O_2 ⟶ 1 NADP + 1 H_2O_2

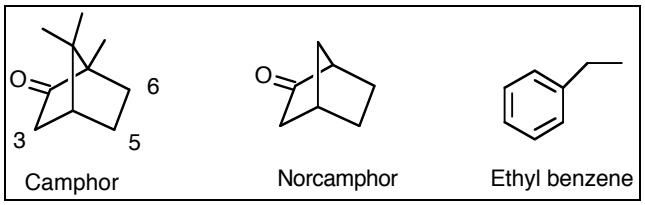
2 NADPH + 1 O_2 ⟶ 2 NADP + 2 H_2O

We will look first at the cycle for CYP101 (P450 cam) with alternative substrates. Note: the rate of superoxide formation in P450 cam is very slow so we will ignore it.)

1. The CYP101 cycle is highly coupled with oxidation of the "natural substrate" camphor.

2. Note then that any branching to alternative products will reduce metabolite formation rates from the optimal rate (totally coupled) and release alternate, sometimes toxic, products. How significant is this?

Coupled and uncoupled turnover of substrates by P450 cam (wild type)



	Consumed		Produced		
Substrate for P450cam	NADH	Oxygen	R-OH	H_2O_2	H_2O*
Camphor amounts	318	280	290	20	0
Norcamphor amounts	577	354	88	88	189
Ethylbenzene rates	52	46	5	40	6

Loida, P and Sligar, S. Biochemistry 32 11530 (1993)
Atkins, W. and Sligar, S. Biochemistry 27 1610 (1988)
*We have corrected for the first water molecule that is formed via the conventional monooxgenase pathway such that the value for water formation reflects the reduction of Compound 1 to water.

Observations:

1. For the "natural substrate" camphor the cycle is almost fully coupled. Only a small amount of hydrogen peroxide is formed. Also there is no water beyond what is made during productive turnover.

2. For nor-camphor we see that the cycle goes to completion much less often (88/(88+88+189)= 24% coupled. The dominant cycle pathway for this substrate is the reduction of the active oxygen to water (52% of total cycles).

3. For ethylbenzene the dominant pathway is the formation of hydrogen peroxide (78%). The cycle only goes to completion 10% of the time.

We can carry out systematic studies to study the linkage between substrate turnover, enzyme structure and products:

1. Series of closely related substrates with well defined enzymes like CYP101 in the hope that we can begin to understand how substrate structure influences coupling.

2. Study the effects of mutations to understand how active site architecture influences coupling.

3. Look at the effects of membrane composition, CPR concentration, the presence of cytochrome b5 and other variables on coupling.

The effects of any particular mutation are unique to the enzyme-substrate fit and may diverge with different substrates… the multistep catalytic mechanism is complex, and there is still little information about what the parameters k_{cat} and K_m really represent in the case of this P450 and different mutations may affect different steps. Parikh, et al Biochem **38**; 5283 (1999).

<u>The ultimate goal of these types of empirical studies is to help us to develop models to help us predict routes and rates of metabolism for the P450s and their substrates.</u>
In order to understand why ROH formation rates are what they are, we will need to understand the factors that promote or de-promote the oxidase pathways such as active site architecture in simpler systems.

Loida, P and Sligar, S. Biochemistry 32 11530 (1993) looked at the turnover of the "un-natural" substrate ethylbenzene by CYP101 wild type and mutants.

Table I: Effect of Active-Site Mutations on Product Formation, Reactant Consumption and Branching Ratios of Uncoupling

P-450$_{cam}$	1-phenylethanol[a]	oxygen[a]	water via oxidase path[a,b]	hydrogen peroxide[a]	NADH oxidation rate[c]
wild-type	5.0 ± 0.5	89 ± 4	11 ± 4	77 ± 4	52 ± 4
single mutants					
T101M	0.1 ± 0.05	76 ± 6	24 ± 6	61 ± 5	14 ± 2
T101I	2.2 ± 0.1	90 ± 4	10 ± 4	83 ± 5	26 ± 3
T185V	5.0 ± 0.5	87 ± 5	13 ± 5	76 ± 4	49 ± 3
T185L	13 ± 1	88 ± 4	12 ± 4	72 ± 3	64 ± 4
T185F	13 ± 1	93 ± 3	7 ± 3	76 ± 4	11 ± 1
V247A	3.0 ± 0.4	52 ± 5	48 ± 5	4 ± 3	30 ± 3
V247M	12 ± 1	87 ± 4	13 ± 4	67 ± 3	40 ± 4
V295I	0.3 ± 0.1	83 ± 4	17 ± 4	68 ± 4	41 ± 2
triple mutant					
T101M–T185F–V247M	13 ± 1	80 ± 6	20 ± 6	52 ± 5	23 ± 2

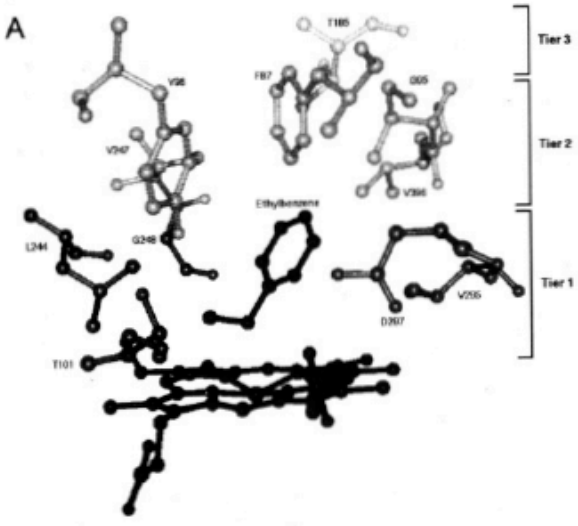
[a] Expressed as a percentage of the NADH equivalents oxidized. [b] Method of calculation from oxygen consumption is outlined under Results. [c] Nanomoles of NADH per minute per nanomoles of P-450. Taken from the data collected for peroxide production and oxygen consumption.

Table II: Substrate Binding Parameters for Active-Site Mutants

	camphor		ethylbenzene	
P-450$_{cam}$	% HS[a]	K_D (μM)	% HS[a]	K_D (μM)
wild-type	91	1.4 ± 0.3	33	90 ± 15
single mutants				
T101M	67	34 ± 4	5	70 ± 18
T185L	62	0.8 ± 0.2	24	44 ± 6
T185F	24	13 ± 2	4	80 ± 20
V247A	95	2.9 ± 0.6	35	60 ± 11
V247M	74	3.3 ± 0.7	31	81 ± 13
V295I	95	1.7 ± 0.3	25	75 ± 10
triple mutant				
T101M–T185F–V247M	40	49 ± 7	5	52 ± 10

[a] The ferric spin-state equilibrium is expressed as the percentage of high-spin form under saturating substrate concentrations.

Autoxidation and Oxidase Activities. Wild-type P-450$_{cam}$ hydroxylates ethylbenzene to give one regioisomer, 1-phenylethanol, with 5% of the reducing equivalents coupled to product formation (Loida & Sligar, 1993). Of the NADH oxidized in the reaction, 77% is used to produce hydrogen



We will also have to understand how substrate structure affects (a) total turnover (b) decoupling (c) enzyme spin states and what substrate features promote/depromote oxygen insertion into substrates.

Sibbesen, O, Zhang, Z. and Ortiz de Montellano. P. Arch. Bioch. Biophys. p 285 (1998)

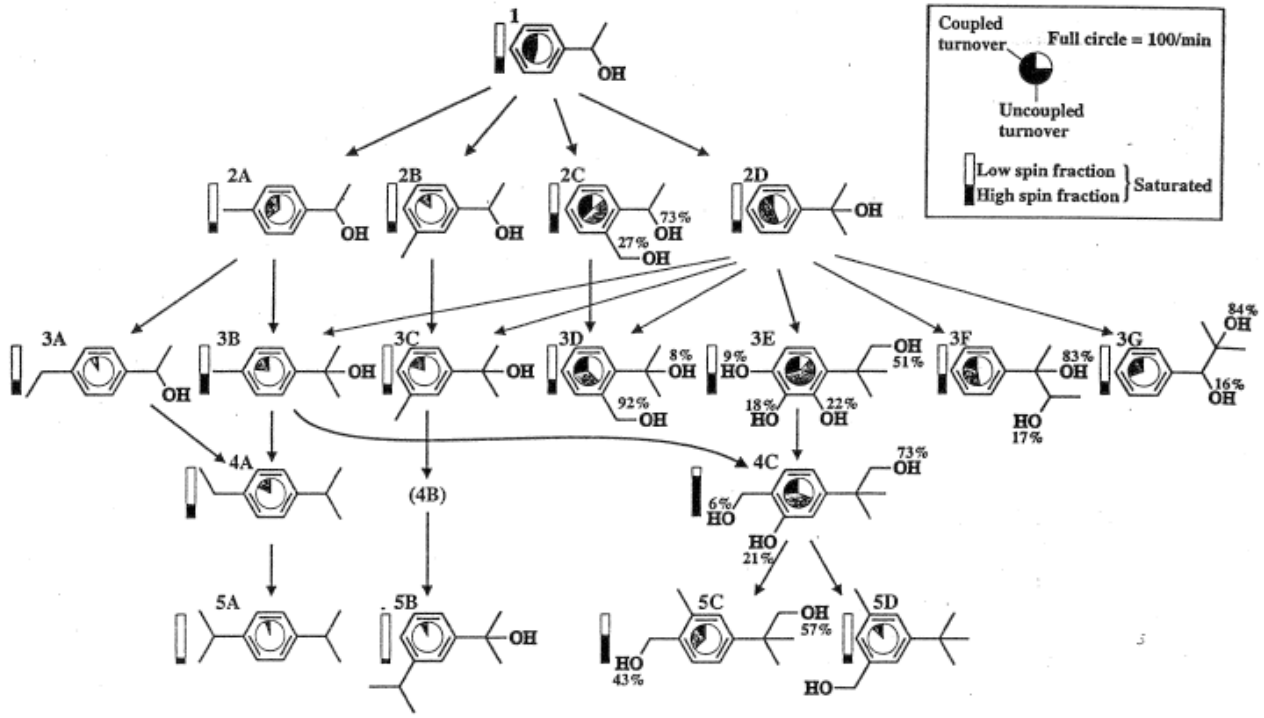
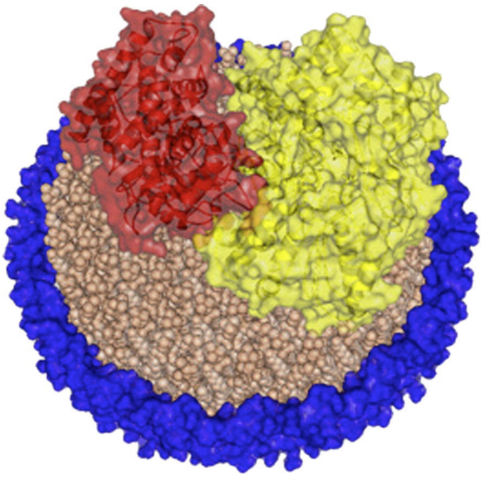


FIG. 2. Spin state changes, uncoupled and coupled turnover, and position of hydroxylation by wild-type $P450_{cam}$ of a series of alkylbenzenes of increasing size. The rows in the figure correspond to compounds having the same number of carbon atoms. The substrates are the alkylbenzenes without the hydroxyl groups. The hydroxyl groups indicate the sites that are hydroxylated in the metabolites, with only one hydroxyl group per metabolic product. The extent of hydroxylation at each site is indicated as a percentage of the total organic products. The bar adjacent to each structure indicates the fraction of the protein in the high (dark) versus low (open) spin state with saturating concentrations of the compound. The extent of coupled (dark wedge) and uncoupled (speckled wedge) turnover is indicated in the circle in the phenyl ring of each compound, where the full circle corresponds to an NADH consumption of 100 μM min^{-1}.

The oxidation by cytochrome $P450_{cam}$ (CYP101) of ethylbenzene and a series of substrates derived from it by addition of one, two, three, or four carbon atoms has been examined. For each of the 18 substrates, the shift in spin state due to substrate binding, the extent of coupled turnover to give organic products and uncoupled turnover to give H_2O_2 and H_2O, and the identities of the organic products have been determined. The same studies have been carried out with the T185L and T185F mutants of $P450_{cam}$ in which the active site volume is decreased. The results show that no detectable correlation exists between the observed spin state change and any other parameter studied. For substrates of equal size, coupled and uncoupled turnover vary widely but both are maximized when the phenyl ring bears one large alkyl substituent or a methyl ortho to the largest alkyl substituent. The presence of substituents in addition to these decreases activity. In the absence of other changes, coupled turnover is correlated with the size of the largest substituent, but no such correlation exists for uncoupled turnover. Decreasing the size of the active site cavity by a T185L mutation generally increases coupled turnover without altering the dependence on the alkyl group size. A T185F mutation causes too great an active site perturbation for structure–activity studies. Substrate oxidation occurs preferentially at 2° or 3° C–H bonds of the largest substituent or on the benzylic methyl ortho to it. Aromatic hydroxylation only competes with the oxidation of nonbenzylic 1° C–H bonds. The extent of coupled turnover is a function of substrate shape, substrate size, and cavity size, but still elusive parameters control the extent of uncoupled turnover.

SAR studies on the mammalian forms: This may not be easy as we are interested in rates, sites of oxidation and catalytic efficiency. Even when we have crystal structures of the mammalian forms. Effects on kcat and catalytic efficiency (substrate clearance) may not be correlated

P450 3A4 in nanodiscs from the Sligar Lab (Biochem Biophys Res Comm (January 4 2013 in press)



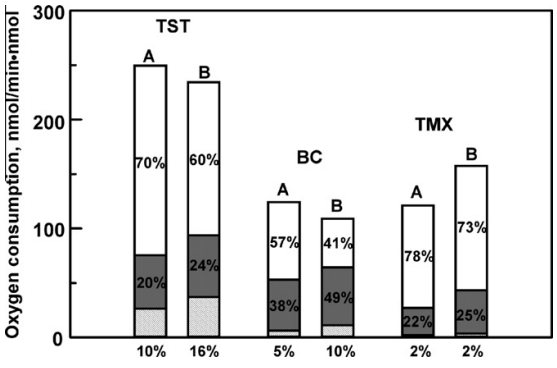


Fig. 3. Uncoupling pathways in CYP3A4 catalyzed metabolism of testosterone (250 μM), bromocriptine (10 μM) and tamoxiphene (80 μM) shown as fractional contributions of oxygen consumption. CYP3A4 is incorporated in Nanodiscs assembled with POPC (A) or with 30% POPS + 70% POPC (B). The fraction of peroxide uncoupling is shown on the top bar (white), the oxidase uncoupling fraction as the middle shaded gray bar and the product forming pathway in the dashed bar at the bottom.

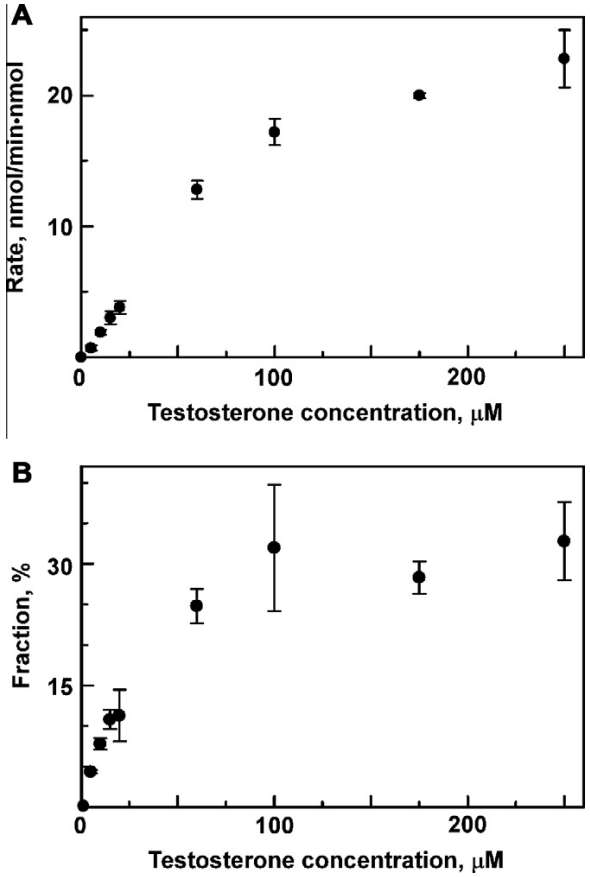


Fig. 2. Rate of testosterone hydroxylation (A) and fraction of Cpd I utilized via the productive pathway (B) measured at different testosterone concentrations.

Conclusions

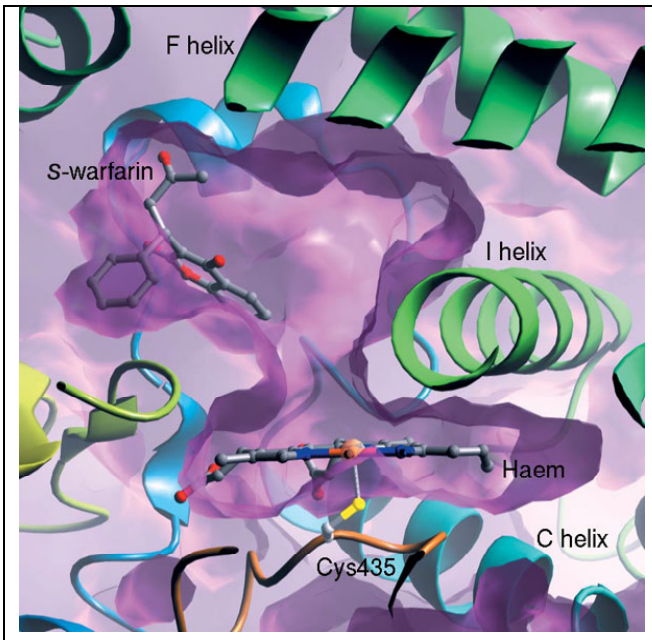
We have documented the oxidase uncoupling pathway in CYP3A4 Nanodiscs as a function of TST concentration.

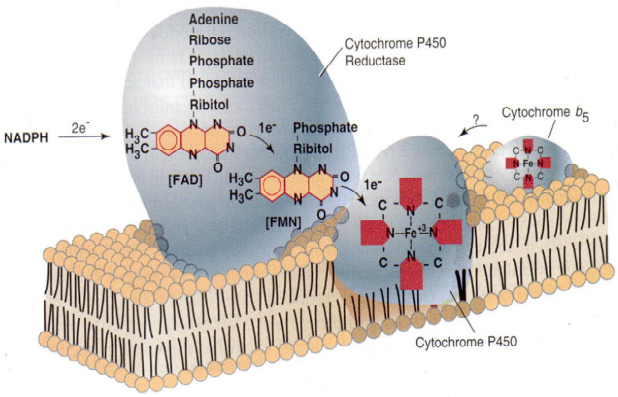
We found that even in the absence of substrate the futile consumption of NADPH results in substantial, irreversible formation of Cpd I.

For all three studied substrates Cpd I predominantly decays via the oxidase pathway, even at substrate saturated CYP3A4.

We also found that the presence of anionic lipids, in this case 30% POPS, improves overall coupling and facilitates product formation for TST and BC by a factor of 1.5–2.

How will we eventually rationalize structure and function to predict affinities, products and rates?
Will we need to include coupling/decoupling factors?





Textbook of Biochemistry with Clinical Correlations, Fourth Edition

Catalytic activity in CYP2C9 and its Leu^{359} variant

Table 1. Kinetic parameters of piroxicam 5′-hydroxylation, phenytoin 4′-hydroxylation, tenoxicam 5′-hydroxylation, mefenamic acid 3′-methylhydroxylation, tolbutamide methylhydroxylation, *S*-warfarin 7-hydroxylation and diclofenac 4′-hydroxylation by wild-type CYP2C9 (Ile^{359}) and its Leu^{359} variant expressed in yeast

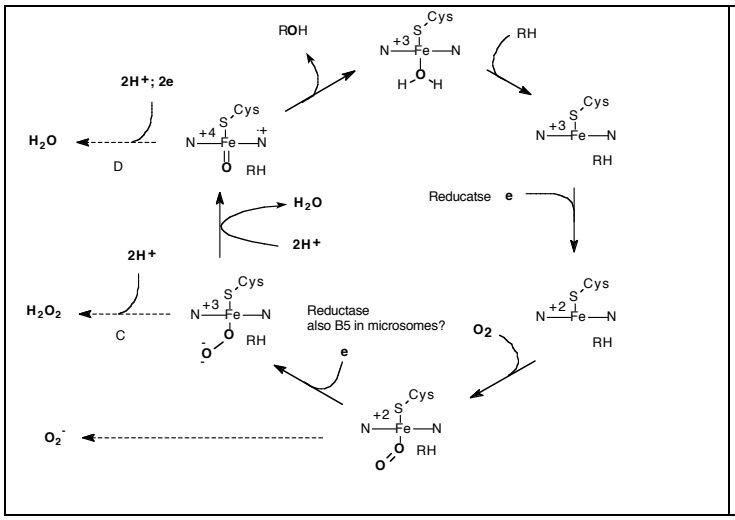
	K_m (μM)	V_{max} (*pmol/min/pmol P450*)	V_{max}/K_m (*μl/min/nmol P450*)
Piroxicam 5′-hydroxylation			
Ile^{359}	40 ± 3	0.408 ± 0.026	10.2 ± 0.6
Leu^{359}	61 ± 33	0.019 ± 0.006***	0.3 ± 0.1***
Phenytoin 4′-hydroxylation			
Ile^{359}	15 ± 4	0.191 ± 0.018	14.0 ± 4.8
Leu^{359}	30 ± 7*	0.015 ± 0.003***	0.5 ± 0.2*
Tenoxicam 5′-hydroxylation			
Ile^{359}	28 ± 3	0.264 ± 0.003	9.44 ± 0.81
Leu^{359}	90 ± 19*	0.034 ± 0.014***	0.41 ± 0.21***
Mefenamic acid 3′-hydroxylation			
Ile^{359}	8.4 ± 0.5	14.9 ± 3.0	1.80 × 10^3 ± 0.42 × 10^3
Leu^{359}	40.8 ± 7.5*	4.2 ± 1.4**	0.10 × 10^3 ± 0.01 × 10^3*
Tolbutamide hydroxylation			
Ile^{359}	151 ± 32	9.2 ± 1.0	61.4 ± 6.1
Leu^{359}	1729 ± 512*	10.0 ± 2.0	5.9 ± 0.7***
S-warfarin 7-hydroxylation			
Ile^{359}	5.8 ± 0.8	0.248 ± 0.018	43.7 ± 7.0
Leu^{359}	21.6 ± 1.5***	0.111 ± 0.012***	5.1 ± 0.6*
Diclofenac 4′-hydroxylation			
Ile^{359}	3.9 ± 0.3	35.6 ± 1.3	9.16 × 10^3 ± 0.29 × 10^3
Leu^{359}	12.6 ± 2.8*	33.3 ± 2.6	2.69 × 10^3 ± 0.76 × 10^{3***}

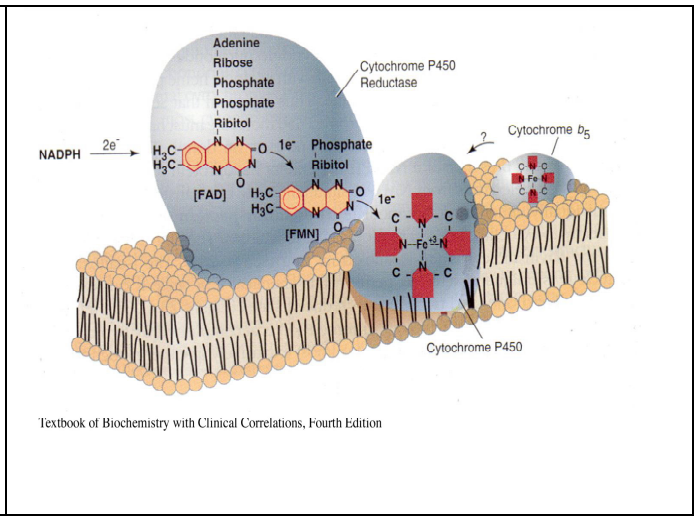
Note that position 359 is not in the binding pocket and that the mutation does not cause major changes in coupling and spin state!

Factors that are often mentioned as being important:

1. Ability of substrates to promote the high spin form (dehydration of the active site)?
2. Distance between the heme iron and site of oxidation on the substrate?
3. "Ease of oxidation" at the site of oxidation?
4. Ability of the substrates to inhibit decoupling reactions or stabilize productive cycle intermediates, particularly compound I?
5. Ability of substrates to promote slow steps in the cycle (second electron transfer)?
6. Ability of allosteric effectors to increase stability of compound I?

The role of cytochrome b5 in microsomal oxidation reactions also represents a serious barrier to predictivity.





Stimulatory effects of cytochrome B_5: See Pharmacol. Therap. 97 139-152 (2003) for a review

Cytochrome B_5 is a small heme containing protein that **donates** (ferrous B_5) and **accepts** (ferric B_5) a single electron. There is ample evidence that some P450 enzymes will form a high affinity complex with B_5. Antibodies to B_5 inhibit the oxidation of a number of substrates in intact microsomes. The implied stimulation of P450 activity by B_5 has been demonstrated in reconstituted lipid vesicles of the enzymes as well as membranes prepared from standard expression systems. The effect is paradoxical in that it is:

(1) substrate and enzyme dependent
(2) not uniformly observed but inhibition is rarely observed
(3) occasionally reported to be obligatory for some substrate enzyme pairs
(4) stimulation of activity is also sometimes observed by with redox-silent apo-B_5.

Two major hypothesis (with many variations) have been forwarded to explain the B_5 effect.

1. Electron transfer/enhanced coupling: Introduction of the second electron in the P450 cycle from B_5 enhances the rate of second electron transfer observed with reductase alone. Supporting evidence includes reduction in superoxide formation in the presence of B_5. decoupling to superoxide and/or enhancing the rate of second electron transfer.

2. An allosteric effect where the structure of P450 is altered when complexed with B_5. This is supported by the effect of apo-B_5. A conformational change in the P450 could alter the substrate binding pocket of P450 in a manner that decreased uncoupling reactions thereby increasing the concentration of the active oxygen or by enhancing the rate of reaction of substrate with the active oxygen by decreasing the mean heme-substrate distance.
Consider the rather confusing titles of two recent articles by Lucy Waskell and co-workers.

Zhang H, Im SC, Waskell L.
J Biol Chem. 2007 Oct 12;282(41):29766-76.

1. Cytochrome b5 increases the rate of product formation by cytochrome P450 2B4 and competes with cytochrome P450 reductase for a binding site on cytochrome P450 2B4.

Zhang H, Hamdane D, Im SC, Waskell L.
J Biol Chem. 2008 Feb 29;283(9):5217-25.

2. Cytochrome b5 inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4.

In Vivo and In Vitro effects of a liver b5 knockout. Recently studies of drug metabolism in mice where cytochrome b5 was conditionally knocked out in liver were carried out by C. Roland Wolf and co-workers. Panel A shows the expression of b5 in various tissues in wild type and HBN (liver b5 knockout) animals. Panel C shows the levels of various enzymes of interest in the livers of control and knockout animals. (JBC 283; 31385 (2008))

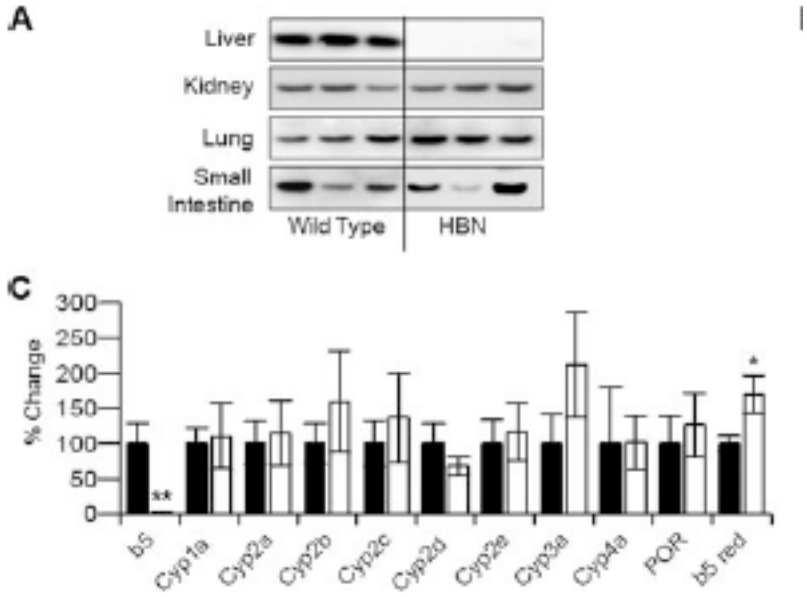


Table 1 below shows the kinetics of oxidation of P450 substrate probes by microsomes from control and knock-out animals.

TABLE 1

Kinetic analyses of the metabolism of probe drugs by hepatic microsomes from HBN mice and wild-type controls

Assays were performed in triplicate for each concentration of substrate. S.D. values given are from the fit of the curve as calculated using the Michaelis-Menten equation (GraFit version 5 (Erithacus Software, Horley, UK)). For tolbutamide, the Hill equation (GraFit version 5) was used to fit the data, giving Hill coefficients (n) of 0.38 and 0.29 for wild type and HBN, respectively.

Substrate	Metabolite	Wild type		HBN	
		K_m	V_{max}	K_m	V_{max}
		μM	pmol/min/mg	μM	pmol/min/mg
Chlorzoxazone	6-Hydroxychlorzoxazone	47 ± 9	1930 ± 100	53 ± 6	387 ± 11
Midazolam	1′-Hydroxymidazolam	2.1 ± 0.3	389 ± 15	2.0 ± 0.3	116 ± 4
Midazolam	4-Hydroxymidazolam	13.3 ± 0.4	146 ± 2	7.7 ± 0.9	7.3 ± 0.3
Phenacetin	Acetaminophen	5.6 ± 1.0	133 ± 6	4.1 ± 0.7	77 ± 3
Metoprolol	α-Hydroxymetoprolol	84 ± 7	483 ± 14	99 ± 9	76 ± 3
Metoprolol	*O*-Desmethylmetoprolol	86 ± 9	1650 ± 2	157 ± 14	531 ± 21
Tolbutamide	Hydroxytolbutamide	11200 ± 3400	211 ± 17	165 ± 48	44 ± 165

Table 2 shows the effects of b5 knockout on oral and iv pharmacokinetics of test drugs in vivo in mice.

TABLE 2

***In vivo* pharmacokinetic data from HBN mice and wild-type controls dosed with a drug mixture**

Adult male HBN and WT mice were administered a drug mixture intravenously; serial blood samples were collected over an 8-h period and analyzed for plasma drug concentrations by LC-MS/MS and key pharmacokinetic parameters calculated. Absolute oral bioavailability was calculated by dividing AUC ($0 \rightarrow \infty$, oral) by AUC ($0 \rightarrow \infty$, intravenous) (supplemental Tables 4 and 5).

Drug	Terminal half-life (intravenous)		Bioavailability (%)	
	Wild type	HBN	Wild type	HBN
Midazolam	83 ± 15	329 ± 132	14	55
Phenacetin	53 ± 11	168 ± 56	0.6	0.8
Metoprolol	96 ± 13	260 ± 65	0.3	0.3
Chlorzoxazone	92 ± 19	257 ± 76	3.7	1.9
Tolbutamide	197 ± 37	321 ± 25	95 (154)[a]	47 (135)[a]

[a] Comparative data where tolbutamide was dosed as a single agent.

Comments: In vitro, cytochrome b5 modulates the rate of cytochrome P450-dependent mono-oxygenation reactions. However, the role of this enzyme in determining drug pharmacokinetics in vivo and the consequential effects on drug absorption distribution, metabolism, excretion, and toxicity are unclear. In order to resolve this issue, we have carried out the conditional deletion of microsomal cytochrome b5 in the liver to create the hepatic microsomal cytochrome b5 null mouse. These mice develop and breed normally and have no overt phenotype. In vitro studies using a range of substrates for different P450 enzymes showed that in hepatic microsomal cytochrome b5 null NADH-mediated metabolism was essentially abolished for most substrates, and the NADPH-dependent metabolism of many substrates was reduced by 50-90%. This reduction in metabolism was also reflected in the in vivo elimination profiles of several drugs, including midazolam, metoprolol, and tolbutamide. In the case of chlorzoxazone, elimination was essentially unchanged. For some drugs, the pharmacokinetics were also markedly altered; for example, when administered orally, the maximum plasma concentration for midazolam was increased by 2.5-fold, and the clearance decreased by 3.6-fold in

hepatic microsomal cytochrome b5 null mice. These data indicate that microsomal cytochrome b5 can play a major role in the in vivo metabolism of certain drugs and chemicals but in a P450- and substrate-dependent manner.

What might be going on with cytochrome b5?

Review: S.-C. Im, L. Waskell / Archives of Biochemistry and Biophysics 507 (2011) 144–153
The interaction of microsomal cytochrome P450 2B4 with its redox partners ,cytochrome P450 reductase and cytochrome b5

Major findings with CYP2B4:

1. Cytochrome b5 first increases and then decreases subtrate oxidation rates: This is commonly observed with other P450s (stimulation then inhibition). The argument here is that cytochrome b5 competes with reductase and inhibits introduction of the first electron in the cycle. Note that the coupling efficiency (+formaldehyde/-NADPH ratio) improves with increasing b5.

Table 3
Effect of cyt b_5 on the rate of NADPH consumption and benzphetamine metabolism by cyt P450 2B4 under steady-state conditions at 30 °C.

Molar ratio P450:CPR:b_5	NADPH nmol/min/nmol of cyt P450	Formaldehyde nmol/min/mol of cyt P450
1:1:0	83 ± 5.5	47 ± 3.3
1:1:0.5	81 ± 2.1	56 ± 0.8
1:1:1	66 ± 3.2	48 ± 1.0
1:1:3	36 ± 6.3	30 ± 3.8
1:1:5	25 ± 1.3	21 ± 1.8

Data from: [28]

2. When the oxyferrous P450 is preformed the decay of this species and the formation of product upon quenching with addition of either reduced b5 or reduced reductase can be monitored. In essence they are measuring the relative ability of b5 and CPR to introduce the second electron. The data indicate that electron transfer from b5 is much faster (2 orders of magnitude).

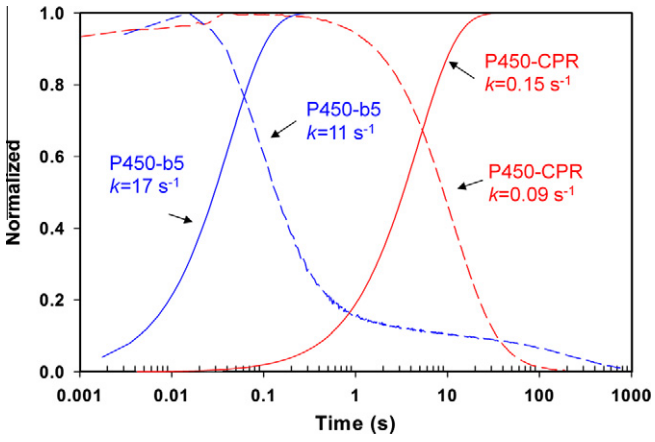
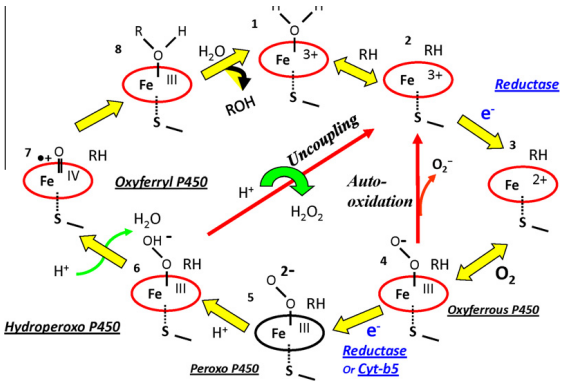


Fig. 7. Comparison of the kinetics of the decay of oxyferrous cytochrome P450 to the ferric species and of norbenzphetamine formation in the presence of substrate, benzphetamine. The ΔA_{438nm} represents the decay of the oxyferrous P450 2B4 to the ferric protein [22]. The data for product formation are from [28]. (—), product formation by P450–b_5; (—), product formation by P450–CPR; (---), ΔA_{438nm} for P450–b_5; (---), ΔA_{438nm} for P450–CPR.



3. The putative docking sites for CPR and b5 have been investigated by site directed mutagenesis studies and by crosslinking studies.

a. It appears that the sites on the P450 overlap which present a conundrum or opportunity.

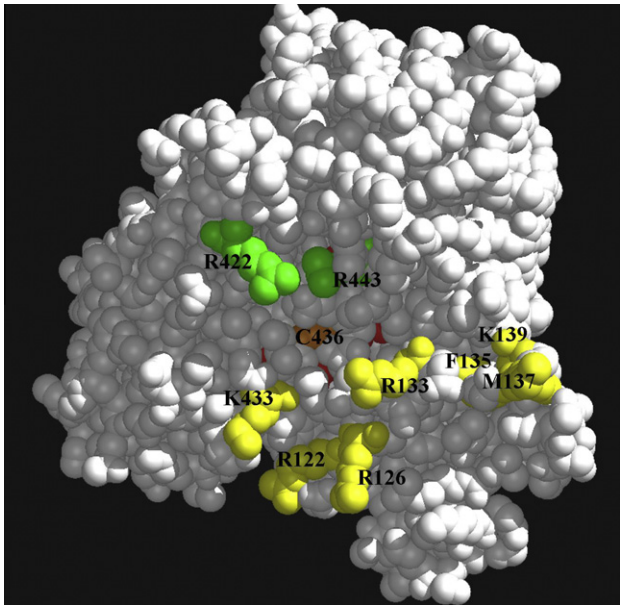


Fig. 2. The binding site for cyt b_5 and cyt P450 reductase on the proximal surface of cyt P450 2B4. The largely buried heme is in red; residues R422 and 443, involved in binding only the reductase, are illustrated in green; residues which participate in binding both cyt b_5 and reductase in and near the C-helix (R122-K139) and K433 are shown in yellow. The figure was generated using the Midas Plus software system from the Computer Graphics Laboratory, University of California, San Francisco, [53]. (Reprinted with permission of Elsevier, Biochem. Biophys. Res. Commun. 338 (2005) 499–506.)

b. It also seems that reductase can bind to P450 in an open and a closed conformation.

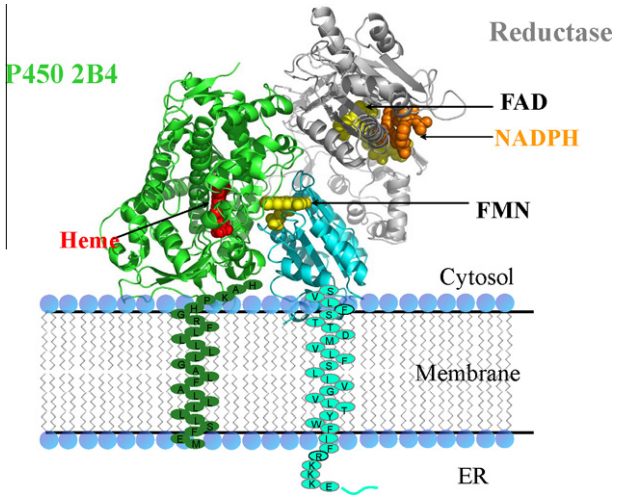
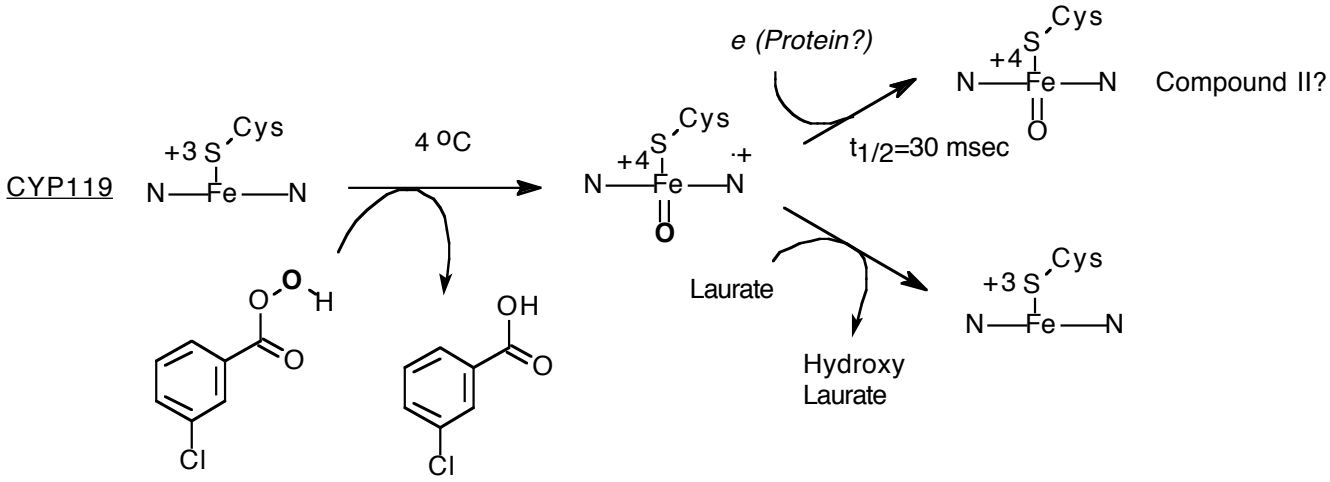


Fig. 5. Cytochrome P450–reductase model complex bound to a membrane. The reductase is molecule A (PDB code 3ES9) of the open conformation of reductase.

Characterization of Compound I

Compound I can be generated by the low temperature treatment of CYP119 with MCPBA.
UV-Vis Spectrum CYP119 Compound I JBC 277: 9641 (2002)

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.



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. Note that additional reducing equivalents are not available to reduce CPD 1 to water.

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.
More recently the technique has been improved and good spectroscopic characterization as well as substrate turnover has been observed.

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

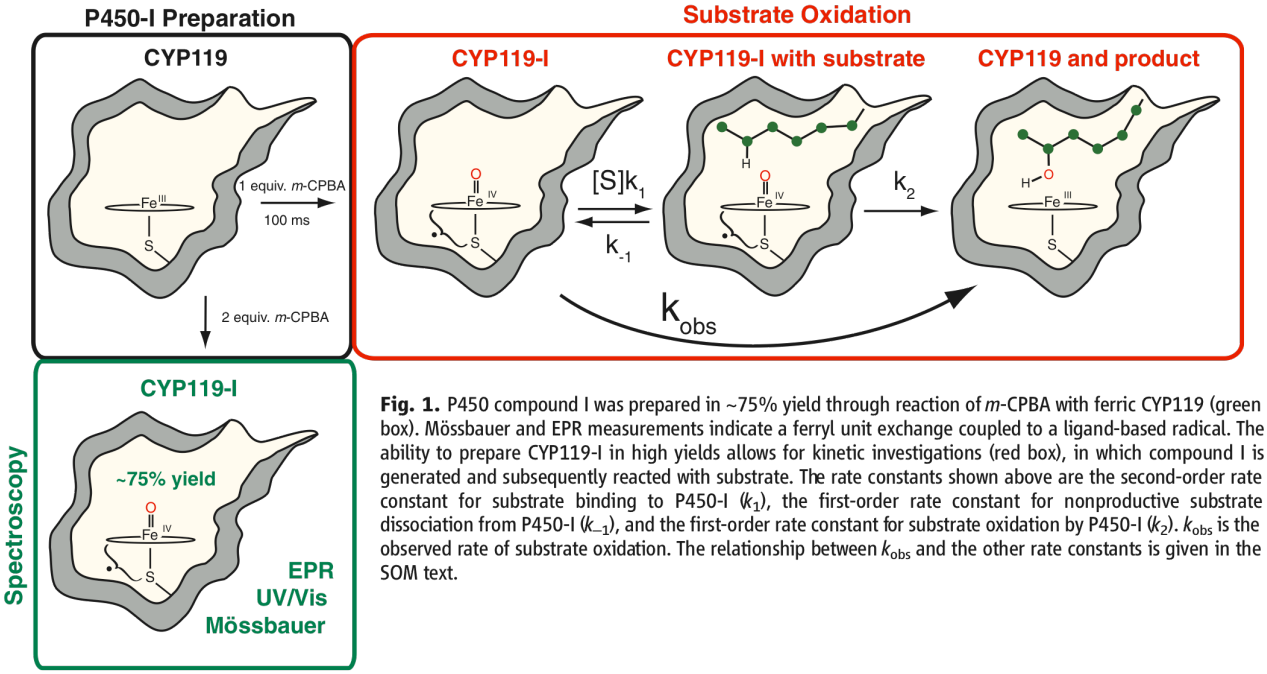


Fig. 1. P450 compound I was prepared in ~75% yield through reaction of *m*-CPBA with ferric CYP119 (green box). Mössbauer and EPR measurements indicate a ferryl unit exchange coupled to a ligand-based radical. The ability to prepare CYP119-I in high yields allows for kinetic investigations (red box), 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.

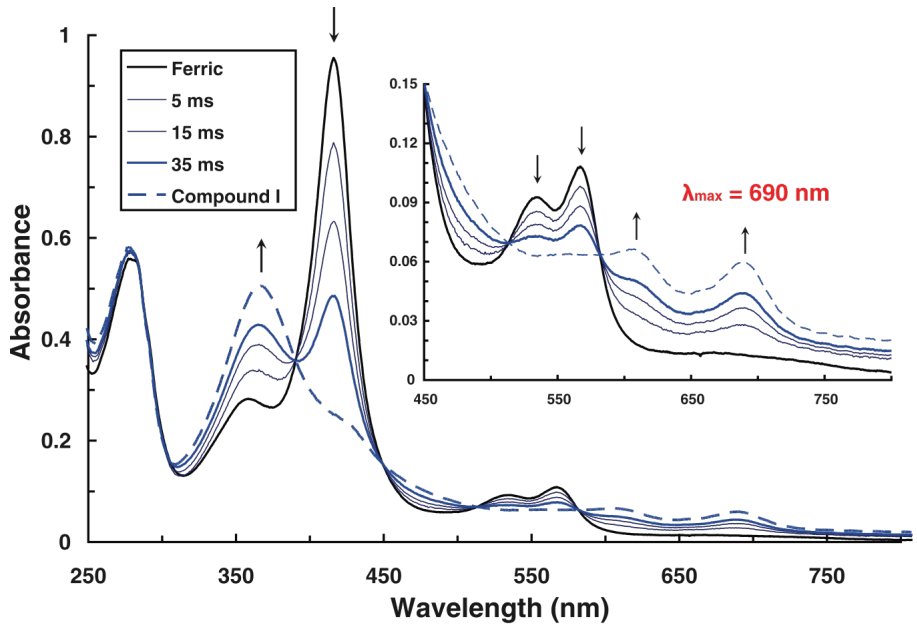
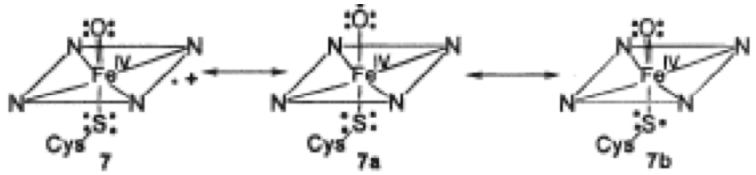


Fig. 2. UV/visible spectra obtained from the stopped-flow mixing (1:1) of 20 μM ferric CYP119 with 40 μM *m*-CPBA at 4°C. The blue traces correspond to spectra taken 5, 15, and 35 ms after mixing. Maximum yield of P450-I was ~70% at 35 ms. At later times, isosbestic points were lost, and a portion of 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*).

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). This rate is much faster than normal turnover indicating that substrate oxidation is not rate limiting.
6. Further studies using deuterium isotope effect studies confirm that the reaction with substrate is limited by the rate of binding of substrate to the pre-formed Compound I enzyme.