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Cytochrome P450 Organization and Function Are Modulated by Endoplasmic Reticulum Phospholipid Heterogeneity

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Nonstandard Abbreviations: CPR, NADPH-cytochrome P450 reductase; P450, cytochrome P450; *b*5, cytochrome *b*5; ER, endoplasmic reticulum; DRM, detergent-resistant membranes; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; SM, sphingomyelin; PB, phenobarbital; PLD, phospholipase D; DAG, diacylglycerol; DLPC, dilauroylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; CL, cardiolipin; l_o, liquid ordered domain; l_d, liquid disordered domain; GPI-anchored, glycosylphosphatidylinositol-anchored; TIMs, Triton-insoluble membranes; MβC, methyl-β-cyclodextrin; PrP^c, cellular prion protein; PrP^{Sc}, scrapie prion protein; Aβ, amyloid-β peptide

Abstract

Cytochrome P450s comprise a superfamily of proteins that catalyze numerous monooxygenase reactions in animals, plants and bacteria. In eukaryotic organisms, these proteins not only carry out reactions necessary for the metabolism of endogenous compounds, but also are important in the oxidation of exogenous drugs and other foreign compounds. Eukaryotic cytochrome P450 system proteins generally reside in membranes, primarily the endoplasmic reticulum or the mitochondrial membrane. These membranes provide a scaffold for the P450 system proteins that facilitate interactions with their redox partners as well as other P450s. This review focuses on the ability of specific lipid components to influence P450 activities, as well as the role of the membrane in P450 function. These studies have shown that P450s and NADPH-cytochrome P450 reductase appear to selectively associate with specific phospholipids, and that these lipid-protein interactions influence P450 activities. Finally, due to the heterogeneous nature of the endoplasmic reticulum as well as other biological membranes, the phospholipids are not arranged randomly, but associate to generate lipid microdomains. Together, these characteristics can affect P450 function by (1) altering the conformation of the proteins, (2) influencing the P450 interactions with their redox partners, and (3) affecting the localization of the proteins into specific membrane microdomains.

Introduction

Cytochromes P450 (P450s) are clinically of great importance because they are responsible for the phase I metabolism of a majority of xenobiotics (Bertz and Granneman, 1997). Variability in P450 activities can be due to modulation of P450 protein levels, inhibition of activities by other chemicals (Lin and Lu, 1998), and genetic differences resulting from both structural and expression polymorphisms (Guttendorf and Wedlund, 1992). P450 primarily catalyzes monooxygenation reactions of both xenobiotics and endogenous substrates (Guengerich, 1992). These enzymes function as the terminal component of an electron transport chain that includes interactions with the redox partners, NADPH-cytochrome P450 reductase (CPR) and cytochrome b_5, as well as other P450 enzymes (Hildebrandt and Estabrook, 1971;Lu, et al., 1969;Reed, et al., 2010;Backes, et al., 1998). The components of this electron transfer chain are anchored in the endoplasmic reticulum (ER) membrane, which orients the proteins for more efficient electron transfer (Black and Coon, 1982). Phospholipid, which was originally thought to be an obligatory component of this system (Strobel, et al., 1970), has been shown to not be required (Muller-Enoch, et al., 1984), but in most cases, supports P450 activities. Phospholipid serves as a matrix for incorporation of P450 and CPR (Causey, et al., 1990), promotes the proper orientation of the P450s with their redox partners and generally facilitates substrate binding (Ingelman-Sundberg, et al., 1983;Taniguchi and Pyerin, 1988). Consequently, study of the interactions among P450 system components as well as the interactions between P450 enzymes in their lipid environment have become increasingly relevant for a more complete understanding of P450 function. This review examines the lipid components comprising the ER, as well as the effect of lipid composition on the P450 system.

Structure of the Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a complex network of lipid, containing many membrane bound proteins, including those of the P450 enzyme system (Glaumann and Dallner, 1968). Electron microscopy shows the ER membrane to be 50-80 Å thick (Yamamoto, 1963) with a surface area 37 times that of the plasma membrane (Weibel, et al., 1969). The lipid composition of rabbit liver microsomes was reported to be approximately 60% phosphatidylcholine (PC), 20% phosphatidylethanolamine (PE), 1% phosphatidylserine (PS), 1% phosphatidic acid (PA), 10% phosphatidylinositol (PI), 4% sphingomyelin (SM) and 5% cholesterol (Brignac-Huber, et al., 2011). These values are similar to those found in liver microsomes of humans (Waskell, et al., 1982) and rats (Glaumann and Dallner, 1968;Dallner and Ernster, 1968;Davison and Wills, 1974). Additional studies have characterized asymmetry of the phospholipid species and reported that approximately 40% of PC, 90% of the PE, 14% of the SM, 90% of the PS, and 20% of the PI are located on the outer leaflet of the membrane (Nilsson and Dallner, 1975). While phospholipids of the rough and smooth ER have been reported to be very similar (Glaumann and Dallner, 1968), cholesterol has been found to be twice as high in smooth ER (Glaumann and Dallner, 1968;Pascaud, 1958). Moreover, the ER is a dynamic organelle that can proliferate in response to xenobiotic exposure (Remmer and Merker, 1963).

The lipid bilayer has been shown to play an essential role in protein function at the plasma membrane and similar findings have been reported for the ER. It is estimated that the ER from rat liver is composed of approximately 70% protein and 30% lipid by weight (Glaumann and Dallner, 1968). *In vivo* studies have suggested that phospholipids in the immediate vicinity of P450 molecules are more organized than the bulk membrane and that the P450 is enclosed in a “phospholipid halo” possibly affecting the diffusion of substrates and products to and from the P450 molecule (Stier and Sackmann, 1973). The exact functional role of the interaction between the phospholipid and the P450 system has been the subject of many studies, which

have provided a better understanding of the system *in vivo*. These studies have employed reconstitution of the proteins into compositionally defined mixtures of phospholipids, as well as natural membranes that are heterogeneous in nature with regard to both lipid and protein content. Numerous lipid species have been used in reconstituted systems that have led to differences in P450 stability and catalytic activities (Ingelman-Sundberg, et al., 1981;Ingelman-Sundberg, et al., 1996;Ahn, et al., 2005;Yun, et al., 1998;Brignac-Huber, et al., 2011). This is not surprising, considering the ER membrane is composed of a variety of lipids. Although PC was found to be a major component that facilitates P450 interactions with their redox partners (Strobel, et al., 1970) it is known that the other ER lipids play a critical role in ER membrane structure and monooxygenase function.

Phosphatidylcholine

Phosphatidylcholine is reported to be the major phospholipid constituent of the ER bilayer (Glaumann and Dallner, 1968) with about half of this lipid species located in the outer leaflet (Nilsson and Dallner, 1975). Most phosphatidylcholine species contain one unsaturated acyl chain that increases membrane fluidity at room temperature. The cylindrical shape of PC facilitates its spontaneous incorporation into a bilayer (van Meer, et al., 2008). PC has been the most studied phospholipid in the context of the P450 system, due to its prevalence in the ER membrane. Numerous studies both *in vivo* and *in vitro* have strengthened our understanding of the role of PC in the monooxygenase system.

In vitro studies by Strobel et al. established PC as an important lipid constituent in the P450 monooxygenase system, illustrating that optimal electron transfer from CPR to P450 was dependent on the presence of this microsomal lipid. A number of synthetic PC variants produced similar P450 activities when substituted for the microsomal lipid, with dilauroylphosphatidylcholine (DLPC) producing the highest activity (Strobel, et al., 1970). This

finding led to the use of DLPC as a standard lipid for many P450 studies. While DLPC is commonly used to prepare preformed lipid vesicles, it was illustrated rather early that certain methods of lipid preparation and protein addition can prevent the enzymes from integrally incorporating into the lipid bilayer (Ingelman-Sundberg and Glaumann, 1980;Autor, et al., 1973;Reed, et al., 2006). Consequently, many studies have characterized different methodologies in reconstituting P450 and CPR into PC lipid membranes. Reconstituted systems prepared with longer chain fatty acids, as typically found *in vivo*, and with the use of detergent, allowed for the more efficient physical incorporation of CPR and P450 into the lipid vesicles. Ultimately, the integration of P450s into phospholipid bilayers affected membrane binding and catalytic characteristics of the P450 enzymes, and was dependent on the method of protein incorporation/membrane preparation utilized (Taniguchi, et al., 1979;Ingelman-Sundberg and Johansson, 1980;Reed, et al., 2006;Reed, et al., 2008)

The PC lipid component of the ER can be altered by xenobiotic exposure. Young (Young, et al., 1971) examined changes in microsomal phospholipid and protein components in rats after phenobarbital (PB) treatment. The investigators found an increase in PC levels as a result of the methylation of PE. Although this study did not focus on P450 levels, they did report a significant increase in the total microsomal protein content after induction (Young, et al., 1971). A more detailed study by Davison and Wills reported that both phenobarbital (PB) and 20-methylcholanthrene caused an increase in PC levels days after injections, with the proportion of linoleic acid of both PC and PE being significantly increased by PB treatment. This increase in linoleic acid corresponded to increased concentrations and activities of P450 and CPR (Davison and Wills, 1974). This group suggested that PC may be more significant than PE for drug metabolism. An additional but more recent finding suggests that CYP1A2 possesses phospholipase D (PLD) activity, converting PC to phosphatidic acid. Phosphatidic acid serves as a precursor for the formation of diacylglycerol (DAG), an important molecule in signal

transduction events. CYP1A2-mediated PLD activity was not dependent on the presence of CPR and was not inhibited by the use of specific CYP inhibitors, suggesting that there may be an alternate active site that catalyzes this reaction. This group suggests that the PLD activity of CYP1A2 could affect membrane organization of the ER by metabolizing PC to other lipid products (Yun, et al., 1999). .

Phosphatidylethanolamine

Phosphatidylethanolamine comprises about 25% of the ER bilayer *in vivo* and is almost exclusively located in the outer leaflet (Nilsson and Dallner, 1975). The presence of this phospholipid has a profound effect on protein organization and function.

Phospholipid packing into bilayers is a function of the general shape of the molecules. This shape is dependent on the size of the polar head group as compared to the size of the phospholipid side chains. Side chains that are unsaturated will displace a larger area in the nonpolar region of the membrane, whereas saturated fatty acids would occupy a smaller space. For example, PC, which has polar and nonpolar regions of similar size would better pack into a bilayers. In contrast, PE, which has a smaller polar head group, would be shaped more like a cone. This would lead to a more negative curvature of the membrane and generate non-lamellar phases in the purified form (Luzzati and Husson, 1962). These non-lamellar-forming lipids affect the fluidity of the membrane and have been reported to modulate the activities of numerous proteins, including rhodopsin (Epand, 1998), protein kinase C (Epand, 1987), and CYP11A1 (Schwarz, et al., 1997), as a few examples.

Generally speaking, the addition of PE tends to increase the fluidity of the membrane, which can be deduced by changes in the temperature of the transition from the lamellar to non-lamellar phases. The temperature of this transition, as measured using ^{31}P-NMR, is generally lower for PE than PC, and is further decreased by an increase in unsaturation of the fatty acid

chains and the addition of cholesterol (Dekker, et al., 1983). In another ^{31}P-NMR study, Bayerl and coworkers (Bayerl, et al., 1985) used hexane phosphonic acid diethyl ester (PAE) as a ^{31}P-NMR probe. Using an upfield shift in the ^{31} P-NMR signal as an indicator of protein binding to the membrane, systems where CPR and P450 were reconstituted into PC vesicles did not produce this upfield shift; however, the upfield shift was observed when PE was included in the reconstituted system. Based on this result, the authors concluded that P450 and CPR interact specifically with PE. Interestingly, this shift was not observed when only one of the proteins was present, suggesting that PE may play an integral role in the interaction between these proteins.

The importance of PE in reconstitution of P450s was first described by Bosterling (Bosterling, et al., 1979) in which the reconstitution of CYP2B4 and CPR was made with a lipid mixture of PC, PE and PA. As opposed to the PC membranes frequently used (Taniguchi, et al., 1979), this group found that the mixed system prevented vesicle aggregation possibly due to the structural stability presented by the PC/PE mixture and the negative surface charge due to the presence of PA. This system also produced hydrogen peroxide at levels comparable to those in microsomes, which led this group to conclude that their mixed system more closely mimicked the *in vivo* membrane (Bosterling, et al., 1979). Ingelman-Sundberg and coworkers showed that CYP2B4 was more active when reconstituted into mixtures of dioleoyl-PC and dioleoyl-PE (DOPE), with the reaction rates increasing as the fraction of DOPE was elevated. Furthermore, there was a direct relationship between the presence of DOPE in the reconstituted system and the rate of first electron transfer (Ingelman-Sundberg, et al., 1981). This led the investigators to suggest that the presence of PE led to more efficient electron transfer, possibly through a more efficient interaction between CPR and CYP2B4.

CYP1A2 and CYP3A4 have been studied in PE-containing membranes. Ahn and coworkers (Ahn, et al., 2005) reported that the addition of PE to PC-vesicles caused an increase in CYP1A2 stability and insertion into the membrane, with a concomitant decrease in CYP1A2

sensitivity to trypsin digestion. Likewise, the time-dependent decrease in catalytic activity seen in PC membranes was stabilized by the inclusion of PE in the reconstituted system. The addition of diacylglycerol, another non-lamellar prone lipid, to the PC:PE reconstituted system furthered augmented these results (Ahn, et al., 2005). CYP3A4 studies suggested a role for domain formation by PE. In these studies, CYP3A4 activity in PC-containing reconstituted systems was unaffected by the addition of PE; however, when the anionic phospholipids PA or PS, the addition of PE to the PC/PA or PC/PS reconstituted systems increased CYP3A4 activities. This led the investigators to suggest that the presence of PE in a ternary system of PC/PE/PA or PC/PE/PS led to the formation of anionic lipid-enriched domains, which led to enhanced membrane binding and catalytic activity of CYP3A4 (Kim, et al., 2003).

Anionic Phospholipids

In the above studies, PE elicited many of its effects in conjunction with the anionic phospholipids. Although PI, PS, and PA make up only a small percentage of the ER bilayer lipids (Dallner and Ernster, 1968;Waskell, et al., 1982;Brignac-Huber, et al., 2011), they provide a negatively-charged environment that has been suggested to be an effector of P450 catalyzed reactions (Ingelman-Sundberg, et al., 1981). In general, anionic phospholipids produce a negatively charged interface that has been shown to facilitate protein insertion into membranes (de Kruijff, 1997). Moreover, these phospholipids play a key role in regulation of cellular functions of the plasma membrane as well as intracellular organelles (Buckland and Wilton, 2000).

Ingelman-Sundberg et al. first established that the minor ER phospholipids play an important role in the P450 system when replacement of PC with microsomal lipids caused an increase in CYP2B4 activity (Ingelman-Sundberg and Johansson, 1980). Further experiments by this group incorporated different lipids into reconstituted systems, including PC, PE and a mixture of PE with negatively charged PS. Not only did PE stimulate 7-ethoxycoumarin and p-

nitroanisole metabolism (as mentioned above), but these activities were further stimulated by the presence of negatively charged PS (Ingelman-Sundberg, et al., 1981).

The importance of PE in facilitating CPR:P450 complex formation was further examined by Blanck et al., who reported that a 3:1 lipid mixture of PE:PS produced a dissociation constant for the CPR:P450 complex that was comparable to systems made with microsomal lipid extracts, which was 10 times lower than in neutral dioleoylphosphatidylcholine (DOPC) membranes (Blanck, et al., 1984). These results could be due to the negative charge imposed by the PS or may be a function of PE in the mixed system.

The involvement of negatively charged phospholipids in the incorporation of P450 system proteins was supported by ^{31}P-NMR studies showing that PS and PI were associated with CPR upon purification (Balvers, et al., 1993). Subsequent *in vitro* experiments illustrated that the addition of PS to these reconstituted systems led to a significantly increased rate of CYP2B1-mediated 7-pentoxyresorufin dealkylation when compared to PC and PC/PE systems, results that are consistent with those of Ingelman-Sundberg using CYP2B4 (Ingelman-Sundberg, et al., 1981). However, these results could not be exclusively attributed to the negative charge of the phospholipid since the presence of PI did not illicit the same results (Balvers, et al., 1993). Das and Sligar more recently came to similar conclusions when they found that the redox potential of CPR was more negative when 50 mol% PS was added to PC containing nanodiscs (Das and Sligar, 2009), illustrating that the anionic environment favored electron transfer from CPR.

Other investigators have found that the P450s, in the absence of CPR, have a greater affinity for negatively charged phospholipids. Enhanced membrane insertion mediated by anionic phospholipids was reported for CYP1A2 (Ahn, et al., 1998), CYP3A4 (Kim, et al., 2003) and CYP2B1 (Kim, et al., 2007). The CYP1A2 study also demonstrated that the anionic lipids

increased cumene hydroperoxide-mediated CYP1A2 activity with PA having the greatest effect. These results were accompanied by an observed structural change in CYP1A2 (Ahn, et al., 1998). Altogether, this particular study illustrated that anionic lipids could effectively change the catalytic potential of CYP1A2 and was not only a function of altered affinity for CPR.

More recently, Sevrioukova and Poulos characterized a citrate-binding site that was located near the F-G loop of CYP3A4 (Sevrioukova and Poulos, 2015). This is an area where anionic membrane phospholipids may interact with the P450. The presence of citrate affects the ability of substrates to bind and produce a low-to-high spin conversion. This effect is substrate-dependent with the testosterone-induced low-to-high spin conversion being significantly enhanced; however, the effect of citrate on the 7-benzyloxy-4-(trifluoromethyl) coumarin-induced conversion was much less prominent. Citrate did not appear to influence the oligomeric state of CYP3A4. CYP3A4 activities and first electron transfer to CYP3A4 were also stimulated by citrate (when CPR was present). However, CPR reduction by NADPH was also stimulated by citrate, making it difficult to parse out the specific effects of citrate on the P450 itself. Taken together, it is plausible that citrate and anionic phospholipids are interacting at a similar region of the P450 molecule.

The role of the F-G loop on association with the membrane was underscored for CYP2J2. Using MD simulations, McDougle *et al.* showed the proposed binding orientation of CYP2J2 to the membrane, suggesting an interaction among several hydrophobic residues in the F-G loop and the membrane. Based on these results, specific F-G loop mutations were generated, replacing hydrophobic Ile-236, and Phe-239 with Asp and His, respectively. When expressed in E. coli, the mutants localized to the cytosolic fraction to a greater extent than Δ34-CYP2J2, consistent with the MD simulations. These results support the role of the F-G loop of CYP2J2 with its interaction with the membrane.

The studies discussed above have led to the consideration that membrane lipid heterogeneity not only can affect the interactions between protein and membrane, but also can affect protein localization through the formation of distinct lipid domains that can modulate the protein interactions. For instance, it was found that CYP3A4 activity was increased as a function of the concentrations of the anionic phospholipids, PS and PA. These results correlated with an increase in membrane insertion of CYP3A4 and it was concluded that these results were due to the ability of anionic phospholipids to form lipid domains that allowed for proper positioning of the proteins within the lipid bilayer (Ahn and Yun, 1998;Kim, et al., 2003). Similarly, CYP2B1 was shown to modulate membrane properties in reconstituted systems by inducing segregation of anionic phospholipids leading to distinct lipid domains. These domains led to alterations in the activity and conformation of CYP2B1 (Kim, et al., 2007).

Until our recent publications (Brignac-Huber, et al., 2011;Park, et al., 2014;Brignac-Huber, et al., 2013;Park, et al., 2015) there were no studies illustrating the effects of such lipid domains in microsomal tissue and their potential to affect P450 function. However, the existence of ER lipid domains has become more widely recognized, and can significantly influence the localization of P450 system proteins.

Lipid Microdomains

Early concepts of lipid bilayer structure were established by the fluid mosaic model (Singer and Nicolson, 1972), which described the bulk of the phospholipids as being organized discontinuously with a small fraction of the lipid specifically interacting with integrally incorporated proteins. It also suggested the possibility of cooperative effects with the membrane and specific ligands. Recent studies have greatly increased our understanding of the organization of the lipid membrane, which have been shown to play fundamental roles in protein-protein and protein-lipid interactions. The long saturated fatty acid chains of sphingolipids facilitates their association with cholesterol to form heterogeneities in the

membrane that are referred to as liquid-ordered (l_o) domains or lipid rafts. The lipids that surround these ordered "raft" regions tend to be more fluid, being enriched in phospholipids containing unsaturated fatty acids. The l_o domains have been characterized by their resistance to solubilization by non-ionic detergents and have been designated as detergent resistant membranes (Simons and van, 1988;Simons and Ikonen, 1997;Diaz-Rohrer, et al., 2014) . Such ordered membrane regions have been implicated in numerous cellular processes, such as, lipid and protein sorting, transmembrane signaling through glycosylphosphatidylinositol-anchored (GPI-anchored) proteins and secretory and endocytic pathways (Brown and London, 1998). These organized domains have been described primarily in the plasma membrane (Brown and London, 1998;Simons and Ikonen, 1997;Pike, 2004;Pike, 2003), Golgi apparatus (Vetrivel, et al., 2004;Rixon, et al., 2004) and mitochondria (Foster, et al., 2003;Bini, et al., 2003). The potential for sphingomyelin- and cholesterol-enriched l_o domains to be found in the ER was thought to be less likely, due to the low concentrations of these constituents in this organelle (Holthuis, et al., 2001;Prinz, 2002). Consequently lipid microdomain formation has only been identified in the ER more recently.

Sevlever et al. first described organized microdomains of the ER, referred to as Triton-insoluble membranes (TIMs), upon studying the GPI-anchor biosynthetic pathway that occurs in the ER (Sevlever, et al., 1999). These domains were isolated by solubilization with 1% Triton X-100 followed by centrifugation using a discontinuous sucrose gradient (Brown and Rose, 1992). Under these conditions, the TIMs would remain intact and float to the 5%/38% interface of the sucrose gradient, whereas the more disordered membrane regions are solubilized. In a study examining the localization of GPI-anchored proteins, ER samples were isolated and shown to be enriched in several the GPI-anchored intermediates as well as the ER marker calnexin, indicating that the GPI-intermediates localized in the ER. When the ER fraction was then subjected to Triton X-100 solubilization/centrifugation, the TIM fractions were shown to be

enriched in sphingomyelin (85%), cholesterol (73%), and some of the GPI intermediates (Sevlever, et al., 1999). These results show that the ER contains sphingomyelin- and cholesterol-enriched ordered domains. Subsequently these investigators demonstrated that ER-TIMs could be oriented to either the cytoplasmic or luminal face, suggesting that this type of domain is not exclusive to one side of the ER bilayer leaflet (Pielsticker, et al., 2005).

Sarnataro et al. found that specialized ER domains may play a protective role in minimizing protein misfolding. They studied the conversion of cellular prion protein (PrP^{c}) into the infectious misfolded protein, scrapie prion protein (PrP^{Sc}), which plays a role in the pathology of transmissible spongiform encephalopathies. Their results showed that disruption of these domains by cholesterol depletion slowed protein maturation, and increased protein misfolding to the infectious scrapie prion. These results led them to conclude that association of PrP^{c} with cholesterol-enriched ER domains allows for correct protein folding (Sarnataro, et al., 2004).

Lastly, Browman et al. characterized two novel proteins, erlin-1 and erlin-2, which were found to reside in lipid-raft-like domains in the ER. These two proteins were found to be new members of the prohibitin family of proteins by the presence of a conserved prohibitin-homology domain of approximately 160 amino acids. Shared properties of these prohibitin family proteins include detergent insolubility, flotation on sucrose gradients, association with membranes, and the ability to form oligomers (Browman, et al., 2006).

The selective association of proteins into specific domains of the ER has become more widely accepted; however, microdomain localization of P450 system proteins has not been extensively examined. Bae et al. performed a large proteomics study of liver tissue and found 37 microsomal proteins including CYP1A2 and CPR to reside in ER-DRMs (Bae, et al., 2004). More recently, our lab has found similar results, in which approximately 70% of CYP1A2 and

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CPR were found in DRM fractions (i.e. ordered regions) of microsomes from rabbits after detergent treatment with 1% Brij 98 and discontinuous sucrose gradient centrifugation (Brignac-Huber, et al., 2011). Lipid analysis of these membrane fractions showed that the composition of the DRM fractions differed from those of the ER lipids, having higher levels of sphingomyelin and cholesterol, and lower levels of PC and PI (Fig. 1). The stability of the DRMs were dependent on cholesterol as seen by disruption of the domains after cholesterol depletion with methyl-β-cyclodextrin (MβC). After cholesterol depletion, both CYP1A2 and CPR were found in the non-DRM fractions of the sucrose gradient suggesting that cholesterol was an integral component of the CYP1A2-containing ordered microdomains. In addition, microsomal substrate metabolism was significantly lower after cholesterol depletion. Interestingly, reincorporating cholesterol back into the microsomal tissue, after depletion, allowed for re-association of CYP1A2 and CPR into the DRM fractions and recovery of microsomal substrate metabolism. Moreover, incorporation of purified CYP1A2 and CPR into lipid vesicles having a lipid composition that mimicked that of the DRM exhibited a lower apparent K_m for the CPR-CYP1A2 complex as compared to when the proteins were incorporated into PC vesicles (Brignac-Huber, et al., 2011). Our studies suggest that the specific lipid composition of ER-DRMs allows for more efficient substrate metabolism by increased binding efficiency between CYP1A2 and CPR.

Other groups also have suggested that organization of P450s into lipid microdomains could influence P450 function. Cardiolipin (CL), which has been shown to be a constituent of lipid raft-like domains in mitochondria (Sorice, et al., 2009), has been implicated as a functional modulator of CYP2E1 and CYP1B1. Recently, Cho et al. reported that the presence of 30 mol% CL in reconstituted systems allowed for approximately a 90% decrease in CYP2E1 mediated reactive oxygen species (ROS) production (Cho, et al., 2008). The presence of CL did not affect CYP2E1 activity or NADPH oxidation, which led the group to propose that the specific interaction of CYP2E1 with CL decreased ROS production although the molecular mechanism

was undetermined. Similarly, CYP1B1's catalytic activity, thermal stability, and membrane binding were affected by the presence of CL (Jang, et al., 2010). In both studies the membrane-spanning region of the P450s was found to be important in these CL-induced effects as illustrated by experiments with the truncated form of the two proteins. Although the CL concentration used in the above studies was much higher than what is found physiologically in the ER, there is increasing evidence that the ER contains enzymes that play a major role in cardiolipin metabolism (Cao, et al., 2004;Zhao, et al., 2009) and may be physiologically relevant in P450 regulation (Jang, et al., 2010). CL is known to exist primarily in the inner mitochondrial membrane, although low levels have been reported in the ER (Baraud and Maurice, 1980). The low levels of CL in the ER likely diminish its potential role with regard to microsomal P450 function; however, the significant concentrations of CL (approximately 14%) in liver mitochondria (Paradies, et al., 1991) may influence the localization and function of P450s that normally reside in the mitochondria, or even microsomal forms that are alternatively targeted to the mitochondria.

Our lab has conducted studies using a variety of fluorescent-labeled probes to determine (1) if the lipid microdomains observed in the endoplasmic reticulum could be detected in reconstituted systems at lipid compositions similar to those found in microsomes, and (2) whether microdomains from reconstituted systems similarly affected localization of P450 system proteins. Utilizing lipid probes, which have been characterized in model membranes as localizing to liquid ordered (l_o) or liquid disordered (l_d) regions of a membrane, we showed that ordered lipid domains do form in reconstituted systems, and that lipid phase separation was seen at lipid compositions similar to those found in liver endoplasmic reticulum. Furthermore, CYP1A2 was found to reside in the l_o regions of these reconstituted systems in a manner analogous to that observed with endoplasmic reticulum (Brignac-Huber, et al., 2013;Brignac-Huber, et al., 2011;Park, et al., 2015;Park, et al., 2014).

More recently, the localization of other P450 enzymes was examined (Park, et al., 2014). In this study, rabbits were treated with either phenobarbital to induce CYP2B4, or pyrazole to induce CYP2E1. CYP2B4 was shown to distribute roughly equally between both the ordered and disordered membrane regions after phenobarbital treatment. In contrast, CYP2E1 was shown to reside predominantly in the disordered regions of microsomes from pyrazole-treated rabbits. CYP1A2 and CPR localization was not affected by either inducer, remaining in the ordered microdomains. Interestingly, CYP1A1 was found to reside in the disordered regions of the ER. Taken together, these results clearly show that P450 enzymes reside in specific regions of the membrane, and that their localization can influence P450 function.

The partitioning of P450 system proteins into different ER microdomains led to questions regarding the presence of amino acid sequences that directed proteins into either ordered or disordered regions. To address this question, we focused on two closely related P450s, CYP1A1 and CYP1A2 that exhibit a high degree of sequence similarity, but localize into different microdomains. When examining the sequences of these proteins, two regions of variability were noted: (1) the early portion of the NH_2-terminus, and (2) an internal region from amino acids 207-306. To test whether these regions were responsible for their differential localization, NH_2-CYP1A2-CYP1A1 and NH_2-CYP1A1-CYP1A2 chimeric proteins were generated (Fig. 2A). CYP1A2, which resides predominantly in the ordered regions of the ER, could be re-localized to the disordered regions by replacement of 100 amino acids of the NH_2-terminus with that of CYP1A1 (Fig. 2B). Conversely, replacement of the NH_2-terminus of CYP1A1 with the first 107 amino acids of CYP1A2 caused its partial re-localization to the ordered regions (Fig. 3).

The ability of the CYP1A2 NH_2-terminus to only partially cause re-localization of CYP1A1 to the ordered domains suggested that other regions of the protein also may participate in governing their microdomain localization. To address this issue, internal segments of CYP1A2

were replaced by the complementary segment of CYP1A1. Although substitution of amino acids 108-205 did not influence the relative amount of CYP1A2 in the ordered microdomains, substitution of amino acids 206-302 caused a dramatic alteration in chimera localization (Fig. 4). Taken together, these results showed that there are at least two regions that participate in the microdomain localization of CYP1A proteins – the NH_2-terminus, and an internal segment spanning amino acids 206-302.

In conclusion, P450 activities are affected by the membrane environment in which they reside. Some lipid-mediated effects result from direct interactions of P450 system proteins with specific phospholipids. These interactions can affect P450 function by promoting protein incorporation into the membrane, affecting the interactions among P450s and their redox partners, and altering the redox potential of the electron donor CPR. However, other effects on P450 function appear to result from the heterogeneity of the ER membrane, which leads to the formation of lipid microdomains. The illustration in Fig. 5 provides a summary of our current knowledge on how the lipid membrane influences the localization of P450 system proteins. The presence of lipid microdomains can lead to the selective clustering of proteins into the same regions, which could augment the interactions among co-localizing proteins. In contrast, segregation of proteins into different domains could lessen these interactions. In considering the interactions between P450s and their redox partners, microdomain co-localization would be expected to decrease the apparent K_D^{CPR}, which could increase substrate metabolism. In contrast, P450 activities would likely be diminished by segregation of P450 and its electron donors. This raises numerous questions regarding the organization of the P450 system proteins. One question is, “If CPR localizes predominantly in the ordered membrane regions how does it reduce P450s that exist in the disordered membranes?” There are several possible explanations. (1) P450s in the disordered regions are reduced by the smaller fraction of CPR found in the l_d membranes. This situation would limit the CPR available to the P450 in this

region and diminish their ability to metabolize substrates. (2) CPR may reside at the interface between the ordered and disordered microdomains, being accessible to P450s in both regions. (3) As lipid microdomains are dynamic in nature, they could constantly be forming and dispersing, leading to the continual constant creation and dissociation of CPR•P450 complexes. (4) There could be factors that govern the migration of proteins in and out of the l_o phases. The most likely agents that may lead to altered protein migration are the presence of substrate and complexes with other proteins. First, substrate may cause a conformational change that alters the K_D of the complex between CPR and a particular P450 enzyme. Therefore, the substrate would lead to a mass-action alteration in protein partitioning between the microdomains, and change protein localization. A second possible mechanism is by alteration of the supramolecular structure of the P450 system protein complex. The ability of P450 enzymes to form both homomeric and heteromeric complexes has been well established (Reed and Backes, 2012;Davydov, 2011). Any factor that affects the size of these complexes can influence their mobility, and possibly their microdomain localization. A better understanding of the relationship between the lipid microenvironment of the ER and the P450 system can only improve our ability to better appreciate how the membrane affects interactions among P450 system proteins, and consequently, drug and xenobiotic metabolism.

Authorship contributions

Wrote or contributed to the writing of the manuscript: LB-H, JWP, JRR and WLB

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Footnotes

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Figure Legends

Figure 1 – Comparison of the Phospholipid Composition of Liver Microsomes with that of the Ordered Microdomains. Rabbit liver microsomes were subjected to solubilization using 1% Brij 98 followed by discontinuous sucrose density gradient centrifugation to isolate the ordered (DRM) regions of the membrane. The phospholipid composition of the ordered region was determined and compared to that of untreated microsomes. (This research was originally reported by Brignac-Huber et al., 2011).

Figure 2 – Replacement of the NH_2-terminal region of CYP1A2 with that of CYP1A1 changes its membrane localization. The microdomain localization CYP1A2 and chimeric proteins where the NH_2-terminus was substituted with that of CYP1A1 was measured by expression of the proteins in HEK293T cells, and partial membrane solubilization using Brij 98. After Brij treatment, the samples were subjected to centrifugation and the proteins remaining in the membranes were detected in the pellet using PAGE. (A) Schematic of the chimeric proteins generated. (B) Immunoblot of the native CYP1A proteins and the chimeras. Proteins in the pellet remain associated with the membrane, whereas those in the supernatant were solubilized. This research was originally published in The Journal of Biological Chemistry. Park, J.W., Reed, J.R., and Backes, W.L. The Localization of Cytochrome P450s CYP1A1 and CYP1A2 into Different Lipid Microdomains is Governed by their NH_2-terminal and Internal Protein Regions. *The Journal of Biological Chemistry*. (2015) **290,** 29449-29460. © the American Society for Biochemistry and Molecular Biology."

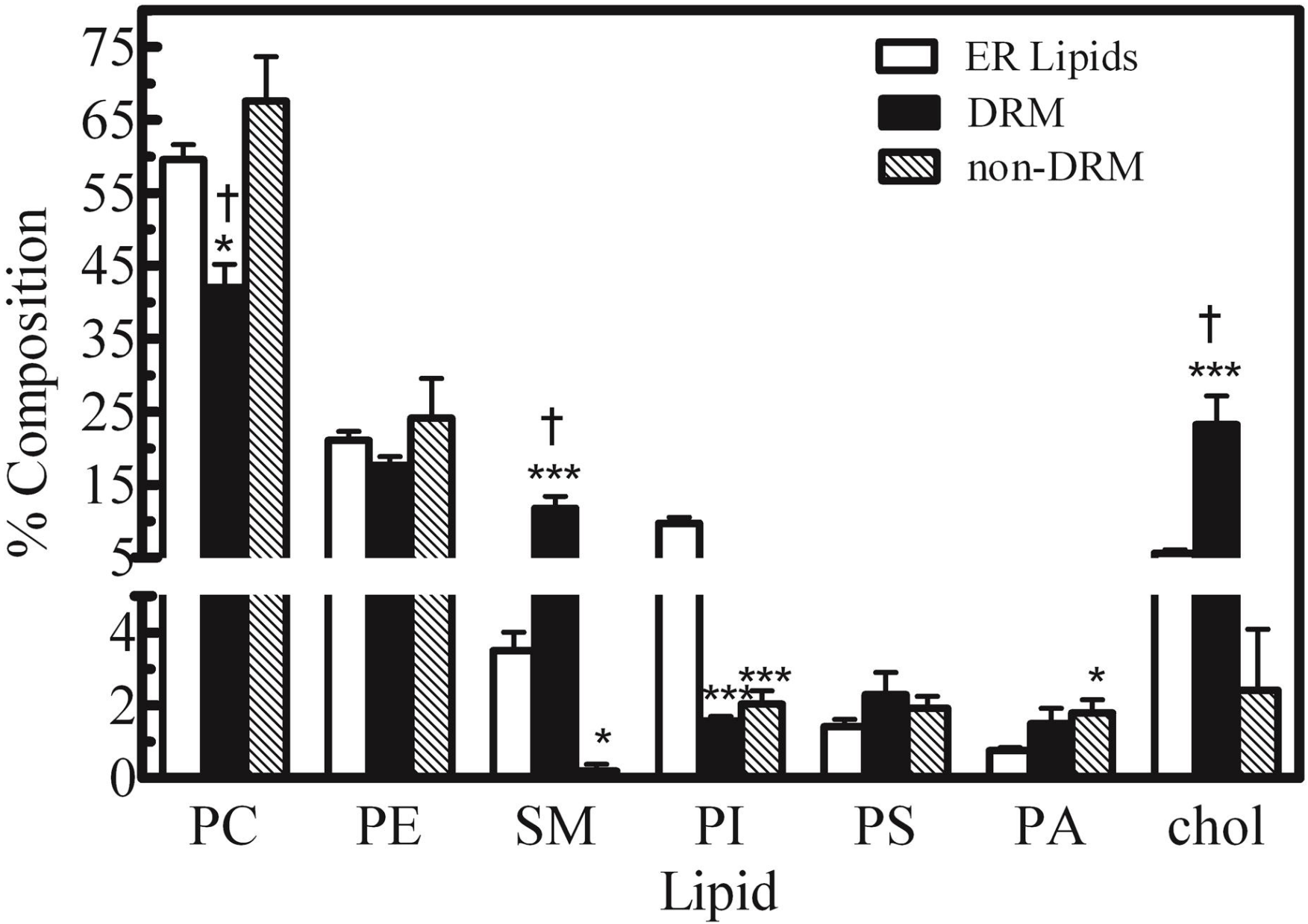
Figure 3 – Replacement of the NH_2-terminal region of CYP1A1 with that of CYP1A2 changes its membrane localization. The microdomain localization CYP1A1 and chimeric proteins where the NH_2-terminus was substituted with that of CYP1A2 was measured as described in the legend to Figure 1. (A) Schematic of the chimeric proteins generated. (B) Immunoblot of the native CYP1A proteins and the chimeras. This research was originally published in The Journal of Biological Chemistry. Park, J.W., Reed, J.R., and Backes, W.L. The Localization of Cytochrome P450s CYP1A1 and CYP1A2 into Different Lipid Microdomains is Governed by their NH_2-terminal and Internal Protein Regions. *The Journal of Biological Chemistry*. (2015) **290,** 29449-29460. © the American Society for Biochemistry and Molecular Biology."

Figure 4 – Importance of the internal regions in directing the microdomain localization of CYP1A proteins. The microdomain localization of chimeric proteins where the internal segment of CYP1A2 was substituted with the corresponding region of CYP1A1 were measured as described in Figure 1. This research was originally published in The Journal of Biological Chemistry. Park, J.W., Reed, J.R., and Backes, W.L. The Localization of Cytochrome P450s CYP1A1 and CYP1A2 into Different Lipid Microdomains is Governed by their NH_2-terminal and Internal Protein Regions. *The Journal of Biological Chemistry*. (2015) **290,** 29449-29460. © the American Society for Biochemistry and Molecular Biology."

Figure 5 – Illustration of P450 system protein localization in the endoplasmic reticulum. Lipid membranes are thought to be segregated into liquid ordered (l_o) and disordered (l_d) domains. The ordered domains (which also are referred to as lipid rafts) tend to be more enriched in cholesterol, and sphingomyelin and tend to pack more tightly and are more resistant to solubilization by detergents (Diaz-Rohrer, et al., 2014). P450 system proteins do not distribute randomly throughout the membrane, but localize in regions of differing phospholipid composition. P450s and CPR have also been reported to be more closely associated with PE and the anionic phospholipids PS and PI. The phospholipids that surround P450 enzymes. The

phospholipid regions with the predominant yellow head groups represent the l_o microdomains with the disordered regions being largely in gray. Cholesterol (aqua) and sphingomyelin (black) are intercalated into the l_o regions. The anionic phospholipids are indicated by the red polar head groups. CYP1A2 and CPR localize predominantly in the l_o regions, whereas CYP1A1 and CYP2E1 are found mainly in the disordered membranes. CYP2B4 distributes between both regions. The figure also illustrates the tendency of different P450 enzymes to form P450•P450 complexes.

Figure 1


ER Lipids
DRM
non-DRM
% Composition
75
65
55
45
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25
15
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4
2
0
*
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PC
PE
SM
PI
PS
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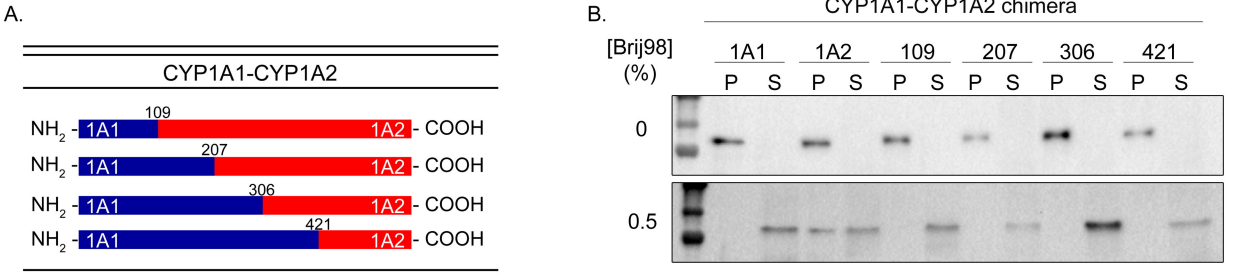
A.
CYP1A1-CYP1A2
109
NH2 - 1A1 1A2 - COOH
207
NH2 - 1A1 1A2 - COOH
306
NH2 - 1A1 1A2 - COOH
421
NH2 - 1A1 1A2 - COOH
B.
CYP1A1-CYP1A2 chimera
[Brij98] (%)
1A1
1A2
109
207
306
421
P S P S P S P S P S P S
0
0.5

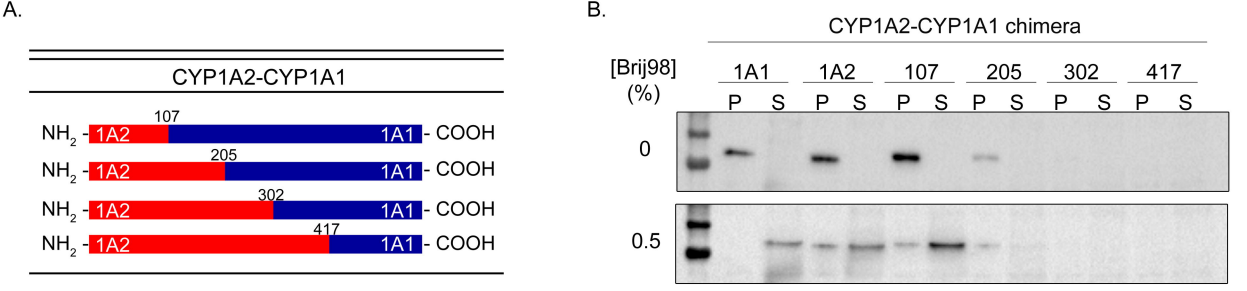
A.
CYP1A2-CYP1A1
NH2 - 1A2 107 1A1 - COOH
NH2 - 1A2 205 1A1 - COOH
NH2 - 1A2 302 1A1 - COOH
NH2 - 1A2 417 1A1 - COOH
B.
CYP1A2-CYP1A1 chimera
[Brij98] (%)
1A1
1A2
107
205
302
417
P S P S P S P S P S P S
0
0.5

Figure 3

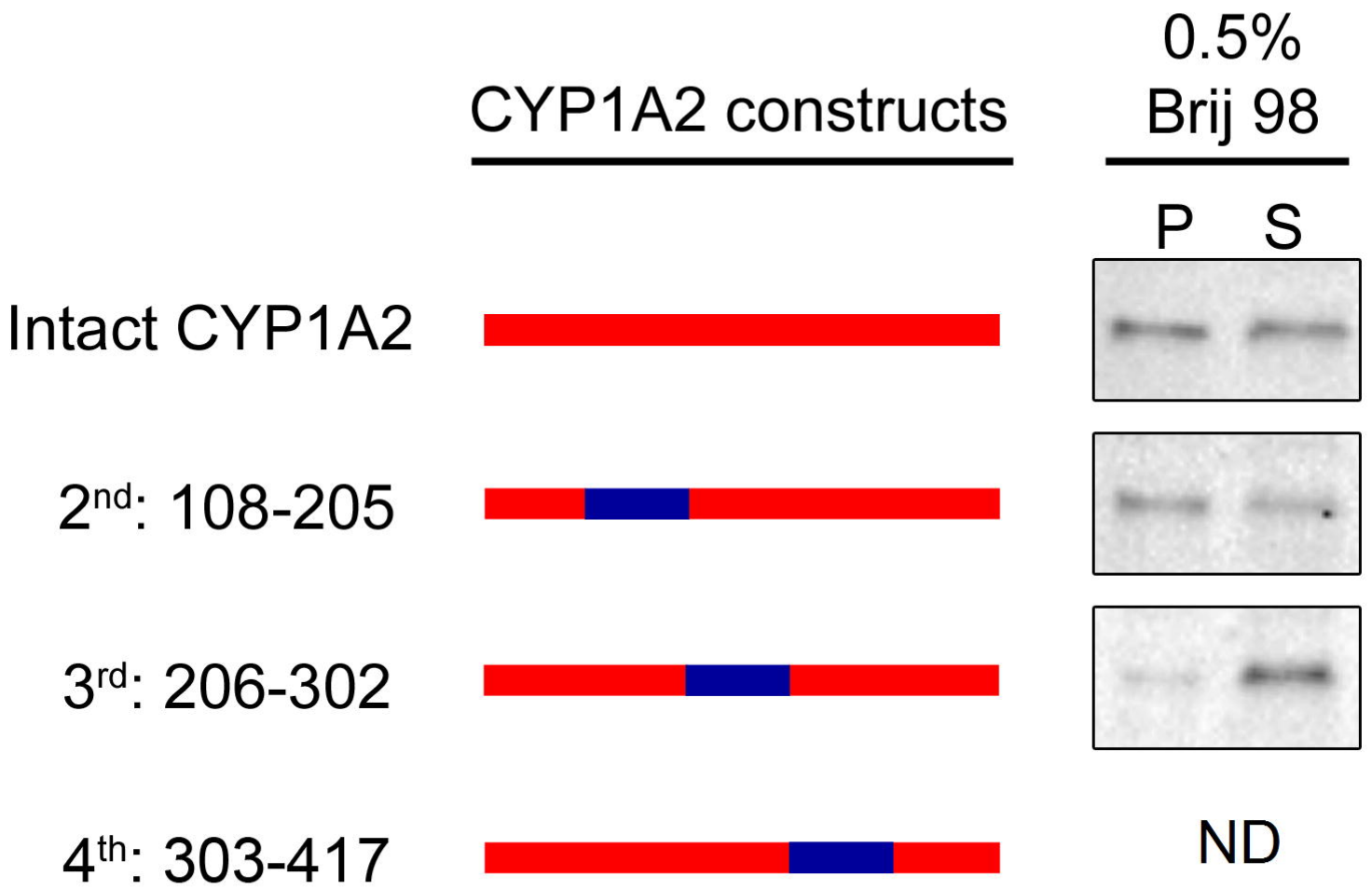
CYP1A2 constructs
0.5%
Brij 98
P
S
Intact CYP1A2
2nd: 108-205
3rd: 206-302
4th: 303-417
ND

Figure 4

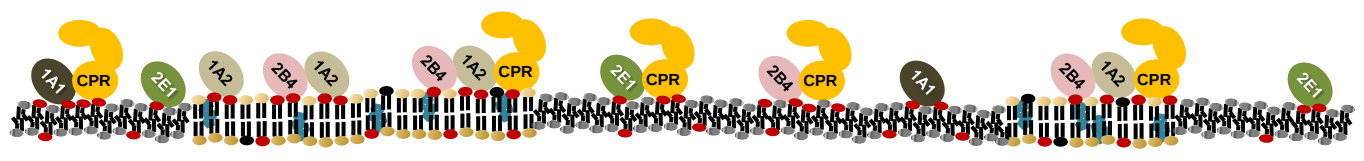
1A1
CPR
2E1
1A2
2B4
1A2
2B4
1A2
CPR
2E1
CPR
2B4
CPR
1A1
2B4
1A2
CPR
2E1

Figure 5