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Formation of P450•P450 Complexes and Their Effect on P450 Function

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Abstract

Cytochromes P450 (P450) are membrane-bound enzymes that catalyze the monooxygenation of a diverse array of xenobiotic and endogenous compounds. The P450s responsible for foreign compound metabolism generally are localized in the endoplasmic reticulum of the liver, lung and small intestine. P450 enzymes do not act alone but require an interaction with other electron transfer proteins such as NADPH-cytochrome P450 reductase (CPR) and cytochrome b₅. Because P450s are localized in the endoplasmic reticulum with these and other ER-resident proteins, there is a potential for protein-protein interactions to influence P450 function. There has been increasing evidence that P450 enzymes form complexes in the ER, with compelling support that formation of P450•P450 complexes can significantly influence their function. Our goal is to review the research supporting the formation of P450•P450 complexes, their specificity, and how drug metabolism may be affected. This review describes the potential mechanisms by which P450s may interact, and provides evidence to support each of the possible mechanisms. Additionally, evidence for the formation of both heteromeric and homomeric P450 complexes are reviewed. Finally, direct physical evidence for P450 complex formation in solution and in membranes is summarized, and questions directing the future research of functional P450 interactions are discussed with respect to their potential impact on drug metabolism.

Keywords

Cytochrome P450; Protein-protein interactions; P450 function; P450•P450 complex formation

1. Introduction

Cytochrome P450 represents a superfamily of enzymes that constitute the major enzyme system responsible for the phase 1 metabolism of drugs and foreign compounds. P450 is a membrane-bound protein in eukaryotic cells. Although some P450 enzymes involved in the metabolism of endogenous substrates are found in the mitochondrial membrane, most of the P450s, particularly those involved in foreign compound metabolism, are located on the cytoplasmic side of the endoplasmic reticulum (Estabrook, Franklin, Cohen, Shigamatzu, &

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Hildebrandt, 1971). As the terminal oxidase of the microsomal electron transport chain, P450 does not generally function alone, but requires interactions with other proteins.

The simplest system that supports NADPH-dependent oxygenation reactions includes three major components: P450, NADPH-cytochrome P450 reductase (CPR) and phospholipid (Lu & Coon, 1968; Lu, Junk, & Coon, 1969; Strobel, Lu, Heidema, & Coon, 1970). In this system, interaction between CPR and P450 is required to allow the reducing equivalents from NADPH to pass from CPR to P450 in two single electron transfer steps (White & Coon, 1980; Loida & Sligar, 1993). However, as membrane-bound proteins, their motion is restricted to the confines of a lipid bilayer, usually that of the endoplasmic reticulum, which localizes and orients CPR and P450 in a manner conducive to a functional interaction.

Other proteins are also known to contribute to monooxygenase function of P450, the most notable being cytochrome b₅. In many cases, the interaction of P450s with their redox partners supplies the electrons necessary to catalyze P450-dependent oxygenations, but in some instances, the interaction plays a conformational role, affecting function by altering a step in the reaction cycle that does not necessarily involve the electron transfer steps (Yamazaki, Johnson, Ueng, Shimada, & Guengerich, 1996; Schenkman & Jansson, 2003). This has been documented with the heme enzyme, cytochrome b₅, which can serve as an allosteric effector without supplying electrons for P450-dependent substrate metabolism (Yamazaki et al., 1996; Porter, 2002).

An interesting feature of the P450 system proteins is that the components are not present in equimolar concentrations. In addition to supplying electrons for P450-mediated metabolism, CPR also functions as a redox partner in reactions catalyzed by heme oxygenase, fatty acid desaturase and, in some cases, cytochrome b₅. Despite its contribution to so many different reactions, CPR concentrations are generally limiting. Early estimates from untreated rat liver microsomes reported about a 20-fold excess of P450 over that of CPR (Estabrook et al., 1971) using cytochrome c reductase activity to quantify CPR levels. When CPR was measured immunochemically, the P450:CPR ratio was about 5:1 (Reed, Cawley, & Backes, 2011). This difference in the relative concentrations of proteins that are known to form a 1:1 molar complex raised important questions regarding how P450s and their associated ER proteins were organized. One interesting early model was proposed by Peterson (Peterson, Ebel, O'Keeffe, Matsubara, & Estabrook, 1976), where multiple P450 molecules were surrounded by a central CPR, which was capable of randomly transferring electrons within the cluster. Those P450s outside the cluster received electrons at a much slower rate.

The relative ratios of P450 and CPR are subject to alterations caused by inducing agents: P450:CPR ratios of about 30:1 have been reported after phenobarbital treatment (Estabrook et al., 1971; Cawley, Zhang, Kelley, & Backes, 2001). Cytochrome b₅ levels are generally in the same range as those of P450s in untreated rats (Estabrook et al., 1971; Gibson & Clarke, 1986), which is a significant excess over CPR. Heme oxygenase-1 (HO-1) levels also are quite variable depending on induction status. In untreated rats, CPR levels are actually in excess of HO-1, with a HO-1:CPR ratio of about 0.2:1; however, treatment with cadmium leads to induction of HO-1 and increases the HO-1:CPR ratio to 3:1 (Reed et al., 2011). With the molar levels of CPR always being limiting when compared to the sum of all proteins that require CPR as their electron donor, changes in the ratio of CPR to individual electron acceptors would be expected to have a profound influence on drug disposition, heme metabolism, and other CPR-mediated functions such as fatty acid metabolism. In fact, various maladies including disordered steroidogenesis, ambiguous genitalia, and Antley-Bixler syndrome have been identified as phenotypes resulting from mutations in the CPR gene that are caused by the impaired abilities of the mutant CPRs to interact with and/or deliver electrons to P450 (Flück et al., 2004; Huang et al., 2005; Xia et al., 2011).

The proteins in the endoplasmic reticulum are concentrated at a relatively high protein density (lipid:protein ratio 23:1) (Depierre & Dallner, 1975; Miller, Herbette, & White, 1996)), which results in many opportunities for protein-protein interactions. This condition raises several questions related to the function of the P450 systems as well for the behavior of other ER-resident proteins: (1) Do P450 proteins interact with each other in the ER membrane? (2) Are these interactions specific or are they simply the result of protein crowding within the membrane? (3) Do the interactions among these proteins actually affect their catalytic function? (4) Can alterations in protein-protein interactions serve as a mechanism whereby P450 function is modulated? This review will summarize studies that were designed to address these questions, focusing on the potential for different P450 proteins to form physical complexes within the endoplasmic reticulum, and their potential to influence P450 function.

2. Mechanisms of P450-P450 interactions affecting metabolism

Early studies with P450s reported that the rates of substrate metabolism by purified enzymes were not consistent with the rates catalyzed by liver microsomes based on the levels of individual P450 expression (West & Lu, 1972; Kaminsky, Guengerich, Dannan, & Aust, 1983; Wood, Ryan, Thomas, & Levin, 1983; Sonderfan, Arlotto, Dutton, McMillen, & Parkinson, 1987). One possible explanation for these findings was that complex formation among different forms of P450 was influencing metabolism by the individual enzymes. In this review, we will refer to specific, physical interactions between P450s *that affect metabolism* as “functional interactions”, and these will be distinguished from interactions that do not result in a change in overall function. Because we are primarily concerned with the manner by which P450-P450 interactions influence the metabolism, disposition, and elimination of compounds, this review begins with a summary of the studies that have examined the effects of these interactions on P450 activities.

2.1. Model 1: Simple competition between two P450s for CPR

There are several mechanisms by which one P450 enzyme can affect the behavior of another form. The simplest mechanism would be one where two P450 enzymes do not actually form a specific P450•P450 complex, but compete for the limiting levels of reductase (Model 1). According to this model, we are assuming that the substrate concentration is saturating for both P450 enzymes, which allows us to simplify the model to focus on the protein-protein interactions. Although the substrate concentration is saturated for both P450s, each P450 would have its own characteristic rate of formation of a particular product. The total rate of substrate metabolism from this mixed reconstituted system would simply be the sum of the rates from the two CPR-P450 complexes, CPR•P450_a and CPR•P450_b. At limiting concentrations of CPR, both P450s would compete for CPR, with catalysis by a particular P450 being governed by (1) its relative affinity for CPR, and (2) the ability of the CPR•P450 complex to oxidize the substrate present. A P450 that binds tightly to CPR and effectively converts substrate to product would be a major contributor to the overall reaction rate.

For example, two P450s compete for CPR as described in Model 1. If the K_{Da} is significantly lower than the K_{Db} , a mixed reconstituted system at subsaturating CPR would have more CPR•P450_a complexes, and consequently more P450_a character. Now, if the substrate is selective for P450_a, then the substrate will be effectively metabolized in the mixed reconstituted system; however, if that substrate is not effectively metabolized by P450_a, then substrate turnover would be lower. That is, a P450 with a smaller K_D for CPR would be able to more effectively compete for the available CPR, and consequently provide a greater contribution to total product formation. The K_D between CPR and a particular P450 is a characteristic of that P450, and could be affected by the presence of a substrate or effector molecule (French, Guengerich, & Coon, 1980; Backes & Eyer, 1989). The K_D for

CPR would not be expected to be affected by the presence of a second P450 if there is no physical P450•P450 interaction.

2.2. Model 2: Formation of a P450•P450 complex that affects CPR binding

In the second potential mechanism, two P450 enzymes can form a physical complex. This complex can lead to a conformational change that alters the relative abilities of the P450s to bind to CPR. As shown in Model 2, there are several species capable of forming product. The binary CPR•P450 complexes are shown in blue and are the same as those described in Model 1. There are also at least two ternary complexes with P450_a and P450_b forming a heteromeric complex, and CPR binding to one or the other P450 moiety of the complex (shown in brown). Finally, there is the potential for the formation of a quaternary complex, with CPR binding to both P450 moieties of the heteromeric complex (shown in green). This would be expected to occur either in the event of a strong cooperative effect, or at saturating CPR levels. Although the P450 complexes are described as dimers in this model, we cannot exclude the potential for the P450s exist in the membrane as higher order complexes.

Any mixed reconstituted system consisting of two different P450 enzymes that are prone to interact will contain P450_a, P450_b, and P450_a•P450_b complexes, with each being capable of binding to CPR (Model 2). Formation of the P450_a•P450_b complex is governed by its dissociation constant K_{Dab} . If the K_{Dab} is small, there will be a greater tendency for the P450 enzymes to exist in a complex, which will decrease the amount of the monomeric P450s. For P450s not in the P450•P450 complex, CPR will associate with each P450 to an extent determined by the enzyme concentrations and the respective binding affinities. Although the K_D values for CPR binding to the P450 monomer will not be affected by the presence of the second P450, the relative amount of each CPR•P450 complex will be dependent on its relative affinity for CPR when at subsaturating levels, similar to that described for the simple competitive situation described in Model 1. However, the formation of a heteromeric P450_a•P450_b complex presents a more complex situation as shown in Model 2 where both moieties of the P450_a•P450_b complex are also capable of CPR binding. Formation of a P450_a•P450_b complex may change the affinity of either one or both moieties of the complex to bind to CPR. For example, this could be seen as tighter binding of CPR to the P450_a moiety of the P450_a•P450_b complex (lower K_{Dcab}) than for CPR binding to monomeric P450_a (K_{Dca}). This would lead to an accumulation of CPR•P450_a•P450_b at subsaturating CPR levels. As the overall rate of substrate turnover by a mixed reconstituted system is the sum of the rates from each of the contributing species, the increase in CPR•P450_a•P450_b will lead to an increase in P450_a character of the reconstituted system. If the substrate used is a P450_a selective substrate, then the overall reaction rate would be higher than that expected based on the single P450 enzyme. On the other hand, if the substrate used is P450_b selective, an inhibition would be observed in the mixed reconstituted system. Interestingly, if the only effect of P450•P450 complex formation is a change in the affinity of the P450s for CPR (and not on any of the rate constants for substrate turnover), then altered activities will only be observed at subsaturating CPR. This is because at saturating CPR, there would be sufficient CPR molecules to associate with all of the P450 molecules. Therefore, in a Model 2 interaction at saturating CPR, the overall rate would be expected to be additive.

2.3. Model 3: Formation of a P450•P450 complex that affects the efficiency of substrate turnover

It is possible for P450•P450 complex formation to alter the rate at which a substrate is metabolized, without significantly affecting the ability of the P450s to associate with CPR. In these cases, the rate constants for substrate turnover will be affected. As an example, let us begin with a mixed reconstituted system described in Model 3. Unlike the case described

for Model 2, P450_a•P450_b complex formation would not affect the relative affinity of CPR for either P450 enzyme; that is, there would be no preferential binding of CPR to either P450 moiety as a consequence of P450•P450 complexation. However, the rate of substrate turnover is affected. This could be seen as an increase in electron transfer or any other catalytic step. If the reaction rate for the complex is faster than that of the uncomplexed enzyme, stimulation in the metabolic rate would be observed. For example, if k_d is larger than k_b , substrate turnover from the mixed system would exceed the sum of the CPR-P450_a plus CPR-P450_b binary systems. In contrast, if the k_d is smaller than k_b , an inhibitory response would be observed. This response, whether synergistic or inhibitory, will be observed both at subsaturating and saturating CPR, in contrast to the response described in Model 2 for alterations in CPR binding affinity.

3. Evidence for alterations in P450 function as a result of P450•P450 interactions

In order to elucidate the functional consequences of P450-P450 interactions, many studies have been conducted in which substrate metabolism by individual P450s and mixtures of P450s were compared. As will be described, studies examining the influence of one P450 on metabolism by another P450 have produced effects consistent with each of the interaction models described above.

3.1. Evidence supporting Model 1 (Simple competition of multiple P450s for limiting CPR)

The earliest study of P450-P450 interactions on metabolism examined the hydroxylation of 3,4-benzpyrene catalyzed by CYP2B1 and CYP1A2 (West & Lu, 1972). Kinetic analysis of the rates of metabolism by reconstituted systems varying the relative amounts of the purified enzymes was shown to be consistent with simple competition for CPR.

The interaction of CYP2A6 and CYP2E1 was also reported in a study that used microsomes from *Baculovirus* expression systems engineered to express CPR and one or both of the P450s (Tan, Patten, Smith, & Yang, 1997). Thus, this study examined P450-P450 interactions in natural membranes, and the results were not influenced by potential artifacts resulting from incorporation of proteins into lipid-reconstituted systems. The study also used substrates that were specific for each enzyme and did not inhibit the other enzyme, so effects on metabolism by the P450s could be compared with and without substrate turnover by the alternate enzyme. The results indicated the mutual inhibition of metabolism by both CYP2A6 and CYP2E1 when both enzymes were present. The inhibition could be alleviated by increasing the relative levels of CPR. Significantly more inhibition was observed when the alternate enzyme was also involved in substrate metabolism indicating either that P450s bind CPR with greater affinity during metabolism or that the supply of electrons from CPR was limited when both enzymes were active. Given these data, the study concluded that the inhibition observed in the ternary system containing both P450s and CPR could be attributed to a simple competition of the P450s for a limiting supply of CPR.

3.2. Evidence supporting Model 2 (P450-P450 complexes that alter the affinity of P450 for CPR)

A much more intriguing effect of P450-P450 interactions involves instances in which metabolism by one or both P450s is influenced by the presence of the other enzyme. There are now many reports of various combinations of P450s that result in inhibition and/or activation of P450-mediated activity when another P450 is present in a reconstituted system. The first evidence for a specific interaction between two P450 proteins was the demonstration of 80% inhibition of the CYP2B4-selective substrate, 7-pentoxoresorufin, when examined in mixed reconstituted systems containing CYP1A2, CYP2B4 and

subsaturing CPR (Cawley, Batie, & Backes, 1995). Interestingly, the degree of inhibition was shown to be dependent on the relative CPR ratio because at a 3:1 CPR:P450 ratio, no inhibition of 7-pentoxoresorufin dealkylation (PROD) was observed. Although these results showed that one P450 could affect the function of a second P450, the specific mechanism was not identified.

These studies were followed by a more complete kinetic analysis of the interaction between CYP1A2 and CYP2B4. In these experiments, lipid reconstituted systems were prepared that contained (1) CYP1A2, (2) CYP2B4, and (3) both P450s. Turnover of CYP2B4- and CYP1A2-selective substrates were then examined as a function of CPR concentration (Backes, Batie, & Cawley, 1998). Whereas CYP2B4-selective PROD was inhibited by the presence of CYP1A2, CYP1A2-selective 7-ethoxoresorufin deethylation (EROD) was synergistically stimulated by the presence of CYP2B4. This effect was much more pronounced at subsaturating CPR and approached additivity at saturating reductase. Mathematical modeling of the kinetic data was consistent with the formation of a CYP1A2•CYP2B4 complex in which the CYP1A2 moiety of the complex had a dramatically increased affinity for CPR (Model 2 where the affinity of CPR binding is affected). The data could not be fit to Model 1 where CYP1A2 and CYP2B4 competed for the available reductase. These results supported the concept that P450s could interact in a manner that modified their function, and raised the possibility that P450s exist in the membrane as subunits of complexes with unique functional characteristics.

Similar results were reported by Davydov (Davydov, Petushkova, Bobrovnikova, Knyushko, & Dansette, 2001) in the absence of a membrane system. In their study, CYP1A2 and CYP2B4 were labeled with different fluorescent labels that reacted with the sulfhydryl residues of cysteine, and the interaction between the enzymes was monitored by FRET in a low-concentration detergent solution. Using this technique, the formations of CYP1A2•CYP2B4, CYP2B4•CYP2B4, and CPR•P450 interactions were detected. Interestingly, when both CYP1A2 and CYP2B4 were combined, CPR selectively associated with CYP1A2. These results are consistent with the kinetic observations showing an activation of metabolism by CYP1A2 and an inhibition of that by CYP2B4 in dilauroylphosphatidylcholine membranes (Backes et al., 1998).

Evidence for these functional interactions between CYP1A2 and CYP2B4 was not limited to reconstituted systems, but was also demonstrated using microsomal preparations from rabbit liver (Cawley et al., 2001). Rabbits were treated with phenobarbital, β -naphthoflavone and a combination of both inducers to enrich microsomes with CYP2B4, CYP1A2 and both P450 enzymes, respectively. Results were analogous to those observed using reconstituted systems, in which CYP2B4-selective activities (PB-treated rabbits) were inhibited by the presence of elevated CYP1A2 (PB plus β -naphthoflavone treatment). CYP1A2-selective activities were not affected by the presence of CYP2B4 in the microsomal preparations. These results show that the functional interactions not only are found in reconstituted systems, but also in the native milieu of the microsomal membrane.

In an effort to determine if the functional interactions between CYP2B4 and CYP1A2 were mediated by electrostatic interactions, the effect of ionic strength on the CYP1A2-mediated inhibition of CYP2B4-selective PROD and 7-ethoxy-4-trifluoromethylcoumarin (7EFC) activities was examined. The study presumed that the P450-P450 complexes would be disrupted by an alteration in the ionic strength of the solvent if electrostatic forces directed the interaction of the proteins. The results showed that CYP2B4-selective PROD activity was substantially inhibited by the presence of CYP1A2 to the reconstituted system, but that the degree of this inhibitory response was attenuated at high ionic strength (Kelley, Reed, & Backes, 2005). Although less dramatic, the inhibition of CYP2B4-selective 7EFC

metabolism was also attenuated with increasing ionic strength, results consistent with the CYP1A2•CYP2B4 complex being stabilized by electrostatic interactions.

Charge pairing also was shown to be an important contributor to the CYP1A2•CYP2E1 complex (Kelley, Cheng, & Backes, 2006). In these experiments, both PROD and EROD activities were synergistically stimulated in mixed reconstituted systems containing both CYP1A2 and CYP2E1 *at subsaturating concentrations of CPR* in low ionic strength solvents. However, this synergistic stimulation was eliminated when the ionic strength of the medium was increased. In more detailed studies in which activities were measured as a function of CPR concentration, synergistic stimulation of EROD was shown to be observed only at subsaturating CPR, and the activity approached additivity at high CPR levels. It is well-known that CPR electron transfer activities (as measured by cytochrome c reduction) are dramatically elevated at higher ionic strength (Phillips & Langdon, 1962). Interestingly, despite elevated electron transfer to cytochrome c, P450-dependent monooxygenase activities measured at high ionic strength were not similarly elevated, demonstrating that the stimulation at subsaturating reductase was due to the P450 components. Again, these data are consistent with the formation of a heteromeric P450•P450 complex that can be disrupted by high ionic strength, supporting the concept that electrostatic interactions are involved in complex formation.

Interestingly, reconstituted systems prepared with CYP2B4 and CYP2E1 did not appear to result in either stimulation or inhibition of EROD or PROD activity (Kelley et al., 2006). This response is consistent with a simple competition between CYP2B4 and CYP2E1 for the available reductase as described in Model 1. The additive response observed at subsaturating reductase was unaffected by alterations in the ionic strength of the solvent. It may be tempting to conclude that these proteins do not form a heteromeric complex; however, these data only show that the *function* of the proteins is not affected by the combination. It is still possible that the proteins form a complex that does not significantly alter their catalytic behavior for the substrates selected. Taken together, the data demonstrate that (1) the combined presence of multiple P450 enzymes can significantly affect their function, (2) not all combinations of P450s alter P450 function, and (3) that the kinetically detectable P450•P450 complexes appear to be stabilized by electrostatic interactions.

3.3. Evidence supporting Model 3 (P450-P450 complexes that alter the rate of catalysis)

Although there is substantial evidence showing that P450•P450 complexes influence the ability of at least one of the P450s in the complex to bind to CPR (as described in Model 2), there is also evidence to support the idea that P450•P450 complex formation actually can alter the rate constants for substrate turnover (as described by Model 3). In a comprehensive study examining the functional interactions of the five major forms of human P450 (CYP1A1, CYP1A2, CYP3A4, CYP2C9, CYP2D6, and CYP2E1), it was found that CYP3A4-mediated metabolism of testosterone was stimulated when the enzyme was co-reconstituted with either CYP1A1 or CYP1A2 but was not affected in reconstituted systems with CYP2C9, CYP2D6, or CYP2E1 (Yamazaki et al., 1997). Furthermore, it was shown that this CYP1A-related effect occurred when using rat, rabbit, and N-truncated forms of CYP1A2. Interestingly, these experiments were done with saturating CPR. Because there was sufficient CPR available to associate with all the P450s in the reconstituted system, the results are consistent with the P450•P450 complex altering the efficiency of substrate turnover as described in Model 3. The potential for CYP1A2 to also stimulate CYP3A4-dependent activities by increasing the affinity of CPR for CYP3A4 (Model 2) remains a possibility; however, verification of this effect would require analysis of the response over a range of CPR concentrations (Backes et al., 1998).

These types of findings are not just limited to the interactions with CYP1A2; several other labs have now reported that CYP2C19 (Hazai & Kupfer, 2005), CYP2D6 (Subramanian, Low, Locuson, & Tracy, 2009), and CYP3A4 (Subramanian, Zhang, & Tracy, 2010) all interact with CYP2C9 to form heteromeric complexes that have altered abilities to metabolize substrates. The effects of CYP2C9-CYP2C19 interaction (Hazai & Kupfer, 2005) are analogous to those reported in studies of the interaction of CYP1A2 with either CYP2B4 (Backes et al., 1998; Reed, Eyer, & Backes, 2010) or CYP2E1 (Kelley et al., 2006). The CYP2C9-CYP2C19 interaction caused the metabolism of the CYP2C19-specific substrates (methoxychlor and S-mephenytoin) to be inhibited, much like metabolism of CYP2B4 in the presence of CYP1A2 (Backes et al., 1998); however, because the data were not examined over a complete range of CPR concentrations, it is uncertain whether this inhibition represents a simple competition (Model 1) or whether a CYP2C9•CYP2C19 complex is formed (as in Model 2). In contrast, the synergistic stimulation of CYP2C9-dependent diclofenac hydroxylation by CYP2C19 was observed at *saturating CPR* concentrations, which is consistent with the CYP2C9•CYP2C19 complex increasing the rate of the CYP2C9-specific reaction (as described by Model 3). In these experiments, the addition of CYP2C19 to a reconstituted system with a CPR:CYP2C9 ratio of 3:1 elevated the rate of diclofenac hydroxylation. Interestingly, where an equimolar CYP2C19:CYP2C9 caused a 55% stimulation of diclofenac hydroxylation, the addition of CYP2C19 beyond the equimolar ratio exacerbated the effect resulting in a 3-fold stimulation observed at a 5-fold excess of CYP2C19. These results again reinforce the concept that the consequences of P450•P450 interactions are dependent on the substrate used.

Recently, an interaction between CYP2D6 and CYP2C9 was established, where the addition of CYP2D6 was shown to inhibit CYP2C9-dependent S-flurbiprofen metabolism even at saturating CPR levels (Subramanian et al., 2009). Again, the data is consistent with a Model 3 type of interaction. Despite the ability of CYP2D6 to influence a CYP2C9-dependent activity, the converse was not observed, *i.e.*, the addition of CYP2C9 did not affect CYP2D6-dependent dextromethorphan metabolism.

In a similar study by this group, the presence of CYP3A4 caused a significant inhibition of CYP2C9-dependent metabolism of flurbiprofen and naproxen. In contrast, CYP2C9 did not influence CYP3A4-dependent metabolism (Subramanian et al., 2010). Supporting the assumption that this change was caused by an interaction between the P450s, the effects were dependent on the concentrations of both CYP3A4 and CPR, and inhibition was detected even at saturating CPR. Additionally, the K_D 's for the CPR•CYP2C9 and CPR•CYP3A4 are similar – 33 nM and 20 nM, respectively (Shimada, Mernaugh, & Guengerich, 2005; Locuson, Gannett, Ayscue, & Tracy, 2007; Subramanian et al., 2010). These results rule out the possibility that these proteins are simply competing with limiting concentrations of CPR. Taken together, these studies provide ample evidence that different P450s can form complexes that actually change the catalytic rate reflected by an alteration in the rate constant for substrate turnover. This is likely due to a conformational change in one or both of the interacting P450s that allow either an enhancement in the rate of electron transfer or to some other catalytic step. Although the interactions described by Model 3 do not require a change in affinity of either P450 for CPR, it is quite possible that many P450•P450 interactions involve both a change in CPR-P450 affinity and the rate constant for substrate turnover.

4. Formation of homomeric P450•P450 complexes affecting function

Demonstration that different P450 enzymes form heteromeric complexes leads to the possibility that P450s also have the potential to form homomeric complexes. Homomeric complex formation has been examined using a kinetic approach, measuring the rate of p-

nitrophenol metabolism by CYP2E1 as a function of CPR concentration at a series of CYP2E1 concentrations (Jamakhandi, Kuzmic, Sanders, & Miller, 2007). These kinetic data could not be fit to a simple model allowing only a binary CPR•CYP2E1 complex. Instead, the data were best fit to a model where homo- and hetero-oligomers of both CPR and CYP2E1 were present, in addition to the simple binary complexes. The mathematical model producing the best fit included catalytically inactive complexes of the CYP2E1 dimer, a trimer comprised of two CYP2E1 with one CPR, and a tetramer comprised of two CPR and two CYP2E1. The only catalytically active complex implicated by this model was the binary CPR-CYP2E1 complex.

Homomeric oligomerization of CYP2B4 was also demonstrated by a hyperbaric study of the ferrous-CO complex of the P450 in solution and in proteoliposomes (Davydov, Knyushko, & Hoa, 1992). When the CYP2B4-CO complex was subjected to more than 2 kbar of pressure, approximately 65% of the enzyme converted from the active complex absorbing at 450 nm to an inactive form absorbing at 420 nm. The other 35% of CYP2B4 was refractory to the increase in pressure even up to 6 kbar. When the CYP2B4 solution was treated with 0.2% Triton N-101 (conditions that were shown to dissociate enzyme hexamers to monomers (Davydov, Karyakin, Binas, Kurganov, & Archakov, 1985)), 95% of the CYP2B4 was susceptible to the pressure-induced conformational change associated with inactivation. Thus, the data were consistent with an aggregate of CYP2B4 in which some enzyme units were protected from pressure-induced conformation changes in the absence of detergent. Given the established evidence that CYP2B4 is stable as a hexamer in solution (Guengerich & Holladay, 1979; French & Coon, 1979; Tsuprun et al., 1986; Myasoedova & Tsuprun, 1993; Viner, Novikov, Ritov, Kagan, & Alterman, 1995) (discussed in detail below), the researchers proposed that four of the six enzyme units (67%) were arranged to be susceptible to the pressure-induced inactivation. When the enzyme was incorporated into proteoliposomes, approximately 74% of the enzyme could be inactivated by an increase in pressure. Thus, the enzyme still demonstrated the properties of an aggregate in a lipid milieu. However, from the larger proportion of inactivated enzyme, it was suggested that the CYP2B4 existed as a tetramer in the proteoliposomes with three of the four units being sensitive to pressure changes.

The aggregation of CYP3A4 was studied by measuring the extent and rate of first electron transfer under conditions that would variably affect the degree of self-association of the P450 (Davydov, Fernando, Baas, Sligar, & Halpert, 2005; Davydov et al., 2010). In the first study, CYP3A4 reduction by sodium dithionite was measured under conditions where CYP3A4 oligomer formation was favored (*i.e.*, oligomers in solution and P450-rich proteoliposomes), and under conditions favoring monomerization of the P450 (*e.g.*, lipid-rich proteoliposomes and Nanodisc-incorporated CYP3A4). When oligomers were favored, CYP3A4 reduction was multiphasic; however, conditions favoring monomerization simplified the reaction, leading to monomeric reduction kinetics (Davydov et al., 2005). A similar response was observed when electrons were passed to CYP3A4 through CPR. When measured under conditions favoring the formation of CYP3A4•CYP3A4 complexes, NADPH-dependent reduction was shown to be a multiphasic process; however, conditions conducive to monomer formation generated a monophasic reduction.

In a subsequent study of CYP3A4 (Davydov et al., 2010), the soluble flavin domain of cytochrome P450 BM3, known as BMR was used to reduce the CYP3A4. BM3 is a bacterial protein that has both CPR and P450 domains in a single polypeptide chain. The BMR-mediated reduction of CYP3A4 is dependent on a protein-protein interaction. Thus, unlike the findings using the chemical reductant, dithionite, which showed complete reduction, only partial reduction of the P450 was observed with BMR. This could best be explained by the aggregation or complex formation between CYP3A4 molecules in a manner that

prevented some of the P450s from interacting with BMR. In fact, under conditions that were most similar to the microsomal lipid:protein ratio, only 55% of the enzyme was reduced. The reducibility of the CYP3A4 could be increased when high lipid:protein ratios were used or when the enzyme was incorporated into Nanodiscs (conditions favoring dissociation of CYP3A4 oligomers). The study also went further to show that the effects of high lipid:P450 ratios were attributable to disaggregation of the enzyme by labeling a CYS-depleted mutant of CYP3A4 with BODIPY-iodoacetamide (a probe that serves as the FRET donor in an interaction with the CYP3A4 heme group). Thus, fluorescence of the labeled P450 was influenced by both intra- and intermolecular interactions of the enzyme. The fluorescence of the labeled CYP3A4 increased with increased lipid:protein ratio, indicative of less interaction among the macromolecules of CYP3A4 present in the reconstituted system.

Both of these studies provided convincing evidence that CYP3A4 exists as an aggregate within the lipid bilayer when reconstituted at low lipid:P450 ratios (similar to microsomes). The measurement of the dithionite-mediated reduction of CYP3A4 demonstrated at least three populations of P450 within the aggregate that were determined by their relative positions in an asymmetrical complex of the CYP3A4. Two protein conformations were involved in the substrate-dependent spin equilibrium, whereas enzyme in the third position of the aggregate was not affected by substrate and may have corresponded to the population of CYP3A4 not reduced by the flavin domain of P450 BM3 (Davydov et al., 2010). This response is consistent with the findings from a flash photolysis study by Friedman's group showing that CYP3A4 existed in at least three conformations that were affected by the presence of substrate (Koley, Robinson, Markowitz, & Friedman, 1994; Koley, Buters, Robinson, Markowitz, & Friedman, 1995; Koley, Robinson, & Friedman, 1996).

5. Formation of physical P450•P450 complexes

Although the functional studies described above suggest that the physical interaction of certain combinations of P450 affect their catalytic activities, these data are indirect. Conversely, there is substantial physical evidence for the formation of P450•P450 complexes. The first of these studies demonstrated the tendencies of P450s to aggregate in solution. Later work provided physical evidence that P450 enzymes also aggregate in membrane environments. This section will review the physical evidence for the formation of P450•P450 complexes.

5.1. Hydrodynamic characteristics of P450s in solution

A basic characteristic of the eukaryotic P450s is their tendency to aggregate, a quality that is largely attributed to the hydrophobic nature of their membrane binding regions. This region has historically led to difficulties in P450 purification, requiring the need of detergents to sufficiently disperse the aggregated proteins to facilitate their separation. The tendency to aggregate has also led to difficulties in the crystallization of these proteins, where modifications of the N-terminal membrane-binding domain were necessary to allow for their crystallization (Cosme & Johnson, 2000).

Early data supporting the existence of multimeric associations of P450 were derived from studies demonstrating that the purified enzymes had strong tendencies to aggregate in the absence of a lipid milieu. Data derived from analytical ultracentrifugation (Guengerich & Holladay, 1979; Tsuprun et al., 1986), gel filtration chromatography (French et al., 1980; Myasoedova & Berndt, 1990b; Myasoedova & Tsuprun, 1993; Viner et al., 1995), and electron microscopy (Tsuprun et al., 1986; Myasoedova & Tsuprun, 1993) indicated that each of the rabbit P450s, CYP2B4 and CYP1A2, existed as hexamers to nonamers in solution, although it was shown that CYP1A2 also tended to form larger aggregates in the absence of glycerol (Guengerich & Holladay, 1979). The aggregates could not be disrupted

even after one unit of the P450 oligomers was covalently immobilized to gel filtration columns and washed with buffer containing 0.8% sodium cholate (Myasoedova & Berndt, 1990b) (Myasoedova & Tsuprun, 1993). By using various detergents to solubilize the homomeric complexes of P450 (Guengerich & Holladay, 1979; Wagner, Dean, & Gray, 1984; Myasoedova & Berndt, 1990b; Myasoedova & Tsuprun, 1993; Viner et al., 1995), it was determined that both hydrophobic and electrostatic forces were involved in self-aggregation of the enzymes as nonionic detergents were only able to disperse the aggregates to dimers, with complete solubilization requiring a strong, amphipathic detergent such as sodium dodecyl sulfate.

Analytical ultracentrifugation data suggested that the predominant forces driving the aggregation were hydrophobic (Guengerich & Holladay, 1979), and the prevailing notion held by P450 researchers at the time was that the N-terminal hydrophobic sequence (approximately 30 amino acids long) on the mammalian P450s was responsible for both the hydrophobic aggregation and the binding of the enzymes to the lipid membrane. Subsequently, truncated P450 enzymes were expressed without the N-terminal sequence. Despite the absence of the respective N-terminal sequences, it was found that CYP2E1 (Pernecky, Larson, Philpot, & Coon, 1993), CYP2B4 (Pernecky, Olken, Bestervelt, & Coon, 1995; Scott, Spatzenegger, & Halpert, 2001), CYP1A2 (Dong, Yamazaki, Guo, & Guengerich, 1996), and CYP2C3 (von Wachenfeldt, Richardson, Cosme, & Johnson, 1997) still tended to aggregate and associate with lipid membranes with little impairment of catalytic abilities. In the cases of CYP2E1, CYP2B4, and CYP1A2, the truncated enzymes formed aggregates that were as large as those associated with the wild type enzymes in the absence of detergent. However, with the exception of the shortened CYP1A2, the aggregates of the truncated forms were more easily solubilized by detergent treatment than the full-length enzymes. Furthermore, truncation of CYP2C3 dramatically reduced the size of the P450 oligomer from octameric to dimeric (von Wachenfeldt et al., 1997). Thus, the N-terminal sequence of P450s seems to have variable roles in imparting the properties of membrane binding and oligomerization. As discussed below, the N-terminal sequences of some P450 enzymes may be involved in specific interactions involving the same (Hu, Johnson, & Kemper, 2010) or different P450 enzymes (Subramanian et al., 2010).

5.2. P450 aggregation in membranes: Rotational studies

Despite the demonstration of P450 aggregation in solution, it was still not clear whether such associations would be maintained when the proteins were present in a lipid bilayer. However, given the resistance of the aggregates to dilution and solubilization, the potential of P450s to interact when in membranes was a real possibility. This was the conclusion from investigators who compared the formation of covalently-linked aggregates of purified and lipid-incorporated CYP2B1 (Baskin & Yang, 1980) and CYP2B4 (Myasoedova & Berndt, 1990b) using various bifunctional, cross-linking reagents. In the CYP2B4 study, the P450 was incorporated into monolamellar, bilayer lipid vesicles of different compositions. The same sizes of cross-linked aggregates of CYP2B4 were observed in the presence and absence of lipid suggesting that the associations observed with the purified proteins were analogous to those present in lipid membranes.

There is now a preponderance of physical evidence indicating that the enzymes exist predominantly as complexes in biological membranes. Several early studies used a variety of techniques to examine the mobility and rotation of P450 in microsomes. In studies using optical rotatory dispersion (ORD) to study the time-resolved anisotropy of the 450 nm absorbance of the ferrous P450-CO complex after photolysis with plane polarized light (McIntosh, Kawato, Freedman, & Cherry, 1980; Kawato, Gut, Cherry, Winterhalter, & Richter, 1982), immobilized P450 in microsomes was demonstrated by persistent anisotropy of the signal, and the results indicated that at least 50% of the P450 in the microsomes was

immobile. In one of these studies (McIntosh et al., 1980), the ORD was monitored before and after cupric phenanthroline was used to cross-link cysteine groups of the P450 in the microsomes. In this study, the authors found that the rotation of the enzyme was not affected by cross-linking with cupric phenanthroline. Because cross-linking would be expected to form larger and larger protein aggregates with time due to random collision and this in turn, would be expected to change the optical rotary dispersion of the proteoliposomes, these findings indicated that the P450s in the vesicles were already aggregated when the cross-linker was added.

In another study using electron paramagnetic resonance (EPR) (Schwarz, Pirrwitz, & Ruckpaul, 1982), cysteine groups on the P450 in microsomes were selectively labeled with a spin-labeled maleimide. The out of phase EPR signal of the spin-labeled P450 was measured to determine rotational correlation times. Using theoretical considerations based on the rotation of a sphere comparable to the size of a CYP2B4 monomer in solution, the effective rotational correlation time determined from the EPR analysis of the microsomes suggested a high degree of aggregation of the P450 in the biological membranes. Unlike the study using cupric phenanthroline to cross-link cysteine residues (described above), when the amine groups of microsomes were cross-linked with glutaraldehyde, the P450 was completely immobilized.

Since these early studies providing evidence for P450 aggregation in microsomes were published, researchers have used a variety of techniques to demonstrate homomeric P450-P450 interactions in well-defined model membrane systems. As mentioned above, evidence for self-aggregation of CYP2B4 was gained from cross-linking studies in proteoliposomes made up of monolamellar, bilayer lipid vesicles made of either phosphatidylcholine or a mixture of phospholipids extracted from microsomes (Myasoedova & Berndt, 1990a). The ORD technique described above was used to assess the mobility of CYP2B4 (Kawato et al., 1982; Yamada et al., 1995) and CYP1A2 (Yamada et al., 1995) in several types of vesicle model systems. In both studies, the lipid model systems also displayed rotational parameters indicative of P450 aggregates. Furthermore, when P450 was incorporated at lipid:protein ratios similar to those observed in microsomes, a portion of the total P450 population was immobilized. However, there was a significantly higher proportion of immobile P450 in the microsomes (Kawato et al., 1982). Although the composition of the lipid vesicles did not seem to greatly influence the mobility of the enzymes, both the mobility and the rates of P450 rotation were increased at higher lipid:P450 ratios. CYP1A2 was much less mobile and rotated with much slower rate than CYP2B4 suggesting that the former existed in larger aggregates in the lipid bilayer. However, the CYP1A2 could be mobilized at higher lipid:P450 ratios in the presence of 800 mM KCl (Yamada et al., 1995). The enzyme under these conditions still rotated with the characteristics of an enzyme aggregate in the lipid bilayer.

Another study using ORD examined the homomeric interaction of CYP2B4 when reconstituted with different amounts of CPR (Gut, Richter, Cherry, Winterhalter, & Kawato, 1982). This study showed that CPR was able to disrupt homomeric complexes of CYP2B4 when it was reconstituted at equimolar concentrations to the P450 (Gut et al., 1982). This finding is significant considering that liver microsomes contain limiting amounts of CPR and thus, have conditions that are favorable for P450 aggregation to occur. This finding also provides a perspective to interpret metabolism studies of P450-P450 interactions when CPR concentrations are in excess to those of the P450.

The EPR analysis performed in microsomes (described above) also was used to study the rotation and mobility of spin-labeled CYP2B4 in lipid vesicles having a phospholipid composition similar to that of microsomes (Schwarz, Pirrwitz, Meyer, Coon, & Ruckpaul,

1990). Again by comparing the measured rotational correlation times to the theoretical rotation of a sphere roughly the size of a CYP2B4 monomer, it was postulated that the enzyme rotated as an aggregate (probably hexameric) both in solution and when the P450 was integrally incorporated in a lipid bilayer. The data in the EPR study were consistent with the N-terminal, hydrophobic tails of the CYP2B4 units in the oligomer aligning with one another to span the membrane and form a central channel between the enzymes, roughly 3.5 nm in diameter. Slower rotation in the lipid bilayer compared to the enzyme in solution was attributed either to friction of the globular enzyme as it moved over the membrane surface or to partial immersion of the globular part of the enzyme in the lipid bilayer. Because the studies described above using the N-truncated enzymes demonstrated that these forms still associate with membranes, it is likely that the globular parts of the P450s are partially immersed in the lipid bilayers. This is consistent with a report from White's group using x-ray crystallography to show that CYP2B4 is deeply embedded in the membrane (Miller et al., 1996).

The rotation and mobility of CYP2B4 was also studied by time-resolved polarized fluorescence after the cysteine groups of the P450 were selectively labeled with diiodofluorescein iodoacetamide (Greinert, Finch, & Stier, 1982). The results were again consistent with the P450 existing as a disc-shaped aggregate, referred to as a "rotamer", in the lipid bilayer. Interestingly, rotation of the fluorescent-labeled CYP2B4 in the membrane slowed when benzphetamine was added to the lipid reconstituted enzyme, and it was postulated that substrate binding caused the enzymes comprising the rotamer to be more deeply immersed in the lipid bilayer. It was hypothesized that this change in the association of CYP2B4 with the membrane might facilitate reduction of the P450 by the P450 reductase and may result in P450-mediated metabolism being more efficient in terms of coupling substrate oxidation to NADPH consumption (Greinert et al., 1982). It also stands to reason that this effect caused by substrate binding could also influence interactions between P450 enzymes within an aggregate.

5.3. Formation of specific P450•P450 complexes in reconstituted systems: Crosslinking and fluorescence studies

There are several advantages in using reconstituted systems for the examination of P450•P450 interactions. First, reconstituted systems allow us to control (1) the specific proteins being incorporated into the membrane, (2) the relative ratios of the proteins, (3) the lipid to protein ratios and (4) the lipid composition. This allows for a more detailed mechanistic analysis and limits the potential for non-specific effects. Consequently, we can more conclusively establish that specific protein-protein interactions are occurring and under what conditions they may be observed.

Despite these advantages, most methods commonly used to reconstitute proteins into lipids do not actually anchor proteins in the membrane. In most of these protocols, proteins are mixed with preformed lipid vesicles. This method generally does not lead to complete incorporation of protein into the lipid. Additionally, the effectiveness of reconstitution is dependent on the lipids used, the manner of sonication (probe versus bath), the duration of sonication, the specific proteins being reconstituted, and their incubation conditions (*e.g.*, temperature, duration of preincubation of proteins with the lipids, and concentration of the proteins when mixed with lipid) (Causey, Eyer, & Backes, 1990; Reed, Kelley, & Backes, 2006; Reed, Brignac-Huber, & Backes, 2008). Under the most favorable conditions, protein addition to preformed vesicles generally leads to the anchoring of less than 50% of CPR and P450, which differs from the more efficient anchoring of these proteins observed in the endoplasmic reticulum and other natural membranes. In contrast, when the proteins are present at the same time that the membrane vesicles are being formed, much higher levels of incorporation are observed (Reed et al., 2006; Reed et al., 2008). Three methods have been

shown to lead to anchoring of P450 proteins into reconstituted lipid systems; detergent dialysis, detergent/gel filtration, and the detergent/Bio-Beads methods (Ingelman-Sundberg & Glaumann, 1980; Reed et al., 2006; Reed et al., 2008). In each method, the protein and lipid are solubilized with a detergent. The detergent is subsequently removed by dialysis, gel filtration or adsorption. As the detergent is removed from the solution, membranes form around the proteins, leading to their efficient incorporation. Upon examination, these proteins are found to integrally localize in the membrane (Ingelman-Sundberg & Glaumann, 1980; Reed et al., 2006; Reed et al., 2008; Reed et al., 2010) – characteristics that more closely duplicate those found in natural membranes.

The detergent/Bio-Beads method for membrane incorporation was recently used to clearly establish that one P450 not only affects the function of a second P450 enzyme, but also that these P450s form physical heteromeric complexes in the membrane (Reed et al., 2010). This was accomplished by incorporation of CYP1A2 and CYP2B4 into both separate and common vesicles. Three reconstituted systems containing (1) CPR/CYP1A2, (2) CPR/CYP2B4, and (3) CPR/CYP2B4/CYP1A2 were prepared as depicted in Figure 1. Substrate metabolism was then measured with each of these reconstituted systems, and in an assay containing a combination of the CPR/CYP1A2 and CPR/CYP2B4 vesicles. The idea being tested was: Do CYP1A2 and CYP2B4 need to be in the same membrane to interact? If this is the case, then the synergistic stimulation of CYP1A2 activities (and inhibition of CYP2B4 activities) will only be observed when both proteins are incorporated into the same vesicles. A mixture of the CPR/CYP1A2 vesicles and CPR/CYP2B4 vesicles should only produce an additive effect. These results were borne out experimentally (Reed et al., 2010). Reinforcing this conclusion, formation of physical CYP1A2•CYP2B4 complexes were demonstrated by a combination of cross-linking and co-immunoprecipitation. Again, the results showed that physical complexes between CYP1A2 and CYP2B4 were only observed when both proteins were incorporated into the same vesicles.ⁱ

Tracy's group (Subramanian et al., 2010), not only showed that the presence of CYP3A4 affects CYP2C9-selective activities, but also that CYP2C9 and CYP3A4 form a physical complex. This was demonstrated by co-immunoprecipitation using antibodies specific to each P450. In these experiments, mixtures of CYP2C9 and CYP3A4 were immunoprecipitated with anti-CYP2C9 antibody and dot blotted using anti-CYP3A4. CYP3A4 was successfully co-immunoprecipitated when both proteins were present. Interestingly, no evidence of CYP2C9•tCYP3A4 complex was observed when truncated CYP3A4, lacking the N-terminal membrane binding region, was used.

5.4. Formation of specific P450•P450 complexes in cellular systems

Hydrodynamic studies support the idea that P450s have a propensity to aggregate in solution, and rotational diffusion (optical or fluorescence) studies suggest that P450s exist in membranes as part of multiprotein complexes. However, quaternary structures of P450s in membranes could be specific or simply the result of crowding of many proteins into the endoplasmic reticulum. The kinetic and functional experiments described in the beginning of this review support the idea that many of these complexes are specific, demonstrating the P450•P450 complex either alters catalysis (Model 3) or changes the affinities of the P450s for CPR (as in Model 2). Despite the demonstration of functional changes resulting from the combination of P450s into reconstituted systems, there is a need to establish that these changes result from the formation of specific complexes.

There are several recent reports showing that P450s form specific complexes within membranes. Some of the earliest physical evidence for P450•P450 complexes was provided by Alston *et al.* (Alston, Robinson, Park, Gelboin, & Friedman, 1991). In a novel study that was probably underappreciated when published, the researchers cross-linked CYP1A-

induced rat liver microsomes with Sulfo-SADP, a heterobifunctional cross-linker that reacts with primary amines through a succinimidyl group on one end and strongly but nonspecifically through a photoreactive aryl azide on the other. After cross-linking, the CYP1A1 was immunoprecipitated by using a Sepharose-bound specific antibody. The study demonstrated a specific interaction of CYP1A1 with CYP3A1/2. The specificity was illustrated by the inability of CYP1A1 to crosslink with either CYP1A2 or CYP2E1. Interestingly, the CYP1A1•CYP3A1/2 complex appeared to influence metabolism of benzo[a]pyrene by the CYP1A enzymes but not testosterone by the CYP3A.

Recent studies from the Kemper lab have used fluorescence resonance energy transfer (FRET) (Szczesna-Skorupa, Mallah, & Kemper, 2003) and bimolecular fluorescence complementation (BiFC) (Ozalp, Szczesna-Skorupa, & Kemper, 2005) techniques to demonstrate that both rabbit CYP2C2 and CYP2C1 self-associate when expressed in the endoplasmic reticulum of living cells. In the FRET study, COS cells were transfected to express two CYP2C2 chimeras, one having the C-terminal end of the P450 linked to yellow fluorescent protein and another having it attached to green fluorescent protein. A FRET interaction was demonstrated by confocal microscopic imaging of the cells when the two CYP2C2 fusion proteins were co-expressed, but was not observed when the N-terminal anchor sequence was removed from CYP2C2. Additionally, transfection of the closely-related CYP2C1 N-terminal region (residues 1–28) also showed a significant FRET response. These studies showed that the N-terminal sequence of CYP2C2 was necessary for the association observed in the cells.

A contrasting response was observed when CYP2E1 was examined. No FRET response was observed when CYP2E1-GFP and CYP2E1-YFP were co-transfected into Cos-1 cells, suggesting that the protein does not form FRET-detectable homomeric complexes. This result is in contrast to the functional data reporting a CYP2E1•CYP2E1 interaction (Jamakhandi et al., 2007). CYP2E1 and CYP2C2 also were unable to form a FRET detectable complex. Control experiments showed complex formation between CPR and CYP2C2, and between CPR and CYP2E1.

The biomolecular fluorescence complementation (BiFC) results were consistent with the FRET conclusions. This method takes advantage of the ability of cleaved N-terminal (NY) and C-terminal (CY) portions of YFP to associate and generate a functional fluorescent protein (Ozalp et al., 2005). In this study, fusion proteins were generated e.g. – CYP2C2-NY and CYP2C2-CY. In the event that CYP2C2 forms a high-affinity complex, the YFP fragments are more likely to associate and fluorescence. CYP2C2 was shown to self-associate when the two fusion proteins were expressed in Cos-1 cells, even when expressed at low levels. Using the CYP2C1(1–28)-NY and CYP2C1 (1–28)-CY constructs, the N-terminal regions of CYP2C1 were shown to form complexes with each other. CPR was also shown to form a complex with either CYP2C2 or CYP2E1. Again, consistent with the FRET experiments described above, there was no evidence for the formation of CYP2E1•CYP2E1 complexes. Taken together, these studies in living cells demonstrate that some P450 enzymes are capable of forming specific P450•P450 complexes, and that the N-terminal regions of the enzymes appear to be important contributors.

The importance of the P450 N-terminal region was emphasized in a subsequent report, where homomeric CYP2C8 complexes were detected in natural membranes by cross-linking cysteine residues followed by immune blotting (Hu et al., 2010). By selective insertion and deletion of cysteine residues in different parts of the enzyme, it was shown that CYP2C8 dimers were formed, and the interface of the dimer involved regions in both the N-terminal anchor region and secondarily in the F-G loop of the enzyme as the N-terminal region was required for stable dimer formation. Although the studies do not preclude the formation of

higher order complexes, which were also shown in this study, they do clearly demonstrate that CYP2C8 closely associates through closely aligned Cys-24 residues of CYP2C8 monomers. These studies extend those showing heteromeric complex formation through the N-terminal region in reconstituted systems (Subramanian et al., 2010) by identifying the potential for the alignment of the N-termini of two CYP2C8 monomers in living cells (Hu et al., 2010).

6. Summary

There is a basic difficulty in examining the quaternary structure of membrane bound proteins in that their removal from the membrane exposes the hydrophobic membrane-binding regions of these proteins, leading to the potential for non-specific interactions. Despite these difficulties, early functional assays, cross-linking experiments and rotational studies, in addition to more recent co-immunoprecipitation, mutagenesis and FRET experiments, have established that P450s form both homomeric and heteromeric complexes in membranes. Complex formation among some of these P450s is a specific high affinity process and not simply the result of random aggregation; however, it also is likely that less specific P450 aggregates form in these membranes. These complexes appear to be stabilized by a combination of hydrophobic and electrostatic interactions, and not only occur in reconstituted systems, but also in natural membranes. Finally, many of these P450•P450 interactions have been shown to affect P450 function, either by inhibition or synergistic stimulation of a specific monooxygenase activity.

Although a more complete understanding of these interactions will require the examination of combinations of numerous P450 enzymes, several basic features have become clear. First, it seems that both CYP1A2 and CYP2C9 tend to form heteromeric complexes with other forms of P450 that influence catalysis of other P450 enzymes. CYP1A2 interactions appear to be specific because effects on metabolism were observed with binary, P450-reconstituted systems that combined CYP1A2 with either CYP2E1 or CYP2B4, but not with binary systems using CYP2E1 and CYP2B4. The greater propensity of CYP1A2 to form functional, heteromeric P450-P450 complexes, relative to CYP2B4, may be caused by a putative, P450-unique charged zone on its surface that is able to electrostatically pair with oppositely-charged zones on other P450s. In fact, preliminary data from our lab demonstrate that CYP1A2 also associates with itself in a manner that influences catalysis (unpublished). This putative charged region may also be responsible for its greater relative ability to aggregate compared to that of CYP2B4. Similar to CYP1A2, functional P450-P450 interactions involving CYP2C9 have now been reported with CYP2C19, CYP2D6, and CYP3A4. Thus, it seems that CYP2C9 (and potentially other CYP2Cs) may also modulate the total P450-mediated metabolism by mixed P450 systems through its interactions with other forms of P450. It also is clear that specific interactions may be involved in homomeric associations of other CYP2C proteins such as CYP2C1, CYP2C2, and CYP2C8. Interestingly, CYP2E1 did not appear to associate with itself or with CYP2C1/2 in FRET and BiFC studies using living cells. Thus, the homomeric aggregation of CYP2E1 demonstrated in the global kinetic study (Jamakhandi et al., 2007) may arise either as a consequence of the purification/reconstitution process, the expression levels in the cells used in the FRET and BiFC studies were too low for CYP2E1 to aggregate, or the fluorescence probes were too far apart to elicit a significant fluorescent signal. Taken together, these data demonstrate that P450s exist in the membrane as subunits of a multienzyme complex. Consequently, the composition of these P450 complexes, which can be altered by numerous physiological and pharmacological factors, may have a significant effect on overall monooxygenase function.

Despite the evidence for the existence of P450•P450 complexes and the propensity for complex formation to affect monooxygenase function, there are still numerous questions that need to be addressed. First, which P450s tend to form complexes, and are there cellular conditions that promote the formation of these complexes? Do these complexes affect function? Which specific regions are responsible for complex formation? Are there certain regions that mediate the formation of non-specific complexes whereas other regions form complexes that also affect activity? What are the mechanistic consequences of P450•P450 interactions? Can they alter substrate selectivity, its regioselectivity, and/or the degree of uncoupling of the monooxygenase reaction? Do polymorphisms in particular P450 enzymes affect their ability to complex with other P450s and does this affect monooxygenase function? Are there functional changes that result from P450 interactions with other proteins that reside in the endoplasmic reticulum? Is there a relationship between complex formation and the regional localization of these proteins in the ER membrane? Information regarding these questions will be important in our understanding of how the components of the P450 system are organized and how they function physiologically.

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Abbreviations used

7-EFC	7-ethoxy-4-trifluoromethylcoumarin
BiFC	bimolecular fluorescence complementation
CPR	NADPH-cytochrome P450 reductase
CYP	designation for specific cytochrome P450 enzymes
EPR	electron paramagnetic resonance
EROD	7-ethoxyresorufin-O-deethylation
FRET	fluorescence resonance energy transfer
HO-1	heme oxygenase-1
ORD	optical rotatory dispersion
P450	general term for cytochromes P450
PROD	7-pentoxoresorufin dealkylation
tCYP3A4	CYP3A4 lacking its N-terminal membrane-binding region

Reference List

- Alston K, Robinson RC, Park SS, Gelboin HV, Friedman FK. Interactions among cytochromes P-450 in the endoplasmic reticulum. Detection of chemically cross-linked complexes with monoclonal antibodies. *Journal of Biological Chemistry*. 1991; 266:735–739. [PubMed: 1985961]
- Backes WL, Batie CJ, Cawley GF. Interactions among P450 enzymes when combined in reconstituted systems: formation of a 2B4-1A2 complex with a high affinity for NADPH-cytochrome P450 reductase. *Biochemistry*. 1998; 37:12852–12859. [PubMed: 9737863]
- Backes WL, Eyer CS. Cytochrome P-450 LM2 reduction. Substrate effects on the rate of reductase-LM2 association. *Journal of Biological Chemistry*. 1989; 264:6252–6259. [PubMed: 2495281]
- Baskin LS, Yang CS. Cross-linking studies of cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase. *Biochemistry*. 1980; 19:2260–2264. [PubMed: 6769473]

- Causey KM, Eyer CS, Backes WL. Dual role of phospholipid in the reconstitution of cytochrome P-450 LM2-dependent activities. *Molecular Pharmacology*. 1990; 38:134–142. [PubMed: 2164629]
- Cawley GF, Batie CJ, Backes WL. Substrate-dependent competition of different P450 isozymes for limiting NADPH-cytochrome P450 reductase. *Biochemistry*. 1995; 34:1244–1247. [PubMed: 7827074]
- Cawley GF, Zhang S, Kelley RW, Backes WL. Evidence supporting the interaction of CYP2B4 and CYP1A2 in microsomal preparations. *Drug Metab Dispos*. 2001; 29:1529–1534. [PubMed: 11717170]
- Cosme J, Johnson EF. Engineering microsomal cytochrome P450 2C5 to be a soluble, monomeric enzyme. Mutations that alter aggregation, phospholipid dependence of catalysis, and membrane binding. *Journal of Biological Chemistry*. 2000; 275:2545–2553. [PubMed: 10644712]
- Davydov DR, Fernando H, Baas BJ, Sligar SG, Halpert JR. Kinetics of dithionite-dependent reduction of cytochrome P450 3A4: heterogeneity of the enzyme caused by its oligomerization. *Biochemistry*. 2005; 44:13902–13913. [PubMed: 16229479]
- Davydov DR, Karyakin AV, Binas B, Kurganov BI, Archakov AI. Kinetic studies on reduction of cytochromes P-450 and b5 by dithionite. *European Journal of Biochemistry*. 1985; 150:155–159. [PubMed: 4018075]
- Davydov DR, Knyushko TV, Hoa GH. High pressure induced inactivation of ferrous cytochrome P-450 LM2 (IIB4) CO complex: evidence for the presence of two conformers in the oligomer. *Biochemical and Biophysical Research Communications*. 1992; 188:216–221. [PubMed: 1417844]
- Davydov DR, Petushkova NA, Bobrovnikova EV, Knyushko TV, Dansette P. Association of cytochromes P450 1A2 and 2B4: are the interactions between different P450 species involved in the control of the monooxygenase activity and coupling? *Adv Exp Med Biol*. 2001; 500:335–338. [PubMed: 11764964]
- Davydov DR, Sineva EV, Sistla S, Davydova NY, Frank DJ, Sligar SG, et al. Electron transfer in the complex of membrane-bound human cytochrome P450 3A4 with the flavin domain of P450BM-3: the effect of oligomerization of the heme protein and intermittent modulation of the spin equilibrium. *Biochimica et Biophysica Acta*. 2010; 1797:378–390. [PubMed: 20026040]
- Depierre JW, Dallner G. Structural aspects of the membrane of the endoplasmic reticulum. *Biochimica et Biophysica Acta*. 1975; 415:411–472. [PubMed: 173395]
- Dong MS, Yamazaki H, Guo ZY, Guengerich FP. Recombinant human cytochrome P450 1A2 and an N-terminal- truncated form: Construction, purification, aggregation properties, and interactions with Flavodoxin, Ferredoxin, and NADPH-cytochrome P450 reductase. *Archives of Biochemistry and Biophysics*. 1996; 327:11–19. [PubMed: 8615680]
- Estabrook RW, Franklin MR, Cohen B, Shigamatzu A, Hildebrandt AG. Biochemical and genetic factors influencing drug metabolism. Influence of hepatic microsomal mixed function oxidation reactions on cellular metabolic control. *Metabolism*. 1971; 20:187–199. [PubMed: 4395592]
- Flück CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Jabs EW, et al. Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. *Nat Genet*. 2004; 36:228–230. [PubMed: 14758361]
- French JS, Coon MJ. Properties of NADPH-cytochrome P-450 reductase purified from rabbit liver microsomes. *Archives of Biochemistry and Biophysics*. 1979; 195:565–577. [PubMed: 112928]
- French JS, Guengerich FP, Coon MJ. Interactions of cytochrome P-450, NADPH-cytochrome P-450 reductase, phospholipid, and substrate in the reconstituted liver microsomal enzyme system. *Journal of Biological Chemistry*. 1980; 255:4112–4119. [PubMed: 6768748]
- Gibson GG, Clarke SE. Incorporation of cytochrome b5 into rat liver microsomal membranes. Impairment of cytochrome P-450-dependent mixed function oxidase activity. *Biochem Pharmacol*. 1986; 35:4431–4436. [PubMed: 3790162]
- Greiner R, Finch SA, Stier A. Cytochrome P-450 rotamers control mixed-function oxygenation in reconstituted membranes. Rotational diffusion studied by delayed fluorescence depolarization. *Xenobiotica*. 1982; 12:717–726. [PubMed: 7168192]
- Guengerich FP, Holladay LA. Hydrodynamic characterization of highly purified and functionally active liver microsomal cytochrome P-450. *Biochemistry*. 1979; 18:5442–5449. [PubMed: 117825]

- Gut J, Richter C, Cherry RJ, Winterhalter KH, Kawato S. Rotation of cytochrome P-450. II. Specific interactions of cytochrome P-450 with NADPH-cytochrome P-450 reductase in phospholipid vesicles. *Journal of Biological Chemistry*. 1982; 257:7030–7036. [PubMed: 6806262]
- Hazai E, Kupfer D. Interactions between CYP2C9 and CYP2C19 in reconstituted binary systems influence their catalytic activity: possible rationale for the inability of CYP2C19 to catalyze methoxychlor demethylation in human liver microsomes. *Drug Metab Dispos*. 2005; 33:157–164. [PubMed: 15486075]
- Hu G, Johnson EF, Kemper B. CYP2C8 exists as a dimer in natural membranes. *Drug Metabolism and Disposition*. 2010; 38:1976–1983. [PubMed: 20699412]
- Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, et al. Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. *Am J Hum Genet*. 2005; 76:729–749. [PubMed: 15793702]
- Ingelman-Sundberg M, Glaumann H. Incorporation of purified components of the rabbit liver microsomal hydroxylase system into phospholipid vesicles. *Biochimica et Biophysica Acta*. 1980; 599:417–435. [PubMed: 6773567]
- Jamakhani AP, Kuzmic P, Sanders DE, Miller GP. Global analysis of protein-protein interactions reveals multiple CYP2E1-reductase complexes. *Biochemistry*. 2007; 46:10192–10201. [PubMed: 17685587]
- Kaminsky LS, Guengerich FP, Dannan GA, Aust SD. Comparisons of warfarin metabolism by liver microsomes of rats treated with a series of polybrominated biphenyl congeners and by the component-purified cytochrome P-450 isozymes. *Archives of Biochemistry and Biophysics*. 1983; 225:398–404. [PubMed: 6311109]
- Kawato S, Gut J, Cherry RJ, Winterhalter KH, Richter C. Rotation of cytochrome P-450. I. Investigations of protein-protein interactions of cytochrome P-450 in phospholipid vesicles and liver microsomes. *Journal of Biological Chemistry*. 1982; 257:7023–7029. [PubMed: 7085615]
- Kelley RW, Cheng D, Backes WL. Heteromeric Complex Formation between CYP2E1 and CYP1A2: Evidence for the Involvement of Electrostatic Interactions. *Biochemistry*. 2006; 45:15807–15816. [PubMed: 17176103]
- Kelley RW, Reed JR, Backes WL. Effects of ionic strength on the functional interactions between CYP2B4 and CYP1A2. *Biochemistry*. 2005; 44:2632–2641. [PubMed: 15709776]
- Koley AP, Buters JT, Robinson RC, Markowitz A, Friedman FK. CO binding kinetics of human cytochrome P450 3A4. Specific interaction of substrates with kinetically distinguishable conformers. *Journal of Biological Chemistry*. 1995; 270:5014–5018. [PubMed: 7890608]
- Koley AP, Robinson RC, Friedman FK. Cytochrome P450 conformation and substrate interactions as probed by CO binding kinetics. *Biochimie*. 1996; 78:706–713. [PubMed: 9010599]
- Koley AP, Robinson RC, Markowitz A, Friedman FK. Kinetics of CO binding to cytochromes P450 in the endoplasmic reticulum. *Biochemistry*. 1994; 33:2484–2489. [PubMed: 8117709]
- Locuson CW, Gannett PM, Ayscue R, Tracy TS. Use of simple docking methods to screen a virtual library for heteroactivators of cytochrome P450 2C9. *J Med Chem*. 2007; 50:1158–1165. [PubMed: 17311370]
- Loida PJ, Sligar SG. Molecular recognition in cytochrome P-450: mechanism for the control of uncoupling reactions. *Biochemistry*. 1993; 32:11530–11538. [PubMed: 8218220]
- Lu AYH, Coon MJ. Role of hemoprotein P-450 in fatty acid omega-hydroxylation in a soluble enzyme system from liver microsomes. *Journal of Biological Chemistry*. 1968; 243:1331–1332. [PubMed: 4385007]
- Lu AYH, Junk KW, Coon MJ. Resolution of the cytochrome P-450-containing omega-hydroxylation system of liver microsomes into three components. *Journal of Biological Chemistry*. 1969; 244:3714–3721. [PubMed: 4389465]
- McIntosh PR, Kawato S, Freedman RB, Cherry RJ. Evidence from cross-linking and rotational diffusion studies that cytochrome P450 can form molecular aggregates in rabbit-liver microsomal membranes. *FEBS Lett*. 1980; 122:54–58. [PubMed: 7215544]
- Miller JP, Herbert LG, White RE. X-ray diffraction analysis of cytochrome P450 2B4 reconstituted into liposomes. *Biochemistry*. 1996; 35:1466–1474. [PubMed: 8634277]

- Myasoedova KN, Berndt P. Cytochrome P-450LM2 oligomers in proteoliposomes. *FEBS Letters*. 1990a; 275:235–238. [PubMed: 2261995]
- Myasoedova KN, Berndt P. Immobilized cytochrome P-450LM2. Dissociation and reassociation of oligomers. *FEBS Letters*. 1990b; 270:177–180. [PubMed: 1699791]
- Myasoedova KN, Tsuprun VL. Cytochrome P-450: hexameric structure of the purified LM4 form. *FEBS Letters*. 1993; 325:251–254. [PubMed: 8319808]
- Ozalp C, Szczesna-Skorupa E, Kemper B. Bimolecular fluorescence complementation analysis of cytochrome p450 2c2, 2e1, and NADPH-cytochrome p450 reductase molecular interactions in living cells. *Drug Metab Dispos*. 2005; 33:1382–1390. [PubMed: 15980100]
- Pernecky SJ, Larson JR, Philpot RM, Coon MJ. Expression of truncated forms of liver microsomal P450 cytochromes 2B4 and 2E1 in *Escherichia coli*: influence of NH₂-terminal region on localization in cytosol and membranes. *Proc Natl Acad Sci USA*. 1993; 90:2651–2655. [PubMed: 8464872]
- Pernecky SJ, Olken NM, Bestervelt LL, Coon MJ. Subcellular localization, aggregation state, and catalytic activity of microsomal P450 cytochromes modified in the NH₂- terminal region and expressed in *Escherichia coli*. *Archives of Biochemistry and Biophysics*. 1995; 318:446–456. [PubMed: 7733676]
- Peterson JA, Ebel RE, O'Keeffe DH, Matsubara T, Estabrook RW. Temperature dependence of cytochrome P-450 reduction. A model for NADPH-cytochrome P-450 reductase:cytochrome P-450 interaction. *Journal of Biological Chemistry*. 1976; 251:4010–4016. [PubMed: 819436]
- Phillips AH, Langdon RG. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *Journal of Biological Chemistry*. 1962; 237:2652–2660. [PubMed: 14486217]
- Porter TD. The roles of cytochrome b5 in cytochrome P450 reactions. *J Biochem Mol Toxicol*. 2002; 16:311–316. [PubMed: 12481306]
- Reed JR, Brignac-Huber LM, Backes WL. Physical incorporation of NADPH-cytochrome P450 reductase and cytochrome P450 into phospholipid vesicles using glycocholate and Bio-Beads. *Drug Metabolism and Disposition*. 2008; 36:582–588. [PubMed: 18048487]
- Reed JR, Cawley GF, Backes WL. Inhibition of cytochrome P450 1A2-mediated metabolism and production of reactive oxygen species by heme oxygenase-1 in rat liver microsomes. *Drug Metab Lett*. 2011; 5:6–16. [PubMed: 20942796]
- Reed JR, Eyer M, Backes WL. Functional interactions between cytochromes P450 1A2 and 2B4 require both enzymes to reside in the same phospholipid vesicle: evidence for physical complex formation. *Journal of Biological Chemistry*. 2010; 285:8942–8952. [PubMed: 20071338]
- Reed JR, Kelley RW, Backes WL. An evaluation of methods for the reconstitution of cytochromes P450 and NADPH P450 reductase into lipid vesicles. *Drug Metab Dispos*. 2006; 34:660–666. [PubMed: 16415125]
- Schenkman JB, Jansson I. The many roles of cytochrome b5. *Pharmacol Ther*. 2003; 97:139–152. [PubMed: 12559387]
- Schwarz D, Pirrwitz J, Meyer HW, Coon MJ, Ruckpaul K. Membrane topology of microsomal cytochrome P-450: saturation transfer EPR and freeze-fracture electron microscopy studies. *Biochemical and Biophysical Research Communications*. 1990; 171:175–181. [PubMed: 2168169]
- Schwarz D, Pirrwitz J, Ruckpaul K. Rotational diffusion of cytochrome P-450 in the microsomal membrane-evidence for a clusterlike organization from saturation transfer electron paramagnetic resonance spectroscopy. *Archives of Biochemistry and Biophysics*. 1982; 216:322–328. [PubMed: 6285833]
- Scott EE, Spatzenegger M, Halpert JR. A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function. *Archives of Biochemistry and Biophysics*. 2001; 395:57–68. [PubMed: 11673866]
- Shimada T, Mernaugh RL, Guengerich FP. Interactions of mammalian cytochrome P450, NADPH-cytochrome P450 reductase, and cytochrome b(5) enzymes. *Archives of Biochemistry and Biophysics*. 2005; 435:207–216. [PubMed: 15680923]

- Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK, Parkinson A. Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. *Archives of Biochemistry and Biophysics*. 1987; 255:27–41. [PubMed: 3592665]
- Strobel HW, Lu AYH, Heidema J, Coon MJ. Phosphatidylcholine requirement in the enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon, and drug hydroxylation. *Journal of Biological Chemistry*. 1970; 245:4851–4854. [PubMed: 4393962]
- Subramanian M, Low M, Locuson CW, Tracy TS. CYP2D6-CYP2C9 protein-protein interactions and isoform-selective effects on substrate binding and catalysis. *Drug Metab Dispos*. 2009; 37:1682–1689. [PubMed: 19448135]
- Subramanian M, Zhang H, Tracy TS. CYP2C9-CYP3A4 protein-protein interactions in a reconstituted expressed enzyme system. *Drug Metabolism and Disposition*. 2010; 38:1003–1009. [PubMed: 20215413]
- Szczesna-Skorupa E, Mallah B, Kemper B. Fluorescence resonance energy transfer analysis of cytochromes P450 2C2 and 2E1 molecular interactions in living cells. *Journal of Biological Chemistry*. 2003; 278:31269–31276. [PubMed: 12766165]
- Tan YZ, Patten CJ, Smith P, Yang CS. Competitive interactions between cytochromes P450 2A6 and 2E1 for NADPH-cytochrome P450 oxidoreductase in the microsomal membranes produced by a baculovirus expression system. *Archives of Biochemistry and Biophysics*. 1997; 342:82–91. [PubMed: 9185616]
- Tsuprun VL, Myasoedova KN, Berndt P, Sogra ON, Orlova EV, Chernyak VY, et al. Quaternary structure of the liver microsomal cytochrome P-450. *FEBS Letters*. 1986; 205:35–40. [PubMed: 3743769]
- Viner RL, Novikov KN, Ritov VB, Kagan VE, Alterman MA. Effect of different solubilizing agents on the aggregation state and catalytic activity of two purified rabbit cytochrome p450 isozymes, CYP1A2(LM4) and CYP2B4(LM2). *Biochemical and Biophysical Research Communications*. 1995; 217:886–891. [PubMed: 8554612]
- von Wachenfeldt C, Richardson TH, Cosme J, Johnson EF. Microsomal P450 2C3 is expressed as a soluble dimer in *Escherichia coli* following modification of its N-terminus. *Archives of Biochemistry and Biophysics*. 1997; 339:107–114. [PubMed: 9056240]
- Wagner SL, Dean WL, Gray RD. Effect of a zwitterionic detergent on the state of aggregation and catalytic activity of cytochrome P-450LM2 and NADPH-cytochrome P-450 reductase. *Journal of Biological Chemistry*. 1984; 259:2390–2395. [PubMed: 6421810]
- West SB, Lu AYH. Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds and endogenous substrates. V. Competition between cytochromes P-450 and P-448 for reductase in 3,4-benzpyrene hydroxylation. *Archives of Biochemistry and Biophysics*. 1972; 153:298–303. [PubMed: 4650614]
- White RE, Coon MJ. Oxygen activation by cytochrome P-450. *Annu Rev Biochem*. 1980; 49:315–356. [PubMed: 6996566]
- Wood AW, Ryan DE, Thomas PE, Levin W. Regio- and Stereoselective Metabolism of Two C₁₉-Steroids by Five Highly Purified and Reconstituted Rat Hepatic Cytochrome P- 450 Isozymes. *Journal of Biological Chemistry*. 1983; 258:8839–8847. [PubMed: 6863312]
- Xia C, Panda SP, Marohnic CC, Martásek P, Masters BS, Kim JJ. Structural basis for human NADPH-cytochrome P450 oxidoreductase deficiency. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:13486–13491. [PubMed: 21808038]
- Yamada M, Ohta Y, Bachmanova GI, Nishimoto Y, Archakov AI, Kawato S. Dynamic interactions of rabbit liver cytochromes P450IA2 and P450IIB4 with cytochrome b5 and NADPH-cytochrome P450 reductase in proteoliposomes. *Biochemistry*. 1995; 34:10113–10119. [PubMed: 7640265]
- Yamazaki H, Gillam EMJ, Dong MS, Johnson WW, Guengerich FP, Shimada T. Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with cytochrome P450 3A4 and other forms: Effects of cytochrome P450-P450 and cytochrome P450-b₅ interactions. *Archives of Biochemistry and Biophysics*. 1997; 342:329–337. [PubMed: 9186495]
- Yamazaki H, Johnson WW, Ueng YF, Shimada T, Guengerich FP. Lack of electron transfer from cytochrome b₅ in stimulation of catalytic activities of cytochrome P450 3A4 - Characterization of a reconstituted cytochrome P450 3A4 NADPH-cytochrome P450 reductase system and studies

with apo-cytochrome b₅. Journal of Biological Chemistry. 1996; 271:27438–27444. [PubMed: 8910324]

Figure 1.
Experimental strategy used to demonstrate that both P450s must reside in the same membrane in order to interact. (reproduced from J. Biol. Chem. (2010) 285, 8942–8952)

Model 1. Simple Competition between Two P450 Enzymes for CPR

In this model, each of the P450 enzymes has its own inherent ability to associate with CPR (defined by the Michaelis constants K_{Da} and K_{Db}) as well as their rates of substrate turnover (defined by the rate constants k_a and k_b). Neither the K_D nor the rate constant for either P450 are affected by the presence of the second P450 enzyme; however, at subsaturating CPR levels both P450s will compete for the available CPR with overall substrate turnover being dependent on these constants. The species labeled in blue are capable of converting substrate to product.

Model 2. Formation of a P450•P450 complex that affects CPR binding

In this model, P450_a and P450_b are capable of forming a physical complex that affects the ability of CPR to bind. P450_a and P450_b monomers each have their characteristic abilities to bind and metabolize substrate as described in Model 1. However, formation of the P450_a•P450_b complex can affect the ability of either moiety of the complex to associate with CPR (i.e. K_{Dcba} or K_{Dcab}). According to this model, the rate constants for substrate turnover are not affected. At subsaturating CPR concentrations the reaction rate can either be stimulated or inhibited by altering the fraction of CPR bound to a particular P450 enzyme. At saturating CPR concentrations, an additive effect would be observed because all of the P450s would be complexed with CPR. The forms capable of substrate turnover include the monomeric P450s (shown in blue), the P450_a•P450_b complexes associated with a single CPR (shown in brown), and the CPR•P450_a•P450_b•CPR complex (shown in green). The latter complex would only be expected to accumulate at saturating CPR levels or in the event of a significant cooperative effect. The dissociation constants affected by P450•P450 complex formation are shown in red.

Model 3. Formation of a P450•P450 complex that affects the efficiency of substrate turnover

This model is similar to Model 2 in that P450_a and P450_b can form a physical complex. However, rather than the P450_a•P450_b complex affecting CPR binding affinity, the rate constants for substrate turnover are affected. This would cause either an inhibition or synergistic stimulation of substrate turnover that would be observed at both subsaturating and saturating CPR concentrations. The CPR-bound P450 moieties that would contribute to substrate turnover are the monomers (blue), the complexes with a single CPR (brown), and the complexes with CPR on each of the P450s (green). The rate constants affected by P450•P450 complex formation are shown in red.

ⁱ Potentially, P450s in separate membranes could interact; however, our data shows that they do not. These results support the idea that the membrane helps to orient the proteins in a manner that facilitates these P450•P450 interactions.