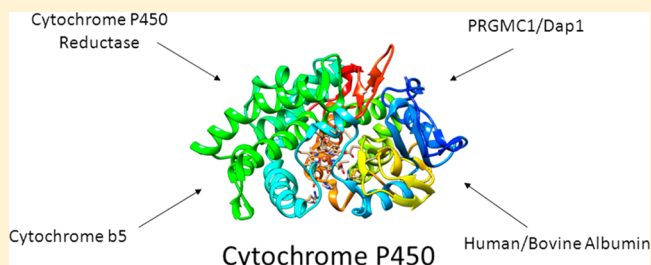# Role of Protein−Protein Interactions in Cytochrome P450-Mediated Drug Metabolism and Toxicity

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ABSTRACT: Through their unique oxidative chemistry, cytochrome P450 monooxygenases (CYPs) catalyze the elimination of most drugs and toxins from the human body. Protein−protein interactions play a critical role in this process. Historically, the study of CYP−protein interactions has focused on their electron transfer partners and allosteric mediators, cytochrome P450 reductase and cytochrome b5. However, CYPs can bind other proteins that also affect CYP function. Some examples include the progesterone receptor membrane component 1, damage resistance protein 1, human and bovine serum albumin, and intestinal fatty acid binding protein, in addition to other CYP isoforms. Furthermore, disruption of these interactions can lead to altered paths of metabolism and the production of toxic metabolites. In this review, we summarize the available evidence for CYP protein−protein interactions from the literature and offer a discussion of the potential impact of future studies aimed at characterizing noncanonical protein−protein interactions with CYP enzymes.

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1. INTRODUCTION

Protein−protein interactions are essential to the function of the ubiquitous xenobiotic-metabolizing cytochrome P450 monooxygenases, or CYPs.[1] Classic studies have demonstrated the importance of CYP interactions with the accessory proteins cytochrome P450 reductase (CPR) and cytochrome b5 (b5)

for both electron transfer and allosteric modulation.[2−4] However, it has recently become clear that CYP enzymes may participate in additional interactions with other protein partners, such as progesterone receptor membrane component 1 (PGRMC1), human serum albumin (HSA), and even other CYP isoforms. These interactions have been shown to alter CYP function and, in some cases, lead to the production of toxic metabolites. In this review, we attempt to summarize the available evidence from the primary literature and offer some future perspectives on the study of noncanonical protein−protein interactions with cytochrome P450 enzymes.

2. CYTOCHROME P450 INTERACTIONS WITH CYTOCHROME P450 REDUCTASE AND CYTOCHROME B5

2.1. Cytochrome P450 Reductase. Cytochrome P450 reductase (CPR) was identified as the primary CYP electron transfer partner by the late 1960s.[2,5] The term *cytochrome P450 reductase* is a bit of a misnomer, as this enzyme participates as a reducing partner in a number of different reactions, including those catalyzed by heme oxygenases I and II, squalene monooxygenase, and certain fatty acid desaturases.[6−9] Initially characterized as a cytochrome *c* reductase, CPR was eventually found to be localized in the endoplasmic reticulum, where it functions as a redox partner with CYPs that are also located there. CPR is a diflavin (FMN, FAD) containing reductase that receives its electron-reducing equivalents from NADPH.[10−13]

The CPR electron transfer protein shuttles two electrons to the CYP during the reaction cycle, with the second electron transfer thought to be the rate-limiting step.[1,14,15] CPR is known to be post-translationally modified and has an apparent molecular weight between 76 and 80 kDa, depending on post-translational modifications, the species-specific isoform, and detergent solubilization procedures.[2,13] In addition to the FMN, FAD, and NADPH binding domains, CPR also contains a 60 amino acid hydrophobic N-terminal membrane anchoring region, which is essential for full activity with native redox partners, such as cytochrome P450 enzymes.[3]

A number of important crystal structures of CPR and several of its mutants have been produced, including those demonstrating important conformational changes that take place upon reduction and CYP binding.[10,16−20] The first crystal structure of CPR was obtained using an N-terminally truncated sequence of the rat isoform.[10,16] This structure demonstrated that the NADPH−FAD binding domain also contained an important linker sequence that could help position the FMN and FAD domains in close proximity for efficient electron transfer. The linker sequence distinguishes CPR from the closely related, single-domain ferrodoxin reductases. It is worthwhile to note that this particular N-terminally truncated construct was not able to support CYP reduction, even though it successfully reduced cytochrome c. More recent X-ray crystal structures have provided some insight into CYP−CPR interactions.[6,18] The crystal structure of a CPR mutant with a four amino acid deletion in the hinge region between the FMN binding domain and the rest of the protein demonstrated remarkable flexibility in this region by crystallizing in three different conformations.[18] Although this particular mutant was not able to catalyze intramolecular electron transfer between the FAD and FMN prosthetic groups, it was able to successfully reduce CYPs, given enough reducing equivalents. The three different conformations observed suggest that CPR inherently possesses a high degree of conformational plasticity, especially in the C terminus of the hinge region, allowing it to efficiently interact with a variety of CYP isoforms and other enzymes. Other structures obtained from protein X-ray crystallography and NMR have confirmed this conformational flexibility and the ability of CPR to transition between closed and open forms,[6,21,22] which had also been predicted by computational molecular dynamics studies.[23,24] Another intriguing possibility is that subtle structural differences between CYP isoforms may regulate interaction with CPR and cytochrome b5.[25,26]

Seminal work examining structural and functional variation in the CPR gene has been conducted by both the Masters[10,16,17,27,28] and Kranendonk[29−31] groups, underscoring the importance of CPR in drug disposition and disease. Several mutations in the gene encoding CPR are known to be associated with specific disease states, such as a rare form of congenital adrenal hyperplasia known as Antley−Bixler syndrome and reduced steroidogenesis.[32,33] Amino acid changes that are implicated in these conditions include Y181D, A457H, Y459H, V492E, and R616X.

Surprisingly, despite the large number of CYPs present in the human genome (57, excluding possible pseudogenes; http://drnelson.uthsc.edu/hum.html), there is only one gene encoding CPR. Additionally, the total CYP content of the human liver is estimated to be between 5- and 25-fold higher than that of CPR, suggesting differential expression.[34−36] The fact that CYPs and CPR interact in a 1:1 stoichiometry raises an unusual conundrum: exactly how do CYPs and CPR interact to promote efficient oxidation of substrates? From a structural perspective, it stands to reason that all of the different CYP isoforms must share a common functional CPR binding surface. Indeed, several studies have proposed that the binding interaction between CYP and CPR takes place between the positively charged proximal surface near the CYP heme and the negatively charged FMN and FAD/NADPH domains of CPR, suggesting that their interaction is primarily driven by electrostatics.[37−39] However, there is some controversy in this regard, as other studies have suggested that hydrophobic interactions are more predominant.[40,41] These discrepancies may be the result of the different experimental approaches employed (e.g., X-ray crystallography vs site-directed mutagenesis and stopped-flow fluorescence spectroscopy).

Surprisingly, the K_d for the CYP−CPR complex is remarkably low, between 5 and 110 nM, depending on the particular isoform used and whether substrate is present, implying a very high-affinity interaction.[42,43] Upon initial examination, a tight-binding interaction may seem counter-intuitive; however, a rapid k_{on} or k_{off} rate would allow the system to work more efficiently due to swift equilibration.[35] Clearly, the interaction can be modulated by the presence of substrates and/or inhibitors, as well as other CYP isoforms, as noted below. Another compounding factor is the variety of methods, often indirect, that have been used to determine CYP−CPR affinities, making it difficult to compare various experimental conditions.

Separate from its function as an electron transfer partner, CPR may also act as an allosteric effector to induce conformational changes in CYPs that modulate the rate of electron transfer.[44−46] A number of useful structural studies conducted with the bacterial model CYP isoform, CYP101 (P450cam), have been useful to inform our understanding of how CPR might interact with mammalian CYPs in order to modulate electron transfer. Pochapsky and colleagues identified an X-Pro cis−trans isomerization redox switch in CYP101 (P450cam) that is triggered by binding to the reduced form of CYP101's cognate electron transfer partner, putidaredoxin (Pdx).[47] These conformational changes account for most of the significant structural perturbations observed by NMR upon complex formation.[47] Conformational plasticity may be one mechanism by which the binding affinity of a CYP and its redox partner are modulated: if the subset of conformational states occupied by the reduced electron transfer protein is the same as that selected upon binding to a cognate CYP, then the entropic cost (i.e., free energy) of binding would be lowered, resulting in a higher affinity.[48]

In a unique approach to examine CYP−electron transfer partner interactions, Hiruma and colleagues utilized para-magnetic NMR in conjunction with a site-specific lanthanide label to solve the solution structure of the CYP101−Pdx complex.[45] This CYP101−Pdx complex structure was, in turn, validated by simultaneous determination of the X-ray crystal structure of the same complex. In contrast to reports of the interaction between mammalian CYPs and CPR, here hydrophobic networks dominated the binding interface between the two proteins. The data suggested a minor conformational substate (or substates) that could represent an encounter complex between the two proteins, as postulated by an earlier study.[46] Additionally, the retrieved structural parameters were consistent with an electron transfer rate theoretically faster than that observed experimentally, suggesting the involvement of a gating process in electron transfer.[24,45] Similar allosterically

Table 1. Effect of Human P450 Oxidoreductase (CPR) Genetic Variants on Cytochrome P450 Enzyme Activities with Different P450 Substrates[a]

single POR mutations	CYP1A2[51]	CYP2C19[51]	CYP2D6[52]			CYP3A4[55]			
	EOMCC[b]	EOMCC	EOMCC	dextramethorphan	bufuralol	testosterone (6β-hydroxylation)	midazolam (1-hydroxylation)	quinidine	erythromycin
A115V	ND[c]	ND							
Q153R	144	284	128	198	153	129	94	150	76
Y181D	ND	ND							
A287P	ND	ND	ND	27	24	17	17	3	ND
R457H	ND	ND	ND	ND	ND	ND	ND	1	ND
V492E	ND	ND							
A503V	85	113	85	62	53	77	61	89	97
C569Y	6	ND							
V608F	5	ND							

[a]Values represent percent of wild-type. [b]EOMCC: 2H-1-benzopyran-3-carbonitrile,7-(ethoxy-methoxy)-2-oxo-(9Cl). [c]ND, evaluated but undetectable.

induced conformational changes have also been observed with mammalian CYPs and cytochrome b5.[44] Taken together, these studies underscore the dynamic nature between CYPs and their electron transfer partners, both from a functional standpoint as well as a structural one.

Seemingly small structural changes, such as a single amino acid substitution, could have dramatic functional consequences on the ability of CPR to efficiently deliver its electrons to a particular CYP isoform. Recent studies have examined how genetic variation in the human CPR gene[49,50] can manifest itself in the form of alterations in the isoform dependent CYP-mediated metabolism of certain substrates (Table 1). Interestingly, the effects of specific CPR mutations seem to be CYP isoform dependent, suggesting a unique interaction surface between individual CYP isoforms and CPR. In a study examining the effect of the 35 most common CPR mutations on the activity of CYP1A2 and CYP2C19, Miller and colleagues discovered that while most mutations either decreased the activity of both CYP1A2 and CYP2C19, or eliminated activity all together (most notably the A287P and R457H mutations), certain mutations *enhanced* CYP activity.[51] In most cases, the enhancements were specific to the CYP isoform examined and the particular CPR mutation. For CYP1A2, activity enhancement was observed with CPR mutants G213E, R406H, and A462T. In the case of CYP2C19, increased activity was reported for CPR mutants M263V and G504R. Most surprisingly, the Q153R CPR mutation, a rare disease-causing mutation that inhibits steroidogenic activity, increased the activity of both CYP1A2 and CYP2C19 to 144 and 284%, respectively. The authors rationalized these results by suggesting that many of the CPR mutants with reduced activity had decreased FAD binding or lay in a region known to be important for CPR–CYP contacts. The CPR gain-of-function mutants were more difficult to explain, but Miller and colleagues suggest that, at least in the case of the Q153R mutant, electron transfer rates to the CYP may be affected by the close proximity of glutamine residue 153 to the FMN moiety.[51] The change in electrostatic charge in the Q153R mutant may accelerate electron transfer from CPR to the CYP. Whatever the mechanism involved, it is clear that there are important functional consequences for subtle alterations in the interactions between CPR and CYP isoforms. A follow-up study examined the effects of CPR mutations on the ability of CYP2D6 to metabolize different substrates.[52] Interestingly, for any particular CPR mutation, different effects were seen

depending on the substrate used. The Q153R CPR mutation mentioned above had a slight stimulatory effect on the CYP2D6-mediated metabolism of 2H-1-benzopyran-3-carbonitrile,7-(ethoxy-methoxy)-2-oxo-(9Cl) (EOMCC), yet it almost doubled the rate of metabolism of dextromethorphan. Similar effects were seen with other CPR mutant/substrate combinations. This observation may speak to the promiscuous nature of drug-metabolizing CYPs and the multiple substrate binding sites within the active site.[53,54] Additional work with the CYP3A4 isoform also indicated that it was susceptible to functional variation due to CPR mutations.[32,55,56] Consistent with the previous findings for CYP2D6 and CYP1A2, CYP3A4 activity is decreased in combination with most of the CPR mutants examined, but it is increased with the Q153R mutant.[55] This effect was again substrate-specific for the substrates testosterone and quinidine, and nominal activity was observed for midazolam and erythromycin oxidation.

A study utilizing the full-length CYP and CPR mutant isoforms simultaneously expressed in insect Sf9 cells via a baculovirus vector recapitulated the results obtained with the bacterially expressed isoforms (referenced above).[56] A result unique to this study was the observation that some of the reduced-function CPR mutants regained activity when coexpressed with CYP isoforms in the Sf9 insect cells. This suggests that mutant CPR association with a CYP may help to stabilize the CPR protein into an active conformation, thereby rescuing activity. This is an intriguing possibility, given the postulate by Pochapsky regarding the ability of a CYP to select the active conformation of a particular electron transfer partner.[46]

Additional studies have pointed to the importance of the N-terminal hydrophobic anchoring sequence in supporting CYP-mediated oxidation. Hayashi and colleagues found that an N-terminally truncated form of rat CPR failed to support CYP1A1-mediated 7-ethoxycoumarin O-deethylation, although it efficiently reduced cytochrome c and rat heme oxygenase I.[57] Furthermore, structural studies have demonstrated that crystallized, yet catalytically active, yeast CPR has a substantially different structure[58] than the conventional N-terminally truncated catalytically inactive rat enzyme,[10,59] reinforcing the importance of this sequence in supporting efficient electron transfer from CPR to CYP.

CPR genetic variants may also have effects on different CYP isoform alleles as well.[60] In a study examining the CYP2C9 alleles CYP2C9.1, CYP2C9.2, and CYP2C9.3, Tracy and colleagues found increased metabolic oxidation of the

substrates diclofenac, flurbiprofen, and tolbutamide with four different mutant CPR enzymes tested (Q153R, A287P, R457H, and A503V).[60] This was in contrast to the predominate effect seen previously with CYP1A2, CYP2D6, CYP2B6, and CYP3A4, where a decrease in activity was reported with these CPR mutants. The authors noted that since the effects they observed led to an increase in the V_{max}, but not a change in the K_m, it was most likely that the increase in activity observed was due to enhanced electron transfer and not an increase in CYP substrate affinity.[60]

Variation in the CPR gene can lead directly to toxicity for certain drugs that are metabolized through reduction.[61−63] The herbicide paraquat produces toxicity through the generation of superoxide anion during redox cycling.[64] This redox cycling process is predominately mediated through CPR.[61] Han et al. examined the effect of six common CPR mutations in patients with impaired steroidogenesis on the redox cycling of paraquat in a CHO cell model system.[61] In five out of the six CPR mutants examined, paraquat toxicity was diminished. Although not explicitly confirmed in this study, the authors inferred that the mutants either affected the catalytic activity or expression levels of CPR, thereby reducing its ability to activate paraquat. In the C569Y mutant, the observed paraquat toxicity was equivalent to that with the wild-type enzyme, implying a rescue of function with this particular mutation. The authors suggest that variation in the CPR gene may be a primary susceptibility determinant in paraquat toxicity. Similarly, in a study investigating the toxicity of mitomycin C, Wang et al. found that CPR mutations may be useful as biomarkers to predict the therapeutic response to this anticancer drug.[62] Mitomycin C is a common chemotherapeutic agent that is metabolically activated by either a one- or two-electron reduction step.[65] CPR is the primary enzyme responsible for the one electron reduction, which produces the semiquinone anion radical that is thought cause damage to cellular DNA. Of six CPR mutants that were examined (Y181D, A287P, R457H, V492E, C569Y, and V608F), cellular toxicity of mitomycin C was decreased in all with the exception of the C569Y mutant, demonstrating that a high degree of CPR functional activity is needed to activate mitomycin C.[62] In particular, the V608F and Y181D mutants lacked any ability to activate mitomycin C, presumably due to altered association with the flavin moiety.

In addition to mitomycin C, the impact of CPR variants has also been examined with tacrolimus,[66] warfarin,[67] and other drugs, illustrating the importance of this particular enzyme in drug metabolism and elimination.

2.2. Cytochrome b5. Cytochrome b5 (b5) is a small (~17 kDa) heme-containing integral membrane protein located on the cytosolic side of the endoplasmic reticulum[4,68,69] and was originally identified in 1950 in silkworm larva.[70] Although the majority of b5 is membrane-bound, a certain fraction is present as a soluble, cytosolic form in erythrocytes, where it catalyzes the reduction of methemoglobin.[71] Although mutations in the b5 gene itself are quite rare, a few have been traced to the congenital abnormality of 17,20-lyase deficiency.[72,73] Despite the fact that it can act as an electron donor to CYP enzymes, b5 is primarily involved in lipid biosynthesis, delivering electrons to microsomal desaturases that synthesize steroids, fatty acids, and plasmalogens.[69] In the early 1970s, Estabrook and Mannering first demonstrated that b5 stimulated the metabolism of several CYP substrates.[74,75] However, at this time, the functional role b5 played in CYP-catalyzed oxidation was still somewhat unclear. Early reports suggested that it could

be involved in delivery of the second electron in the CYP catalytic cycle, thereby accelerating the rate-limiting step in CYP-mediated catalysis.[4,69,74] Remarkably, a more contemporary report from the Wolf laboratory in a hepatic CPR deletion mouse model established that b5 and b5 reductase can act as efficient CYP electron donors, without the need of CPR.[76] Despite its role in electron transfer, the effects seen from b5 are highly variable and dependent on the particular reaction conditions used, as addition of b5 can either inhibit or have no effect on some reactions rather than stimulate them.[42,68,77−82] Furthermore, several studies have demonstrated that addition of apo (heme free) b5 can stimulate the activities of many CYPs, including CYP3A4, CYP2C9, and CYP17.[7,83−85] It has been postulated that, in certain cases at least, b5 exerts the majority of its effects in the absence of electron transfer.[77,86−89] It is still not completely clear if b5 functions solely as an electron donor, allosteric modulator, electron sink, or an uncoupling inhibitor.[75,84,90−93] It is entirely plausible that its function may be contextual, depending on the particular isoform/substrate combination with which it is interacting.

A number of elegant studies over the years have established that electrostatic interactions are the basis for formation of the CYP−b5 complex, much like that for CYP−CPR.[37,94−97] Waskell and colleagues mapped out the binding interface between CYP2B4 and b5 through extensive site-directed mutagenesis of both proteins and identified R122, R126, R133, K139, and K433 of CYP2B4 as residues critical to their interaction.[37]

Recently, a number of structural and dynamic 2D NMR studies have provided additional insight into the CYP−b5 interaction. In a pioneering NMR structural examination of b5 embedded in lipid bicells, Ramamoorthy, Waskell, and colleagues were able to take advantage of rotating frame separated local field (SLF) NMR.[100] The authors employed two-dimensional proton evolved local-field (2D PELF) pulse sequences to accurately measure a broad range of heteronuclear dipolar couplings, allowing for a complete mapping of b5 protein dynamics in a native lipid bilayer environment.[98] Notably, the authors discovered that the hydrophobic N-terminal α helix of b5 precessed in a cone with an approximate 8° fluctuation in the tilt of the helix, which is estimated to be 13°, suggesting that the lipid-embedded hydrophobic N-terminus is more mobile than originally thought.[98] This added flexibility may allow this region of the protein to interact with a diverse variety of CYP isoforms. There is a growing appreciation of how inherent protein flexibility can allow proteins to adopt non-native states, which may allow them to gain new functions or interact with different protein partners.[99] Similar to the approach employed by Soong et al., Ramamoorthy and colleagues utilized a modified INEPT technique, dipolar enhanced polarization transfer (DREPT), to detect an increased range of residual dipolar couplings from isotopically labeled b5 that allowed them to characterize the amino acid side chain motions of b5 while embedded in lipid bicells.[100]

Two-dimensional NMR methodologies have also been useful in providing explanations for the specific rate enhancements seen with certain CYP−substrate pairs. The bifunctional steroidogenic enzyme CYP17A1 performs both steroid hydroxylation, which is unaffected by b5, and an androgen-forming lyase reaction that is enhanced 10-fold in its presence.[101,102] Work by the Scott group examining the interactions between an isotopically labeled b5 construct and

CYP17A1 led to the observation that the CYP17A1−b5 interaction is stronger when the hydroxylase substrate pregnenolone is present in the CYP17A1 active site than when the lyase substrate 17α-hydroxypregnenolone is bound.[44] This suggests that, at least in this case, the b5 reaction rate enhancement may be primarily driven by substrate affinity.[44] In a follow-up study, in which CYP17A1 was isotopically labeled and the b5 construct was unlabeled, titration of b5 into CYP17A1−pregnenolone complex induced a set of conformational substates closely resembling those of the CYP17A1−17α-hydroxypregnenolone complex without b5.[103] This result implied that b5 may also be able to allosterically induce catalytically productive conformations in CYP17A1, even in the absence of substrate.

These studies as well as others[98,104−106] have culminated in a complete structural and catalytic model for the CYP−b5 interaction.[94] Ahuja et al. determined the structure of full-length, membrane bound b5 in the presence of CYP2B4.[94] This structure, determined for the first time in the presence of a phospholipid bilayer, confirmed the electrostatic nature of the CYP−b5 interaction, revealing a large number of charge pairings between surface residues on CYP2B4 and b5 that facilitate the interaction between the two proteins. However, it also hinted at the importance of hydrophobic interactions for the complex that are mediated by the phospholipid bilayer. Interestingly, the authors described an increase in the affinity between CYP2B4 and b5 in the presence of a small molecule substrate or inhibitor, similar to that observed by Estrada et al. for the CYP17A1−b5 interaction.[44] Additionally, their results suggest a pathway for electron transfer between CYP2B4 and b5 mediated through a salt bridge with Arg125 of CYP2B4 and the heme propionates of b5.[94] This study illustrates the power of 2D NMR techniques to obtain detailed structural data for the interactions between two protein partners.

Finn and colleagues created a liver-specific b5 conditional knockout mouse in order to better define the functional importance of the CYP−b5 interaction.[78] *In vitro* metabolism studies demonstrated the complete lack of NADH-mediated metabolism and a 50 to 90% decrease in NADPH-mediated metabolism for most of the drug substrates examined. Additionally, a dramatic increase in the half-life for these particular drug substrates was noted, thus establishing that b5 is a major contributor to the *in vivo* metabolism of many drugs and xenobiotics, albeit in a substrate- and isoform-selective manner.[78] A follow-up study demonstrated altered clearance of several anticancer agents in the same knockout mouse model.[107] Therefore, mutant b5 alleles that are deficient in their ability to interact with, and transfer electrons to, hepatic CYPs could lead to altered clearance rates for certain drugs, which may contribute to drug-induced toxicities.

Furthermore, varying levels of b5 function important for drug oxidation can also produce altered metabolite ratios, potentially increasing the production of toxic products in certain instances. In a study conducted by the Hollenberg group, b5 was determined to be essential for the formation of protein adducts to CYP3A5 from metabolic activation of the contraceptive agent 17α-ethynylestradiol.[79] In the absence of b5, the reactive 17α-oxirene-related species was not formed, leading to CYP enzyme protection. An additional concern from a toxicological point of view is the potential for the generation of reactive oxygen species (ROS) due to increased uncoupling.[108] Indeed, using a reconstituted system containing both lipid and CPR, b5 has been shown to generate superoxide and hydroxyl radicals in

the presence of Cr(VI), leading to double-stranded DNA breaks *in vitro*.[109,110] The fact that CPR was needed to observe the effect suggests the importance of the interaction between b5 and CPR to generate these ROS. Additionally, ROS generated by b5 have been demonstrated to increase lipid peroxidation in reconstituted biological membranes, which may in turn lead to cellular damage and death.[111] Therefore, there are multiple mechanisms through which altered b5 levels and/or function can exert a toxicological effect.

3. CYTOCHROME P450 INTERACTIONS WITH OTHER CYP ISOFORMS

Surprisingly, CYPs also exhibit protein−protein interactions with other CYPs. In the early 1990s, research by Coon, Davydov, and others demonstrated that CYPs can form homomeric complexes.[112−116] Work in the ensuing 2 decades has established that multiple CYP isoforms can interact with one another in both homomeric and heteromeric complexes and that these complexes can often have significant effects on CYP-mediated oxidation of substrates[87,117−122] (for recent reviews, please see references 117 and 118). For simplicity, we have grouped the CYP−CYP interactions into those described by homomeric (two or more molecules of the same isoform) and heteromeric (two or more molecules of different isoforms).

3.1. Homomeric CYP−CYP Interactions. Pioneering studies of homomeric CYP−CYP interactions were conducted by Davydov in the early to mid-1990s, examining the response of CYP2B4 to pressure induced conformational changes.[112−114] Davydov and his colleagues discovered that approximately 35% of the ferrous CYP2B4 CO-bound complex was refractory to conversion into the inactive P420 form upon application of pressures in excess of 2 kbar.[113] However, this refractory population was reduced to less than 5% upon addition of 0.2% Triton N-101, a detergent that created conditions known to produce monomers. These results implied that a significant portion of active CYP (i.e., CYP able to be reduced and bind CO) was present in the oligomeric state. Later work by this same group would demonstrate that sodium dithionite or CPR induced reduction kinetics in CYP3A4 are multiphasic under conditions favoring CYP3A4 oligomers but monophasic under conditions favoring monomers.[123] These studies are suggestive of active CYP arranged in homomeric oligomers in the phospholipid bilayer.

Confirmation of a direct physical interaction between individual CYPs in a live cell membrane was achieved in seminal studies by Kemper and colleagues using fluorescently labeled CYP2E1 and CYP2C2 in cells transfected with murine CYP cDNA.[124] They found that while fluorescence resonance energy transfer (FRET) occurred between individual CYP2C2 molecules in a membrane, it could not be detected between CYP2E1 monomers, representing a homomeric self-association with CYP2C2 but, interestingly, not CYP2E1.[124] The CYP2C2 dimers were later confirmed to exist in murine hepatocyte endoplasmic reticulum membranes, indicating the *in vivo* relevance of such associations.[125]

Taken together, these studies laid the groundwork for analysis of the functional consequences of CYP−CYP homomeric interactions. In an elegant kinetic study conducted by Jamakhandi et al., the authors found that metabolism data for CYP2E1 fit best to either a quaternary (CYP−CPR−CYP−CPR), trimeric (CYP−CYP−CPR), or dimeric (CYP−CYP) complex model where only the binary (CYP−CPR) complex was active.[126] The authors found that simply varying the

individual concentrations of CYP or CPR in a reconstitution system produced contradictory results, which did not correspond to the simplest functional (1:1) complex. Simultaneously, Job titration analysis revealed an asymmetric plot, indicative of higher-order molecular complexes. In an attempt to identify the catalytically relevant species, both CYP and CPR concentrations were titrated simultaneously, resulting in a two-dimensional data surface. When the data were globally fit to 32 different candidate models (including binary, ternary, and quaternary CYP−CPR complexes), it revealed that the most likely model was one composed of the binary complex (CYP−CPR), the quaternary complex (CYP−CPR−CYP−CPR), and the homodimer (CYP−CYP). A second, although less likely, possibility was a model that involved a weakly bound ternary complex (CYP-CYP−CPR). Interestingly, the results implied that the binary complex was the only catalytically active state. Therefore, in this case, even though CYP2E1 was shown to be capable of forming CYP−CYP complexes that could have an effect on catalytic activity, the binary CYP−CPR complex was still shown to be the only active protein−protein complex.

More recently, a comprehensive study by the Backes group examining the effect of homomeric CYP complex formation on the activity of CYP1A2, CYP2B4, and CYP2E1 found that, at least in the case of CYP1A2, CYP−CYP homomeric complexes contribute to altered catalytic activity seen with this enzyme under conditions that would promote dimer formation.[127] To understand how CYP homomeric complexes might affect function, the catalytic activities of several individual CYPs, including CYP2B4, CYP2E1, and CYP1A2, were assessed in reconstituted systems as a function of CYP concentration. Interestingly, although oxidation mediated by CYP2B4 as a function of CYP−CPR best fit to a simple hyperbolic Michaelis−Menten equation, CYP2E1 and CYP1A2 demonstrated atypical (non-Michaelis−Menten) kinetics that fit best to a sigmoidal curve, indicating a high degree of cooperativity with these particular isoforms. This non-Michaelis−Menten kinetic behavior could be converted back to a hyperbola simply by increasing the ionic strength of the buffer, suggesting that the CYP−CYP interaction may have a strong electrostatic character. The authors further went on to verify the CYP−CYP complex in the membrane using a variety of biophysical techniques, including chemical cross-linking and bioluminescence resonance energy transfer (BRET).[127]

In yet another study, CYP−CYP interaction was found to affect enzymatic function by a direct modulation of allosteric (non-Michaelis−Menten) enzyme kinetics. Both homotropic and heterotropic cooperativity have long been known to occur with hepatic drug-metabolizing CYP isoforms, particularly CYP3A4.[53,54,128] Recently, Davydov and colleagues reported that stimulation of CYP3A4-mediated oxidation of 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC) by α-naphthoflavone in human liver microsomes (HLM) was highly dependent on the degree of CYP3A4 oligomerization.[129] The amount of enzyme activation seen was directly correlated with the surface density of the enzyme. While no activation was detectable at high lipid/CYP ratios (≥750), activation reached greater than 225% at a lipid/CYP ratio of 140:1. This suggests that allosteric activation requires at least some degree of homomeric CYP−CYP interaction.

Although a preponderance of physical and functional evidence supports homomeric CYP−CYP complexes, at least in the dimeric form, it is still not entirely clear what role these complexes may play *in vivo* in the detoxification and elimination

of xenobiotic agents, especially under conditions of CYP induction or endoplasmic reticulum (ER) stress. One suggested physiological function basis for the formation of CYP−CYP complexes is their ability to prompt a hypothetical "functional allosteric mechanism" that could lead to a rapid response for detoxification of toxic xenobiotics.[118,130] Future studies with relevant *in vivo* model systems may shed some light in this area.

3.2. Heteromeric CYP−CYP Interactions. The identification of the existence of homomeric CYP complexes has led to interest in the possibility of heteromeric CYP complexes as well. Using the same methodology that revealed homomeric interactions between individual CYP2C2 molecules, Kemper and colleagues identified additional heteromeric interactions between CYP2C2 and CYP2E1.[131] This implied that differences in homo- and heteromeric oligomerization state between isoforms may help explain some of the relative differences seen in activity. Indeed, this was found to be the case in complexes formed between CYP1A2 and CYP2B4 in reconstituted systems.[120,121] In a reconstituted system containing CPR, lipid, and both CYP1A2 and CYP2B4 enzymes, a small increase in catalytic activity for the CYP2B4 substrate benzphetamine was detected. However, activity against another CYP2B4 substrate, 7-pentoxyresorufin-*O*-dealkylation (PROD), was dramatically inhibited.[121] Inhibition of PROD was also dependent on CPR levels, with the inhibitory effect being more pronounced at subsaturating CPR concentrations, suggesting competition for reductase binding between the two isoforms. In a follow-up study, Backes and colleagues determined that it was likely that a ternary complex containing CPR, CYP1A2, and CYP2B4 was formed, for CYP2B4 activity was dramatically reduced at subsaturating reductase concentrations.[120] This result suggested an alternative model where, under substoichiometric conditions, CPR is specifically bound to CYP1A2 and at higher CPR concentrations, reductase binding to CYP1A2 becomes saturated, resulting in the formation of a quaternary complex in which CPR binds to both CYP1A2 and CYP2B4 enzymes.[120]

In a more recent study examining the interaction between CYP2C9 and CYP2C19 in reconstituted systems, Kupfer and colleagues found evidence of a metabolic interaction between even these highly related CYP isoforms.[132] Previously, using *in vitro* systems reconstituted with purified enzyme, CYP2C19 was the most active isoform for methoxychlor-*O*-demethylation.[133,134] However, in HLM, CYP2C9 performed the bulk of methoxychlor-*O*-demethylation. Interestingly, while an equimolar ratio of CPR to CYP2C9 supported maximal rates of methoxychlor-*O*-demethylation, the rate of methoxychlor-*O*-demethylation by CYP2C19 was not fully saturated in the same system; even when reconstituted with a 9-fold molar excess of CPR, CYP2C19 methoxychlor-*O*-demethylation and *S*-mephenytoin hydroxylation kinetics were not fully saturated (as compared with that in HLM). However, when a binary reconstitution system was prepared by mixing CYP2C9 and CYP2C19 enzymes, methoxychlor-*O*-demethylation and *S*-mephenytoin hydroxylation by CYP2C19 were inhibited dramatically, with the amount of inhibition dependent on the amount of CPR and substrate used, yet increasing concentrations of CPR were generally able to overcome the inhibitory effects. By contrast, in the incubation containing only CYP2C9, diclofenac hydroxylation was activated by the presence of CYP2C19, thus demonstrating that CYP−CYP interactions can modulate the catalytic rates of a variety of oxidation reactions but that this modulation is highly dependent on the substrate

undergoing metabolism, with some substrates activating metabolism and others inhibiting it.[132,135]

Surprisingly, Subramanian and colleagues found that while addition of CYP2D6 to a reconstituted incubation of CYP2C9 and (S)-flurbiprofen increased the K_s by 20-fold, it had no effect on the K_m, but it decreased the V_{max} by 50%.[136] One possible explanation for this discrepancy is that affinity constants determined by heme spin state (i.e., optical spectrum) perturbation may mask total ligand binding because they measure only substrate interactions that perturb the heme iron.[137] It is possible that, in the presence of an additional CYP isoform, the spin state shift may be less complete, thereby not reflecting the effects of additional ligand binding. No effects were observed on the metabolism of CYP2D6 substrates. The authors additionally noted that the effects on metabolism were dependent on the order of addition of the enzymatic constituents, with the greatest rate enhancements observed when CPR was added before the second CYP, again suggesting a competition for reductase binding.[136] In yet another study, Yamazaki et al. discovered similar types of enzymatic stimulation between CYP1A2 and other isoforms, including CYP3A4, CYP2C9, and CYP2D6, which indicate a more generalizable phenomenon among the hepatic CYPs.[87]

As more substantial biophysical and functional evidence has accrued demonstrating heteromeric interaction between individual CYP isoforms, attention has shifted to structurally defining the mechanism of this interaction. Two different studies conducted in 2010 by the Backes and Tracy laboratories were instructive in this regard. The Backes group set out to answer the question of whether two different CYP isoforms need to be located in the same membrane to functionally interact, thereby potentially excluding long-range allosteric effects.[138] Their experimental system consisted of incorporating CYP1A2 and CYP2B4 into vesicles with CPR individually and together as a CYP–CYP complex. If the two CYPs functionally interacted in the same membrane, then metabolic stimulation (or inhibition) would be observed only when both CYPs were present in the same vesicle, and a mixture of both the individual CYP1A2–CPR and CYP2B4–CPR vesicles would simply produce an additive effect. Indeed, this is exactly what the authors observed prior to confirming this functional interaction between these two CYPs with cross-linking and coimmunoprecipitation experiments.

Work from the Tracy lab examined the effect of the hydrophobic N-terminal anchor sequence on mediating the interaction between CYP2C9 and CYP3A4.[139] Using a reconstituted enzyme system containing both CYP2C9 and CYP3A4, the authors demonstrated that CYP2C9 mediated (S)-naproxen metabolism was inhibited as much as 80% by the presence of CYP3A4, while the K_m remained unchanged. The degree of inhibition was directly proportional to the CYP3A4 concentration and indirectly proportional to the CPR concentration, suggesting a competition for reductase. Oddly, CYP2C9 did not seem to alter CYP3A4-mediated testosterone metabolism. In order to study the role of the hydrophobic N-terminal anchor sequence, the experiments were performed with both N-terminally truncated and full-length CYP isoforms. There was no evidence for metabolic inhibition when the full-length CYP3A4 and CYP2C9 isoforms were incubated in the presence of the truncated form of the other CYP isoform, but evidence for inhibition was found only when both full length forms were present. This indicated that interaction between the hydrophobic N-termini of the two CYP isoforms was required

for functional inhibition. It is possible that, in this context, the hydrophobic N-termini may orient the CYPs in the membrane. Finally, the authors conducted coimmunoprecipitation studies to demonstrate that the two CYPs were directly interacting in the membrane.

It has now been firmly established that CYP's form both homomeric and heteromeric complexes with functional consequences. The difficult work of defining how these complexes interact structurally and what their *in vivo* roles may be now lies ahead.

4. CYTOCHROME P450 INTERACTIONS WITH OTHER PROTEINS

Of all the protein–protein interactions in which CYPs have been documented to participate, perhaps the most enigmatic is that with nonenzymatic proteins, including the progesterone receptor membrane component 1 (PGRMC1)[140] and bovine and human serum albumin (BSA/HSA).[141] Although these proteins do not provide reducing equivalents to the CYP catalytic cycle, they are still capable of modulating CYP-mediated metabolism. Presently, these mechanisms remain to be fully elucidated; intriguingly, they may involve direct delivery of a drug substrate to the particular CYP. Below, we provide a brief summary of what is currently known regarding these CYP protein–protein interactions.

4.1. PGRMC1–CYP Interactions. Despite its rather precise identification, the progesterone receptor membrane component 1 is a somewhat misleading name, as it neither binds progesterone directly nor does it share any homology with progesterone receptors.[142,143] In contrast, PGRMC1 shares a high degree of structural homology with cytochrome b5.[143] PGRMC1 has been demonstrated to either increase or decrease the rate of CYP-mediated metabolism, depending on the CYP–substrate pair being examined, although perhaps not directly through electron transfer.[144] PGRMC1 also seems to play a role in the development of certain cancers, as recent reports suggest that it may be the inscrutable "sigma-2" receptor, a known biomarker of tumor cell proliferation.[145,146] In mammals, there exist two additional PGRMC1 family isoforms, PRGMC2/Dg6[147] and neudesin,[148] although it is not known if they also interact with CYP enzymes. PRGMC1 is highly expressed in the human liver and typically colocalized in the smooth endoplasmic reticulum (SER) with CYP proteins. It has been shown to interact directly with CYP enzymes, including CYP3A4, CYP2C9, CYP7A1, CYP21A2, and CYP51.[140] PGRMC1 activates progesterone oxidation mediated by CYP21A2[142,149] and is essential for CYP51 activity in the cholesterol synthesis pathway.[140] Min and colleagues reported that the coexpression of PGRMC1 with CYP21A1 enhanced progesterone 21-hydroxylation in COS-7 cells, while a heme-deficient PGRMC1 mutant had no effect.[142] In an experiment with the yeast homologue, Dap1, Hugues et al. showed that both CYP51A1 and CYP61A1 can be positively regulated by the presence of Dap1.[140]

Remarkably, PRGMC1 has been revealed to exhibit isoform-selective effects in its interaction with CYPs.[150,151] Oda found that PGRMC1 generally reduced CYP activities, either through decreased V_{max} and K_m values (in the case of CYP3A4) or simply through a decreased V_{max} (in the case of CYP2C9); however, in the case of CYP2E1, it had no effect.[151] Coimmunoprecipitation studies suggested that the mechanism behind the differential effects may be differences in binding affinity between PGRMC1 and individual CYP isoforms.

Szczesna-Skorupa and Kemper found similar results in their studies of the interactions of PGRMC1 with CYP2C2, CYP2C8, and CYP3A4 in transfected HepG2 cells.[150] In each case, PGRMC1 downregulated CYP activities, which could be rescued by increased expression of CPR. PGRMC1 was found to have a high affinity for CPR, but only in the absence of CYP2C2, indicating competition between PGRMC1 and CYP2C2 for binding to CPR. In contrast to what was observed with the drug-metabolizing CYP isoforms, CYP51 activity was reduced with decreasing levels of PGRMC1, indicating that a separate mechanism may be in place for the interaction of PGRMC1 with CYPs involved in steroid synthesis.

The mechanism of CYP activation or inhibition by PGRMC1 is highly debatable; however, it is conceivable that it may involve drug delivery or sequestration, as a hydrophobic binding pocket has been identified on PGRMC1.[152] In any case, much work remains to elucidate the structural and functional details of the CYP−PGRMC1 interaction.

4.2. Albumin−CYP Interactions. Human serum albumin (HSA) is a 65 kDa monomeric, slightly basic globular protein that circulates as a major component of blood plasma and is the most abundant protein in the human body.[141,153−155] HSA functions primarily as a carrier for steroids, fatty acids, and thyroid hormone. Additionally, it plays a role in stabilizing osmotic pressure by regulating extracellular fluid volume. HSA binds a large variety of drugs and xenobiotic agents and has a major impact on their pharmacokinetics and disposition.[141] Under normal physiological circumstances, HSA does not cross from the portal circulation into hepatocytes. However, it is synthesized in the liver and is present in the SER of hepatocytes,[155−157] so it is conceivable that HSA could come into direct contact with CYP isoforms.

Moreover, the extent of drug or toxin protein binding has long been known to effect *in vitro*-to-*in vivo* extrapolations of disposition.[141,158] Therefore, in an effort to improve *in vitro*-to-*in vivo* predictions, researchers began to add either HSA or bovine serum albumin (BSA, a cheaper and more well-characterized substitute) to both microsomal incubations and recombinant baculovirus-expressed enzymes.[159] This accomplished the desired effect of improving pharmacokinetic predictions by increasing the *in vitro* intrinsic clearance rate, CL_{int} (V_{max}/K_m) to more closely match what is seen *in vivo*. This effect is often mediated through a decrease in the substrate K_m, and it has been observed for a number of different CYP isoform/drug combinations, including CYP2C8,[160] CYP2C9,[161,162] and CYP1A2.[163] However, for some CYP isoforms, including CYP2C19 and CYP2D6, BSA was found to be inhibitory.[164,165] Xu et al. established that the degree of activation or inhibition observed was dependent on the concentration of BSA used, with lower concentrations activating and higher concentrations being inhibitory.[164] In addition, BSA's CYP activation/inhibition profile was demonstrated to deviate from that observed with HSA, indicating the importance of the albumin source for comparison studies and *in vitro*-to-*in vivo* extrapolations.[164] A number of theories have been proposed to account for the effect seen with albumin, including direct drug delivery, allosterically induced conformational change, and inhibitory long-chain unsaturated fatty acid sequestration. Although no single theory fits the data precisely, recent evidence seems to lend credence to long-chain unsaturated fatty acid sequestration.[160,162,166] Of course, because none of these theories are mutually exclusive, it may be that multiple mechanisms are involved rather than a single predominant mechanism with any particular drug/CYP isoform. Lastly, other proteins related to albumin, such as human intestinal fatty acid binding protein (IFABP), have also been demonstrated to improve *in vitro*-to-*in vivo* predictions based on microsomal incubations,[167] suggesting a more generalizable phenomena with this class of proteins.

In addition to clearly defining the particular mechanisms of albumin−CYP interaction, future work in this area will also need to delineate exactly which members of this protein family interact with individual CYP isoforms. A comprehensive understanding of this protein−protein interaction will no doubt further enhance our ability to correlate *in vitro* metabolism to that observed *in vivo*.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The CYP pathway of oxidative transformation of drugs and other xenobiotics is critical for the elimination of toxicity in the host organism. Proper functioning of this pathway relies on effective formation of protein−protein complexes among the CYPs, their electron transfer partners, and other proteins that can influence their function. Mutations in either the CYP or an interacting protein that effect the structure or stability of the CYP or its protein partner can lead to deficiencies or alternative pathways of biotransformation, which in turn may produce toxic metabolites. Therefore, a comprehensive understanding of the many different types and functions of protein−protein interactions that CYPs undergo is necessary to predict and treat such toxicities. New techniques to understand protein−protein interactions that are beginning to be developed will be a boon for research in this area. Additionally, from a pharmacological point of view, modulating protein−protein interactions may provide an avenue for therapeutic intervention.[168] It is likely that additional CYP protein−protein interactions remain to be discovered. Conceivably, CYPs might also interact with phase II, or drug conjugating, enzymes such as glucuronidases, sulfotransferases, or gluthione-*S*-transferases to modulate their activity as well. These sorts of interactions would be logical from a synergistic point of view.

As our understanding of CYP protein−protein interactions increases, we will not only improve our ability to predict and prevent toxic CYP−drug interactions but also gain a new appreciation for the many unique attributes of this versatile enzyme family.

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Notes
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■ ABBREVIATIONS

CYP, cytochrome P450; CPR, cytochrome P450 reductase; b5, cytochrome b5; HLM, human liver microsomes; 2D NMR, two-dimensional nuclear magnetic resonance; Pdx, putidaredoxin; EOMCC, 2H-1-benzopyran-3-carbonitrile,7-(ethoxymethoxy)-2-oxo-(9Cl); SLF, separated local field; 2D PELF, two-dimensional proton evolved local field; INEPT, insensitive nuclei enhanced by polarization transfer; DREPT, dipolar enhanced polarization transfer; BRET, bioluminescence resonance energy transfer; ER, endoplasmic reticulum; SER, smooth endoplasmic reticulum; 7-BFC, 7-benzyloxy-4-(trifluoromethyl)coumarin; PROD, 7-pentoxyresorufin-O-dealkylation; PGRMC1, progesterone receptor membrane component 1; HSA, human serum albumin; BSA, bovine serum albumin; IFABP, intestinal fatty acid binding protein

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