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Review

Role of cytochrome b_5 in catalysis by cytochrome P450 2B4

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Abstract

Cytochrome b_5 has been shown to stimulate, inhibit or have no effect on catalysis by P450 cytochromes. Its action is known to depend on the isozyme of cytochrome P450, the substrate, and experimental conditions. Cytochrome P450 2B4 (CYP 2B4) has been used in our laboratory as a model isozyme to study the role of cytochrome b_5 in cytochrome P450 catalysis using two substrates, methoxyflurane and benzphetamine. One substrate is the volatile anesthetic, methoxyflurane, whose metabolism is consistently markedly stimulated by cytochrome b_5. The other is benzphetamine, whose metabolism is minimally modified by cytochrome b_5. Determination of the stoichiometry of the metabolism of both substrates showed that the amount of product formed is the net result of the simultaneous stimulatory and inhibitory actions of cytochrome b_5 on catalysis. Site-directed mutagenesis studies revealed that both cytochrome b_5 and cytochrome P450 reductase interact with cytochrome P450 on its proximal surface on overlapping but non-identical binding sites. Comparison of the rate of reduction of oxyferrous CYP 2B4 and the rate of substrate oxidation by cyt b_5 and reductase with stopped-flow spectrophotometric and rapid chemical quench experiments has demonstrated that although cytochrome b_5 and reductase reduce oxyferrous CYP 2B4 at the same rate, substrate oxidation proceeds more slowly in the presence of the reductase.

Keywords: Cytochrome P450 2B4; Cytochrome b_5; Cytochrome P450 reductase; Methoxyflurane; Anesthetic; Benzphetamine; Rapid chemical quench; Single-turnover kinetics; Stopped-flow spectrophotometry; Stoichiometry; Site-directed mutagenesis; Binding site

For a number of years our laboratory has attempted to understand the mechanism by which cytochrome b_5 (cyt b_5) influences catalysis by cytochrome P450 (cyt P450). Interest in the role of cyt b_5 in catalysis by cyt P450 began a number of years ago when it was observed that the volatile anesthetic, methoxyflurane, was not significantly metabolized by pure CYP 2B4 [1]. This result was very perplexing because phenobarbital, which was known to induce CYP 2B4, markedly enhanced the metabolism of methoxyflurane in rabbit hepatic microsomes. Subsequently, it was found that in a purified reconstituted system methoxyflurane required cyt b_5 for catalysis by CYP 2B4. In contrast, benzphetamine metabolism was minimally altered in the presence of cyt b_5 under the same conditions. Our results with cyt b_5 were obtained at a time when other laboratories were reporting that cyt b_5 either stimulated, inhibited or had no effect in their reconstituted systems. The consensus of the published investigations was that the effect of cyt b_5 was dependent on the isozyme of cyt P450, the substrate, and experimental conditions. Subsequently, it was shown that the effect of cyt b_5 even varied with the sequence of addition of the pure proteins to the reconstituted system [2,3]. In light of these confusing and unintelligible but nevertheless fascinating studies on the role of cyt b_5 in cyt P450 catalysis, experiments were initiated to elucidate the function of cyt b_5 in a model system using CYP 2B4 as the model cyt P450 and the substrates, methoxyflurane and benzphetamine. The long-term goal of our laboratory has been to elucidate the mechanism of the fascinating effects of cyt b_5 on substrate oxidation by a single cyt P450 isozyme. The literature on the effect of cyt b_5 on microsomal cyt P450 is vast with many incompletely understood phenomena. It is not the purpose of this review to summarize that extensive literature. Rather the focus here will be to briefly describe the major conclusions drawn from our studies on

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the role of cyt b_5 in CYP 2B4 catalysis with the substrates methoxyflurane and benzphetamine, and briefly highlight future directions.

Stoichiometry of the CYP 2B4-catalyzed metabolism of methoxyflurane and benzphetamine with and without cyt b_5

Because of the variable effects of cyt b_5 on catalysis by cyt P450, we elected to attempt to understand its effect with a single cyt P450 and two substrates, one, methoxyflurane, which appeared to require cyt b_5 for its metabolism, and a second, benzphetamine, whose metabolism was minimally altered by cyt b_5. The reasoning is that once the basis of the effects of cyt b_5 in a particular system is well understood, targeted experiments could more rationally be designed to explore its effect with other substrates and cyt P450 isozymes ultimately for a more complete understanding of its role in cyt P450 catalysis. An essential first step in understanding a catalytic mechanism is to understand the stoichiometry of the reaction being investigated. Although this is a standard procedure and obvious after a few moments reflection, it is often ignored. Determination of the stoichiometry of metabolism of the two substrates included measurement of the reactants consumed, NADPH and oxygen, and products produced. Included as products were superoxide, hydrogen peroxide, and the metabolites of benzphetamine and methoxyflurane which is oxidized by two pathways [3]. Previous work had indicated that the activity and relative stoichiometry in a reconstituted system are influenced by protein-to-lipid ratios, the length of preincubation, and the order of addition of the reactants. For both substrates cyt b_5 invariably improved the efficiency of NADPH utilization for product formation ≅6–15% at the expense of the side product, superoxide. It was assumed that the superoxide resulted from the autoxidation of oxyferrous cyt P450. The superoxide attributed to autoxidation of cyt P450 was corrected for the superoxide formed upon autoxidation of cyt P450 reductase. If the catalytic efficiency had increased at the expense of hydrogen peroxide, the conclusion and mechanistic implications would have been different. A decrease in hydrogen peroxide formation by cyt b_5 would have demonstrated that uncoupling had occurred after reduction of oxyferrous cyt P450. Depending on the experimental conditions product formation from methoxyflurane invariably was enhanced, 5- to 7.5-fold, by cyt b_5. On the other hand, product formation from benzphetamine fluctuated minimally, remaining unchanged, slightly increased (1.3-fold) or decreased by ≅40%.

The apparently conflicting observations of increased catalytic efficiency but a diminution in total product formation for benzphetamine are explained by a second effect of cyt b_5. When cyt b_5 was added to the reaction mixture before the reductase, cyt b_5 inhibited total NADPH consumption, which is considered to be a measure of the overall activity of the reconstituted system. It is noteworthy that the inhibitory action of cyt b_5 could be reversed by incubating the reaction mixture overnight at 4 °C [2,3]. The net result of the stimulatory and inhibitory effects of cyt b_5 will dictate whether stimulation, inhibition or unchanged product formation is observed. For example, when total NADPH utilization is inhibited by addition of cyt b_5 to the reaction mixture before the reductase, the efficiently metabolized (≅50%) substrate, benzphetamine, exhibits a net decrease (≅40%) in metabolism not counterbalanced by an enhancement of the efficiency (2–15%) of the reaction. In contrast, with methoxyflurane, whose inefficient metabolism (≅0.5–3% in the absence of cyt b_5) is virtually undetectable in the absence of cyt b_5, inhibition of NADPH consumption was never able to overcome the simultaneous stimulation of product formation (5- to 7.5-fold) due to an increase in the utilization of NADPH for product formation. These studies reconfirmed that the effect of cyt b_5 on cyt P450 catalysis was variable and subject to alteration by a vast number of experimental conditions. In spite of the variability some useful general conclusions were reached. Depending on the experimental conditions cyt b_5 may or may not inhibit catalysis by decreasing NADPH consumption. Nonetheless, it consistently increases the efficiency of substrate oxidation. It remains to be determined how generally this interpretation of our results applies to additional systems [3].

Identification of the binding site for cyt b_5 and cyt P450 reductase on CYP 2B4

Because the buried heme is closest to the protein surface near the heme thiolate ligand on what is known as the proximal surface of cyt P450, it was expected to be the region to which its redox partners would bind. The proximal cyt P450 surface also has a positive charge which had been proposed to interact with the negatively charged surface of its redox partners [4].

To determine whether the proximal surface was the binding site for its redox partners, 25 amino acids distributed over the entire surface of the P450 2B4 model were mutated and tested for their ability: (a) to bind cyt b_5, cyt P450 reductase and substrate, and (b) to stimulate the metabolism of methoxyflurane [5]. Positively charged as well as hydrophobic residues on the proximal surface were mutated to alanine. Of the 25 mutated amino acids only seven (R122, R126, R133, F135, M137, K139, and K433) in the C-helix on the proximal surface, including both basic and hydrophobic amino acids, were shown to be involved in binding cyt b_5. The proteins containing these mutations had a diminished ability to stimulate methoxyflurane metabolism in the presence of cyt b_5 and an increase in the dissociation constant with cyt b_5 (see Table 1). Substrate interacted normally with the seven mutant proteins.

Surprisingly, mutation of the same seven amino acids to alanine also generated mutant proteins which bound cyt P450 reductase poorly as revealed by an increase in the apparent dissociation constant, K_d (app), for the reductase. In addition, mutation of Arg422 near the β bulge and

Table 1
Characterization of the binding site mutants of cyt P450 2B4

P450 residues and location in 2° structure[a]	K_d P450-b_5 complex (μM ± SD)	Ratio of MF metabolism ± cyt b_5	K_d (app) P450-reductase complex (μM ± SD)
Group A			
WT	0.2 ± 0.16	4.9	0.02 ± 0.02
R422A (m-β)	0.3 ± 0.04	5.3	0.28 ± 0.13
R443A (L)	0.2 ± 0.15	3.8	0.46 ± 0.16
Group B			
R122A (B′–C)	1.2 ± 0.16	2.9	0.23 ± 0.08
F135A (C*)	1.6 ± 0.56	2.0	0.14 ± 0.04
M137A (C*)	2.8 ± 1.2	1.1	0.17 ± 0.02
K139A (C*–D)	3.0 ± 1.1	1.5	0.46 ± 0.11
K433A (β)	3.6 ± 0.77	0.3	1.1 ± 0.81
R126A (C)	4.9 ± 4.2	0.7	0.27 ± 0.08
R133A (C–C*)	>10	1.2	1.2 ± 0.57

[a] The secondary structure nomenclature and methods are provided in [5].

Arg443 in the L helix to alanine implicated these two amino acids in binding cyt P450 reductase. Fig. 1 shows the location of these amino acids on the proximal surface of the heme in relationship to the axial cysteine and the buried heme. The V_{max} of the mutant proteins for benzphetamine metabolism was similar to that of the native protein demonstrating that the enzymatic mechanism had not been altered by the mutations. When the crystal structure became available [6], the mutated amino acids were confirmed to be on the surface of CYP 2B4 as predicted by our model of CYP 2B4 [7].

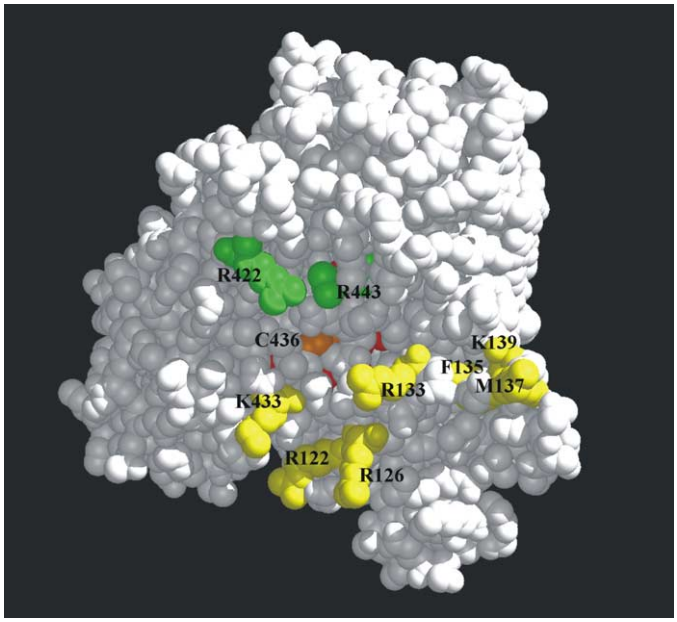

Fig. 1. The binding site for cyt b_5 and cyt P450 reductase on the proximal surface of CYP 2B4. The largely buried heme is in red; residues R422 and 443 involved in binding only the reductase are illustrated in green; residues which participate in binding both cyt b_5 and reductase in and near the C-helix (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.

In view of the fact that mutation of the seven amino acids in the C-helix increased the dissociation constant, K_d, of both cyt b_5 and cyt P450 reductase without altering the V_{max}, a probe for the enzymatic mechanism, we concluded that their binding sites on cyt P450 significantly overlapped and that these two enzymes could not bind simultaneously to CYP 2B4. Rather their interaction with CYP 2B4 would be mutually exclusive. Whether cyt b_5 or reductase is bound to CYP 2B4 will depend on the relative affinity of each redox partner for cyt P450 under a particular set of experimental conditions and the relative active concentration of the redox partner in the reaction mixture. For example, the poorer binding redox partner would have to be present at a higher concentration to successfully compete with a tighter binding partner present at a lower concentration. These mutagenesis studies have caused us to reexamine and ultimately reinterpret our original stoichiometry experiments and those of other investigators which demonstrated that cyt b_5 could inhibit NADPH consumption in a reconstituted system when added to cyt P450 before the reductase. Interestingly, the inhibition could be reversed following an ≅24-h incubation at 4 °C at which temperature hydrophobic forces are minimized [2,3]. The reversible inhibition of CYP 2B4 activity by addition of cyt b_5 prior to reductase can now be understood by hypothesizing that cyt b_5 ($K_d = 0.2$ μM) rapidly binds to cyt P450 initially interfering with the reduction of ferric cyt P450 by cyt P450 reductase. As a consequence NADPH consumption and overall activity are diminished. Subsequent incubation of the reaction mixture at 4 °C for 24 h allows the tighter but slower binding reductase ($K_d = 0.02$ μM) to displace cyt b_5 and ultimately restore activity and NADPH consumption [8]. These results are consistent with the proposal that binding of cyt b_5 and reductase is mutually exclusive and therefore, competitive, and that reductase binds CYP 2B4 tighter than cyt b_5, in agreement with a measured K_d of cyt b_5 of ≅0.2 μM and K_d apparent of reductase of ≅0.02 μM [5]. Unpublished data demonstrate that cyt b_5 binds more quickly than cyt P450 reductase.

Non-identical but overlapping binding sites are frequently encountered in proteins that exchange electrons with multiple partners.

Our conclusions are not in agreement with those reached by Schenkman and colleagues. Based on experiments with a EDC carbodiimide cross-linked cyt b_5–cyt P450 complex these investigators proposed that cyt b_5 and reductase had separate, non-overlapping binding sites on CYP 2B4 [9]. The complex was crosslinked by adding the EDC carbodiimide to a mixture of ferric proteins whereas our conclusions were drawn from experiments conducted under steady-state conditions. Since residues participating in the crosslinking of the cyt b_5–cyt P450 complex were not identified, it has not been possible to compare the identity of the binding sites identified in the mutagenesis and crosslinking experiments. Although the contradictory results cannot presently be understood, steady-state experiments demonstrating that cyt b_5 stimulates cyt P450 optimally at low reductase to CYP 2B4 molar ratios are consistent with cyt b_5 and reductase having overlapping mutually exclusive binding sites [10].

Due to the sensitivity of the activity of cyt P450 and its redox partners to a myriad of experimental conditions including but not limited to: temperature, ionic strength, identity of the buffer, pH, aggregation state of any of the three proteins, order of addition of reactants to the reaction mixture, substrate under study, the purity of the protein preparation, identity of the lipid, lipid-to-protein ratio, presence of detergent, and impurities in the reaction components (such as the NADPH generating system), it remains a challenge to unambiguously understand the role of cyt b_5 in catalysis. Another difficult-to-evaluate complexity is that under steady-state conditions only cyt P450 reductase can provide the first electron. If, as is proposed, CYP 2B4 can only accommodate a single redox partner, it would indicate that their binding would be competitive and the reductase would have to dissociate from the cyt P450 to allow cyt b_5 binding and delivery of the second electron. Nevertheless, single turnover experiments, to be described in a later section, with CYP 2B4 in the presence of either cyt b_5 or reductase will address some of the outstanding issues.

Comparison of the kinetics of the reduction of oxyferrous cyt P450 by cyt b_5 and cyt P450 reductase

The observation that cyt b_5 increases the efficiency of catalysis in a reconstituted system under steady-state conditions by decreasing superoxide formation and the possibility that reduction of oxyferrous cyt P450 by cyt b_5, a reaction also known as delivery of the second electron, faster than cyt P450 reductase prompted us to measure the rate of delivery of the second electron to CYP 2B4 by cyt P450 reductase. Until recently it had not been possible to measure the rate of delivery of the second electron to cyt P450 by cyt P450 reductase for two major reasons; (a) the two flavins in the reductase give rise to nine different forms of the reductase since each flavin can exist in three different oxidation states and (b) the electrons rapidly equilibrate among the different forms of the flavins according to their redox potentials [11]. The combination of these factors rendered the spectral changes in the reductase upon oxidation of the two-electron reduced reductase with cyt P450 too complex to unambiguously deconvolute and interpret. For example, because of the similarity in potential of the FMN hydroquinone (−270 mV) and FAD semiquinone (−290 mV) virtually no change in the absorbance at 585 nm (where FMN semiquinone formation is followed) can be observed when the two-electron reduced native reductase oxidizes to the one-electron reduced FMN semiquinone. However, in a reductase with a single redox active flavin, FMN, oxidation of the FMN hydroquinone to the air-stable semiquinone can readily be observed spectrally. In order to simplify the spectral changes and be able to follow the oxidation of the 2-electron reduced cyt P450 reductase, the FAD cofactor which receives the electron from NADPH was replaced with 5-deazaFAD [12]. Briefly, the T491V mutant of rat cyt P450 reductase, which binds FAD ≅ 100-fold less tightly than the wild type, but otherwise functions normally was used. The decreased affinity of FAD for the T491V mutant reductase made it possible to exchange FAD for 5-deazaFAD which had been synthesized from 5-deazariboflavin. The electron transfer properties of the 5-deazaFAD T491V reductase were characterized. Control experiments demonstrated that the FMN hydroquinone of the 5-deazaFAD containing reductase reduced cytochrome b_5 and cytochrome c as expected for the wild type enzyme [12]. It was necessary to incorporate a FAD analogue into the reductase because without it the reductase was unstable and quickly lost activity. Under the experimental conditions used, 5-deazaFAD remains oxidized throughout the experiments. With the reductase containing the oxidized 5-deazaFAD in place of FAD one can unambiguously measure spectrophotometrically the rate of oxidation of the FMN hydroquinone to the air-stable semiquinone. As expected this is a simple monophasic reaction (Table 2).

Measurement of the rate of second electron transfer from cyt P450 reductase to oxyferrous cyt P450 in the presence of the substrate, benzphetamine, requires premixing of equimolar amounts of the two proteins which are subsequently anaerobically reduced with dithionite. The ferrous cyt P450-2-electron reduced reductase complex is subsequently rapidly mixed with oxygen saturated buffer. Oxyferrous cyt P450 is formed almost completely within the deadtime of the stopped-flow spectrophotometer and is immediately reduced by the cyt P450 reductase in the interprotein complex [13]. Fig. 2 and Table 2 illustrate that cyt P450 reductase oxidizes rapidly with a rate constant of 8.4 s^{-1} whereas oxyferrous cyt P450 oxidizes and turns over significantly more slowly with a rate constant of 0.09 s^{-1}. Such kinetics are typical of a reaction in which an intermediate forms in the slower reacting protein, in this instance, CYP 2B4. These results were totally unexpected

Table 2
Summary of the rate constants and amplitudes for the auto-oxidation and redox reactions of cyt P450, cyt b_5, and 5-deazaFAD reductase in the absence and presence of their redox partners

Syringe 1	Syringe 2	λ (nm)	Species	Phase 1		Phase 2		Phase 3	
		Obs[a]	Obs[a]	A (%)	k_1 (s^{-1})	A (%)	k_2 (s^{-1})	A (%)	k_3 (s^{-1})
2e-reduced 5-deazaFAD[c] reductase	O_2	450	Reductase					100 ± 11	0.007 ± 0.001
		585	Reductase					100 ± 8	0.007 ± 0.001
Cyt b_5^{2+}	O_2	422	Cyt b_5					97 ± 5	0.005 ± 0.0003
$P450^{2+}$	O_2	438	P450	25 ± 3	0.96 ± 0.2	34 ± 6	0.13 ± 0.04	41 ± 7	0.016 ± 0.005
$P450^{2+}$ + 1 mM BP[b]	O_2 + 1 mM BP	438	P450			40 ± 4	0.13 ± 0.05	60 ± 7	0.048 ± 0.004
2e-reduced 5-deazaFAD[c] reductase	Cyt b_5^{2+}	422	Cyt b_5					98 ± 10	0.002 ± 0.0002
		567	Reductase					100 ± 12	0.002 ± 0.0004
$P450^{2+}$-cyt b_5^{2+} + 1 mM BP	O_2 + 1 mM BP	422	Cyt b_5	50 ± 6	9.3 ± 0.7	4 ± 0.1	0.43 ± 0.21	46 ± 7	0.005 ± 0.0003
		438	P450	62 ± 7	10.5 ± 1.5	18 ± 1.1	0.83 ± 0.18	20 ± 3	0.005 ± 0.001
$P450^{2+}$-2e-reduced 5-deazaFAD reductase + 1 mM BP	O_2 + 1 mM BP	598	Reductase	31 ± 6	8.4 ± 1.5	52 ± 6	0.37 ± 0.06	17 ± 0.7	0.041 ± 0.005
		438	P450			86 ± 10	0.090 ± 0.01	14 ± 0.5	0.012 ± 0.002

[a] Observed.
[b] Benzphetamine.
[c] These data from [13].

especially when compared to a control experiment with cyt b_5 which revealed that under identical experimental conditions cyt b_5 and cyt P450 oxidized simultaneously (see Fig. 3 and Table 2). Table 2 from a previous publication provides relevant control and experimental rate constants [13]. The data clearly indicate that the reductase and cyt b_5 both donated an electron to cyt P450 with a similar rate constant, and that CYP 2B4 reacts differently, depending on whether cyt b_5 or reductase is delivering the second electron. Painstaking analysis of the spectra for a cyt P450 intermediate was unrewarding. This suggests that the reduced oxyferrous intermediate possesses a spectrum similar to that of oxyferrous cyt P450 and that proton delivery rather than donation of the second electron may be a rate influencing step.

Under the single turnover conditions of the experiment benzphetamine metabolism was 50% efficient in the presence of cyt b_5 whereas it was only 30% efficient with reductase. In this context, efficiency refers to the fact that only one-half and one-third of the reducing equivalents in cyt b_5 and reductase, respectively, resulted in product formation. The rate constant for decay of oxyferrous cyt P450 ($k = 0.09\ s^{-1}$) was the same as the rate constant for catalysis under single turnover conditions ($0.08\ s^{-1}$) at 15 °C. It is not known what causes the uncoupling of cyt P450 catalysis under the single turnover conditions. The data with cyt b_5 suggest that only 50% of the cyt P450 and cyt b_5 forms a catalytically competent complex while the remainder of the proteins merely undergo autoxidation (Table 2). The identity of the side products with the reductase is more uncertain due to the more complex kinetics of the reaction. A more complete understanding of the mechanism of uncoupling awaits the identification and quantitation of the side products (superoxide versus hydrogen peroxide) under single turnover conditions. If superoxide is formed, it would indicate that autoxidation is occurring while hydrogen peroxide formation would suggest that oxyferrous cyt P450 has been reduced but catalysis has been aborted, possibly the case with reductase. The findings under single turnover conditions support the conclusions of steady-state experiments where cyt b_5 has been shown in several laboratories to increase the efficiency of catalysis [3,14,15]. As stated previously in the case of metabolism of methoxyflurane and benzphetamine, cyt b_5 increases efficiency by decreasing superoxide production. The data also suggest that when Hildebrandt and Estabrook [16] added NADH to hepatic microsomes to reduce cyt b_5 via NADH cyt b_5 reductase they were observing the turnover of oxyferrous microsomal cyt P450 rather than simply the more rapid delivery of the second electron by cyt b_5.

On the basis of these studies we hypothesize that oxyferrous cyt P450 assumes different conformations in the presence of cyt b_5 and reductase. The single turnover experiments indicate that even though oxyferrous cyt P450 receives the electron at the same rate from both of its redox partners subsequent steps in the catalytic cycle

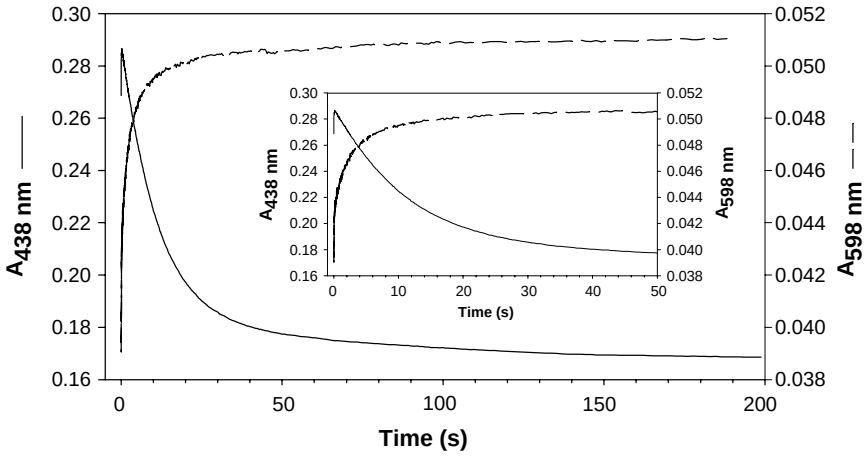

Fig. 2. Kinetics of reduction of oxyferrous CYP 2B4 by 5-deazaFAD reductase. The final concentration of cyt P450 and 5-deazaFAD reductase in the observation cell of the stopped-flow spectrophotometer was 4.7 μM. The solid and dashed lines represent absorbance changes observed at 438 nm (cyt P450) and 598 nm (reductase), respectively. See [13] for experimental details. Reprinted with permission from [13]. Copyright 2003 American Chemical Society.

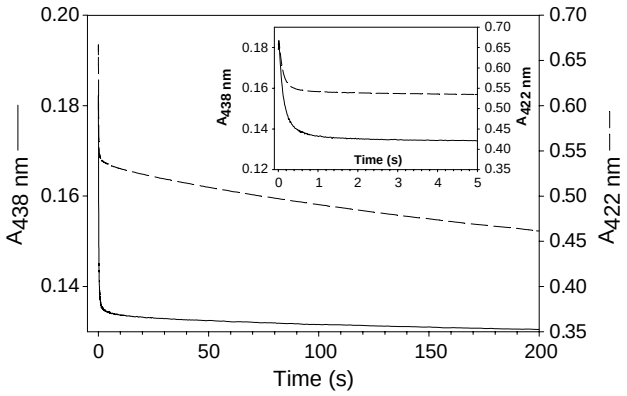

Fig. 3. Kinetics of the reduction of oxyferrous CYP 2B4 by cyt b_5. The final concentration of cyt P450 and cyt b_5 in the observation cuvette was 5 μM. The solid and dashed lines represent absorbance changes observed at 438 nm (P450) and 422 nm (b_5), respectively. See [13] for experimental details. Reprinted with permission from [13]. Copyright 2003 American Chemical Society.

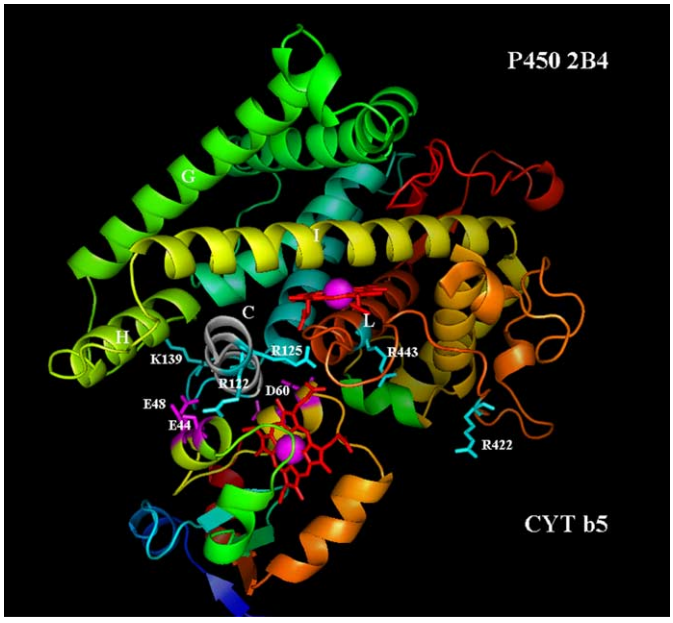

Fig. 4. Model of the putative cyt b_5–CYP 2B4 complex. The complex was modeled using the coordinates of cyt b_5 (pdb 1CYO) and cyt P450 2B4 (pdb 1SUO). C, G, H, I, and L refer to helices in CYP 2B4. The picture was generated using PyMol (www.pymol.org).

do not occur simultaneously. In the presence of reductase, catalysis proceeds more slowly than in the presence of cyt b_5. The data are consistent with the possibility that proton delivery is slower in the presence of reductase presumably because of suboptimal organization and function of the proton delivery pathway. The crystal structures of the low activity mutant oxyferrous cyt P450 ery F and cyt P450cam which exhibit a disturbance in the architecture of the active site proton shuttle machinery illustrate the importance of an organized proton delivery mechanism in cyt P450 catalysis [17–19]. A key question is: how might cyt b_5 and reductase differentially modify the structure of the distal heme pocket? Figs. 4 and 5 illustrate models of complexes of CYP 2B4 with cyt b_5 and the FMN domain of cyt P450 reductase, respectively. Site-directed mutagenesis data that implicated residues in the C-helix in interacting with cyt b_5 and unpublished mutagenesis and double mutant cycle studies, indicating that Asp64 and Val65 on cyt b_5 were in contact with cyt P450 residues Arg122, Arg126, and Lys433, were used to construct the cyt P450–cyt b_5 complex [5]. The complex with reductase was generated by superimposing the heme of the closed conformation of CYP 2B4 and the FMN domain of cyt P450 reductase on the crystal structure of the complex between cyt P450 BM_3 and the FMN domain of the BM_3 reductase [20]. CYP 2B4 residues identified in mutagenesis studies as participating in binding only the reductase (R422 and 443) are in close proximity to the FMN of the reductase in the

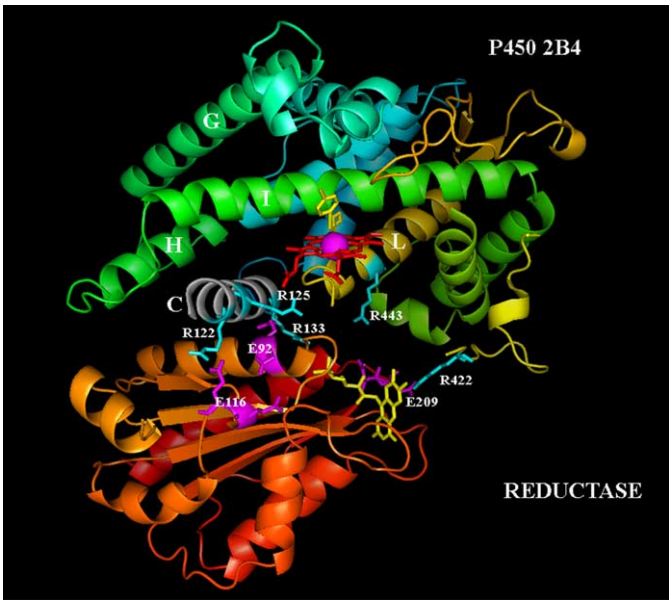

Fig. 5. Model of the putative CYP 2B4-FMN domain of rat cyt P450 reductase complex. The complex was modeled by superimposing the heme of CYP 2B4 and the FMN of rat reductase on the cyt P450 BM_3-reductase crystal structure (pdb 1BVY) using the coordinates of CYP 2B4 (pdb 1SUO) and cyt P450 reductase (pdb 1AMO). C, G, H, I, and L refer to helices in CYP 2B4. The picture was generated using PyMol (www.pymol.org).

model complex. These residues are not close to cyt b_5 in the cyt b_5–CYP 2B4 complex. It should also be noted that as predicted by the mutagenesis data the reductase spans the depression on the proximal surface of cyt P450 whereas the cyt b_5 interacting surface is restricted to one side of the depression (Fig. 1).

Both proteins bind to the highly dynamic C-helix, which undergoes very large conformational changes which are illustrated in the crystal structure of the "open" and "closed" conformations of cyt P450 2B4 [6,21]. The C-helix is in van der Waals contact with the heme and I helix. The I helix forms part of the substrate binding site and its residues play a major role in organizing the proton delivery machinery. It is postulated that cyt P450 undergoes a conformational change on binding cyt b_5 to facilitate proton delivery to the reduced oxyferrous intermediate and subsequent formation of the catalytically active oxyferryl intermediate. Cyt P450 reductase also binds to the proximal surface of cyt P450 but its "footprint" is larger. Nevertheless, it binds close to the heme in a position capable of influencing the structure and reactivity of the proton shuttle in the distal heme pocket as well as the thiolate heme interaction (Figs. 4 and 5). NMR studies and rapid mixing experiments with cyt P450cam and putidaredoxin demonstrate that the interaction of putidaredoxin with oxyferrous and carbon monoxy cyt P450cam produces spectral and structural changes in the cyt P450. It is therefore likely that the microsomal cyts P450 undergo conformational changes on binding their redox partners prior to electron transfer [22,23].

In order to understand the molecular basis of this intriguing observation of the different behavior of oxyferrous cyt P450 in the presence of its two reductants, we sought to measure whether product was formed at different rates under conditions analogous to those in which the spectral observations were recorded. Preliminary experiments which have been performed using a rapid chemical quench apparatus and experimental protocol similar to that described in the stopped-flow spectrophotometric experiments [13] demonstrate that cyclohexane is metabolized to cyclohexanol with a rate constant of 23 and 3 s^{-1} in the presence of cyt b_5 and cyt P450 reductase, respectively, under single turnover conditions. Of note, the reaction is 10% efficient with cyt b_5 but only 3% efficient with cyt P450 reductase at 30 °C. Although the metabolism of benzphetamine which was monitored in the previously described stopped-flow spectrophotometric studies was 50% (cyt b_5) and 30% (reductase) efficient, the trend with cyclohexane as a substrate is identical, more efficient coupling with cyt b_5. Presumably hydrogen peroxide is the side product of the reductase-generated reduced oxyferrous complex which does not give rise to product. Nevertheless, this remains to be experimentally confirmed. Analysis of the reaction kinetics of oxyferrous cyt P450 with cyt b_5 suggests that there are two approximately equal populations of cyt P450, one that accepts an electron from cyt b_5 and undergoes catalysis, and a second that does not. The spectral changes for the cyt P450 which does not undergo catalysis do not provide a clear indication of its fate. The spectral changes with cyt b_5 are more definitive. They suggest that about half of the cyt b_5 donates an electron to cyt P450 while the remainder undergoes autoxidation. A better understanding of these phenomena is under investigation.

In summary, site-directed mutagenesis experiments, modeling studies, the reversibility of cyt b_5 inhibition of catalysis and the enhanced stimulatory effects of cyt b_5 at low molar ratios of reductase are consistent with the proposal that cyt b_5 and cyt P450 reductase bind on overlapping but non-identical sites on the proximal surface of cyt P450 near where the buried heme comes closest to the surface [2,3,5,10]. Both cyt b_5 and reductase interact with residues in the flexible C-helix which constitutes about one-third of the rim of the depression on the proximal side of cyt P450. In addition, the reductase binds to amino acids on the rim of the concavity opposite the C-helix, thereby spanning the depression which has the axial cysteine and heme at its base (Fig. 1) [5]. Currently, it is not possible to reconcile experiments conducted on a cross-linked CYP 2B4–cyt b_5 complex which imply totally separate binding sites for cyt b_5 and reductase on CYP 2B4 with site-directed mutagenesis, modeling, and other experiments which are consistent with non-identical but overlapping and therefore mutually exclusive binding sites [2,3,5,9,20].

The rate of electron transfer from cyt P450 reductase to oxyferrous CYP 2B4 in the presence of the substrate, benzphetamine, has recently been measured [13]. Surprisingly, these experiments revealed that cyt b_5 and the reductase

both transfer the electron to oxyferrous CYP 2B4 at the same rate, but that oxyferrous cyt P450 turns over more slowly in the presence of reductase. The reaction is 50% efficient in the presence of cyt b_5 and 30% efficient in the presence of reductase. Our current hypothesis is that cyt P450 catalysis proceeds via a long-lived reduced oxyferrous intermediate in the presence of cyt P450 reductase. In contrast in the presence of cyt b_5 catalysis proceeds rapidly and comparatively efficiently. This proposal is supported by preliminary rapid chemical quench experiments which indicate that metabolites are formed more rapidly in the presence of cyt b_5 than cyt P450 reductase. A straightforward interpretation of these experiments is that the catalytic machinery in the active site of cyt P450 functions differently in the presence of cyt b_5 and reductase presumably due to changes in its structure induced by its redox partners.

The identity of the hypothetical cyt P450 intermediate formed in the presence of reductase and the side products of this inefficient reaction remain to be established. When this information becomes available, it will provide a better understanding of the mechanism of "Mother Nature's Blowtorch."

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