Published in final edited form as:

Expert Opin Drug Metab Toxicol. 2011 May ; 7(5): 543–558. doi:10.1517/17425255.2011.562194.

Microsomal monooxygenase as a multienzyme system: the role of P450-P450 interactions

Dmitri R. Davydov

Skaggs School of Pharmacy and Pharmaceutical Sciences, UCSD, 9500 Gilman Drive, La Jolla, CA 92093

Abstract

Introduction—There is increasing evidence of physical interactions (association) among cytochromes P450 in the membranes of the endoplasmic reticulum. Functional consequences of these interactions are often underestimated.

Areas covered—This article provides a comprehensive overview of available experimental material regarding P450-P450 interactions. Special emphasis is given to the interactions between different P450 species and to the functional consequences of homo- and heterooligomerization.

Expert opinion—Recent advances provide conclusive evidence for a substantial degree of P450 oligomerization in membranes. Interactions between different P450 species resulting in the formation of mixed oligomers with altered activity and substrate specificity have been demonstrated clearly. There are important indications that oligomerization of cytochromes P450 impedes electron flow to a fraction of the P450 population, which render some P450 species non-functional. Functional consequences of P450-P450 interactions make the integrated properties of the microsomal monooxygenase remarkably different from a simple summation of the properties of the individual P450 species. This complexity compromises the predictive power of the current *in vitro* models of drug metabolism and warrants an urgent need for development of new model systems that consider the interactions of multiple P450 species.

Keywords

cytochrome P450; protein-protein interactions; oligomers; P450-P450 interactions; multiple P450 species; allostery; microsomal monooxygenase; multienzyme system

1. Introduction: monooxygenase as a multienzyme system

The central role microsomal cytochromes P450 play in human drug metabolism makes these enzymes a major subject for studies of drug disposition, adverse drug effects and drug-drug interactions. However, these enzymes are not functional alone, but rather constitute a part of the membrane-bound multienzyme system of microsomal monooxygenase (MMO). The major components of MMO, which has also been called “the microsomal electron transfer chain”, are represented by multiple cytochrome P450 species, NADPH-P450 reductase (CPR), and cytochrome *b*₅. A very important characteristic of MMO is a large excess of cytochromes P450 over CPR, the major electron donor. The molar ratio of P450 to CPR in the liver microsomal membranes of rodents has been reported to be around 10:1 – 30:1 [1–

*ddavydov@ucsd.edu, Tel.: +1 (858) 2460271, Fax: +1 (858) 2460089.

Declaration of interest

D Davydov’s research was supported by NIH grant GM054995.

2], or even as high as 40:1[3]. A recently published study with 150 human liver samples shows this ratio to be in the range of 2:1 to 27:1 with the average level of 7.1:1 [4]. The presence of multiple cytochrome P450 species in the same membrane makes the limiting concentration of CPR of remarkable functional importance. Different P450 species must compete for CPR. Furthermore, even though the system consisting of P450 and CPR is fully functional, the complete MMO system also contains cytochrome b_5 , which is believed to exert an allosteric effect on P450 conformation [5–9] and may also serve as an alternative source of electrons or an “electron shuttle” [10].

A consideration of the interactions of multiple P450 enzymes with CPR and b_5 suggests an important difference between the integral properties of MMO and a simple summation of the properties of the individual P450 species. Increasing evidence of P450 oligomerization in the membrane, and, in particular, demonstration of physical interactions between different P450 species [11–14] implies that the composition of the P450 pool in the membrane may affect the properties of the system in a very complex manner.

This article is written in an attempt to approach an understanding of the function of MMO as a multienzyme system. I will present an overview of the experimental data on oligomerization of microsomal cytochromes P450, and on the interactions between different P450 species in particular. The review summarizes the current knowledge of the functional consequences of P450-P450 interactions and demonstrates their critical importance as a determinant of the functional properties of microsomal drug metabolizing system. I also discuss a possible physiological role of P450-P450 interactions as an element of a hypothetical allosteric mechanism that provides a rapid metabolic response to cell exposure to xenobiotics..

2. Evidence of P450 oligomerization in solution and in the membrane

2.1. Aggregation of purified cytochrome P450 in solution

The tendency of microsomal cytochromes P450 to form aggregates in solution is well known. Reported values of the molecular masses of rabbit CYP2B4 or human CYP3A4 in the absence of detergent (300–700 kDa) correspond to oligomers of 5 – 15 protein molecules [15–21]. The molecular masses of CYP1A2 oligomers are even larger and vary from 300 to several thousand kDa [15,22–23]. No monomeric states of microsomal cytochromes P450 were detected in solution in either sedimentation experiments [15,17,19–20,22], size exclusion chromatography [16,24–25], dynamic light scattering [23–24] or FRET studies [26].

The interaction of the hydrophobic loci at the surface of the membranous cytochromes P450 is an essential component in the mechanisms of their aggregation [15,27–28]. The role of hydrophobic interactions is revealed in the dissociation of the oligomers by detergents [18,21,24–25,28–31]. Sedimentation experiments showed complete dissociation of CYP2B4 aggregates in the presence of $\geq 0.2\%$ non-ionic detergents, such as Emulgen-913 [30,32].

Co-incubation of two different species of microsomal cytochromes P450 in solution in the absence [26,33] or at low concentrations [26] of detergents may result in their heteroassociation with the formation of mixed aggregates (heterooligomers). Thus, association between human CYP2C9 and CYP3A4 has been demonstrated in the coimmunoprecipitation experiments of Subramanian and Tracy [33]. The association of these proteins in solution apparently involves the interaction of their N-terminal domains, since the substitution of either of the two proteins with their N-terminally truncated variant prevented coprecipitation [33].

Interactions between CYP2B4 and CYP1A2 have been studied with FRET using CYP1A2 labeled with N-(1-pyrenyl)maleimide (PM) and CYP2B4 labeled with 7-ethylamino-3-(4'-maleimidilphenyl)-4-methylcoumarin maleimide (CPM) [26]. Co-incubation of the labeled proteins in solution resulted in formation of mixed oligomers revealed in the slow appearance of FRET from PM to CPM, which took several hours to reach completion [26]. Introduction of a low concentration of non-ionic detergent (0.05% of Triton N-101) did not prevent the heteroassociation but considerably increased the rate of subunit exchange [26].

The slow rate of subunit exchange in the absence of detergent simplifies studies of the effect of heteroassociation on the properties of the interacting enzymes, as the progress of heteroassociation may be easily followed in time. A remarkable effect of heteroassociation was demonstrated in the study of pressure-induced transitions in heterooligomers of CYP1A2 and CYP2B4 [34]. When taken separately, these enzymes reveal quite distinct behavior at increasing hydrostatic pressure. While the CYP2B4(Fe²⁺)-CO undergoes a P450→P420 transition at rather low pressures, the CYP1A2(Fe²⁺)-CO is extremely resistant to such inactivation. However, interaction with CYP1A2 renders the behavior of CYP2B4 similar to that of CYP1A2. After several hours of co-incubation with CYP1A2, CYP2B4 becomes protected from pressure-induced inactivation and reveals attenuated compressibility of the heme pocket [34]. A pronounced effect of the interactions between CYP1A2 and CYP2B4 on the conformation of the latter suggests an important specificity in the mechanism of interaction between these enzymes [34].

The major role in P450 aggregation is attributed to the N-terminal membrane anchors [28,35–38]. However, although the truncation of the N-terminal sequence of membranous P450s decreases their aggregation, it is not usually sufficient to produce soluble monomeric enzymes [23,28–29,35–40]. Both association of cytochromes P450 with the membranes and their aggregation in solution apparently involve some hydrophobic regions other than the N-terminal sequence [28,36,38,41]. The most probable candidate for the second site of hydrophobic interactions is the region of F/G loop and its proximity [40,42–44].

The selective effect of CYP1A2-CYP2B4 interactions on the properties of CYP2B4 [34] suggests that the association of cytochromes P450 in solution could not be relegated to unspecific aggregation of their hydrophobic segments. Involvement of other components, such as electrostatic interactions, is implied from the fact that the monomerization of engineered N-terminally truncated cytochromes P450 usually requires high salt concentrations [39,45–46].

A complex mechanism of interactions with participation of several different interfacing regions is consistent with the electronic microscopy of CYP2B4 [19] or CYP1A2 [22], which demonstrates that the predominant state of both proteins in solution is hexamer organized as a double-layered dimer of planar trimers. This organization suggests a difference between the interfacing regions within the trimers and the regions involved in the association of the trimers into a double-layered structure. The most plausible explanation is that the interface between the two layers is formed by the membrane-spanning regions, whereas the lateral contacts in trimers may be formed by the extramembrane segments.

2.2. Studies of P450 oligomerization in the membranes with optical techniques

The first indications of oligomerization of membrane-bound microsomal cytochromes P450 came from the measurements of their rotational mobility, which were extensively used in the studies with various P450s in microsomal, mitochondrial and model membranes [47–56].

The studies of rotational mobility of cytochromes P450 differ in the physical principle of the measurements. The laboratories of Cherry, Richter, Kawato studied the decay of absorbance

anisotropy of ferrous P450 after flash photolysis of its carbonyl complexes with polarized light [47–49,53,57–68]. Depolarization of delayed fluorescence of diiodofluoresceine-labeled cytochromes P450 was used in the studies of Stier and co-authors [51,69–72], while saturation-transfer EPR spectroscopy was employed by Schwarz and co-authors [54–56].

Results obtained with different methods are in reasonable agreement with each other. The values of the rotational relaxation time (ϕ_1) of membrane-bound cytochrome CYP2B4 incorporated into liposomes vary from 50 to 200 μ s [51,55,68] depending on protein concentration in the membrane and other conditions (lipid composition, ionic strength, presence of other proteins, etc.). According to theoretical calculations [51,55], even the shortest relaxation times obtained in these measurement are considerably longer than those expected for the enzyme monomer. Calculations suggest that the rotating species correspond to P450 hexamers [51,55]. It should be noted, however, that the accuracy of these calculations is compromised by uncertainty in the estimates of the viscosity of the membrane lipid, degree of penetration of the protein into the membrane, etc.

An important observation is a strict dependence of ϕ_1 on the concentration (surface density) of P450 in the membrane. An increase in ϕ_1 with increasing concentration of P450 in the membrane suggests a concentration-dependent oligomerization of the protein [68]. Additional evidence of P450 oligomerization is the finding that only a fraction of the heme protein in the membrane is mobile, while rotation of the rest of the enzyme is too slow to be detected [47,51,57,68]. The fraction of the immobile population, which is apparently represented by large oligomers, depends on the protein concentration. For instance, in CYP2B4 incorporated into proteoliposomes, the fraction of mobile protein increases from 35% at lipid:protein (L/P) ratio of 1 w/w (~65:1 mol/mol) to 100% at L/P=30 w/w (~2000:1 mol/mol) [68].

The studies of the effect of various factors on the rotational mobility of cytochromes P450 in microsomes and in model membranes provide important information on possible functional significance of P450-P450 interactions. Oligomerization of microsomal and mitochondrial cytochromes P450 in the membrane was shown to be decreased upon their interactions with CPR [49,58,61] or cytochrome b_5 [58], as well as upon the reduction of P450 [51]. In contrast, interactions of P450 with substrates [51,70] as well as peroxidation of the membrane lipids [50,64] retard the rotation, which is indicative of increased protein aggregation.

In some cases the measurements of rotational diffusion in P450-containing liposomes were complemented with freeze-fracture electron microscopy that allowed detection of intermembrane particles (IMP) of 50 Å or larger [55], which presumably represent the oligomers of the heme protein [55,57]. The formation of IMP increased at high surface density of the protein and this was paralleled with an increase in the rotationally immobile fraction [57].

In general, although the interpretation of the values of rotational relaxation time (ϕ_1) in terms of the size of P450 “rotamers” requires precaution, the dependence of the rate of P450 rotation (ϕ_1) and the fraction of immobile P450 on the surface density of P450 undoubtedly demonstrates a high degree of oligomerization of microsomal cytochromes P450 in microsomal membranes [51,57,68–70].

Another inventive optical technique was used by Ramsden and co-authors, who probed the kinetics of interactions of purified CYP1A2 and CYP2B4 with a lipid bilayer immobilized at the surface of a waveguide with an incorporated diffraction grating [73]. This design allowed the authors to monitor protein incorporation into the membrane by measuring the changes in the refractive index. The kinetics of protein absorption reveal self-association of

both proteins in the lipid bilayer, although in the case of CYP1A2 the effect of oligomerization was observable at considerably lower protein concentrations than in the case of CYP2B4 [73]. These results indicate that, although both proteins appear to form oligomers in the membrane, CYP1A2 has considerably higher tendency to self-associate than does CYP2B4.

2.3. Cross-linking experiments

There are several studies of P450-P450 interactions in membranes that employ chemical cross-linking with bifunctional reagents. In the study of Baskin and Yang [74] cross-linking of proteins in rabbit liver microsomes followed by SDS-PAGE and immunoblotting revealed the aggregates ranging from 2 to 4–5 cross-linked P450 molecules.

Myasoedova and co-authors studied chemical cross-linking of CYP2B4 [75] or CYP1A2 [76] incorporated into proteoliposomes. Interestingly, the patterns of cross-linked species obtained with CYP2B4 and CYP1A2 were markedly similar [76]. In both cases SDS electrophoresis revealed two major bands of the cross-linked protein, which were identified as two forms of the dimer with slightly different mobility. In addition, the study also detected several less pronounced bands of cross-linked aggregates with molecular weights ranging from 150 to 330 kDa.

A meticulous study of self-association of membrane-incorporated CYP2C8 has been recently presented by Hu, Johnson and Kemper. The studies were carried out with the membranous preparations of *E. coli* or the microsomes of AD-293 cells expressing human CYP2C8 or its mutants. The authors cross-linked the sulfhydryl groups of the protein using copper-phenanthroline or bis-maleimidoethane. It was shown that the initial phase of the cross-linking yields dimers specifically cross-linked at residue Cys-24, which is located in the linker between the trans-membrane N-terminal sequence and the catalytic domain. Cross-linking was prevented by substitution of Cys-24 with serine, but rescued in the mutants, where new cysteine residues were introduced into the FG-loop of the catalytic domain [77].

Cross-linking and coimmunoprecipitation techniques have also been used to probe the formation of mixed oligomers of different P450 species. In the study of P450-P450 interactions in rat liver microsomes, Alston and co-authors used protein cross-linking followed by immunopurification and immunoblotting [14]. Detection of cross-linked complexes of CYP1A1 with CYP3A2 in these experiments suggests that the two enzymes interact in the membrane of ER.

In a recent study on the interactions between CYP2B4 and CYP1A2 in the membrane of proteoliposomes, Backes and co-authors cross-linked the oligomers of CYP1A2 and CYP2B4 [11]. Cross-linking followed by immunoblotting revealed the formation of high molecular weight homo-oligomers of either protein studied separately, as well as the hetero-oligomers of CYP2B4 and CYP1A2, when the two proteins were co-incorporated into the same membrane [11].

2.4. Fluorescence-based techniques and other biophysical approaches applied to model membranes

Oligomerization of human CYP3A4 in the membrane of proteoliposomes has been recently probed with a FRET-based technique employing a cysteine-depleted mutant (CYP3A4(C468)) labeled at Cys-468 with BODIPY-FL iodoacetamide [78]. The evanescence of FRET from BODIPY to the heme groups of the neighboring subunits were used to monitor the dissociation of CYP3A4 oligomers upon their incorporation into the lipid bilayer. The increase in the lifetime and augmentation of the intensity of BODIPY

fluorescence that signifies the monomerization were considerably more pronounced at a high lipid:protein molar ratio (L/P ratio) where the ultimate concentration of the heme protein in the lipid bilayer was low. In this study the dependence of the amplitude of the observed effect on the L/P ratio was used to estimate the dissociation constant of CYP3A4 oligomers in the membrane.

It is worthy of note in this context that the conventional per-unit-volume concentration is inapplicable to protein-protein interactions in the membrane because of the bi-dimensional nature of the lateral diffusion. In this case the units of surface density (number of molecules or moles of protein per surface area) should be used in preference to the units of volumetrical concentration [79–80]. Although the experimental determination of the bi-dimensional concentration of proteins in the membranes is problematic, the surface density may be approximately estimated from the L/P ratio using known estimates of the area of bilayer per one phospholipid molecule and the footprint area of the protein [78,80–81]. The dissociation constant of CYP3A4(C468) oligomers found in the above experiments and expressed in the units of surface density is equal to 1.4 pmol/cm², which is approximately equivalent to the L/P ratio of 300 mol/mol.

A very recent study from the same laboratory used luminescence energy transfer (LRET) from erythrosine isothiocyanate (ErIA) covalently bound to the wild type protein to a near-infrared label DY-731 maleimide attached to Cys-468 of CYP3A4(C468) mutant [81]. The time course of incorporation of CYP3A4(C468)DY-731 into the liposomes containing CYP3A4-ErIA was monitored by LRET. The value of K_D for CYP3A4 oligomers determined from the dependence of the LRET amplitude on the surface density of the protein was found to be around 1.5–2 pmol/cm². The K_D values determined in these experiments are consistent with an important degree of P450 oligomerization in the ER membrane of hepatocytes where the surface density of cytochromes P450 has been reported to be in the range of 0.6 – 2.8 pmol/cm² [82].

2.5 Studies of P450-P450 interactions in living cells

FRET and bimolecular fluorescence complementation analysis (BiFC) using the chimeras of P450 with fluorescent proteins or their fragments were recently employed to demonstrate P450-P450 interactions in the living cells. The laboratory of Byron Kemper used these techniques to study oligomerization of rabbit cytochromes P450 2C2 (CYP2C2) and 2E1 (CYP2E1). The use of chimeric constructs, where a fluorescent protein is attached at the C-terminus of the P450 molecule, allowed these authors to demonstrate the formation of the homo-oligomers of CYP2C2 in the ER membranes of COS cells [83]. At the same time, this approach revealed neither self-association of CYP2E1 nor its association with CYP2C2.

Another study from this laboratory employs bimolecular fluorescence complementation analysis (BiFC), where the interaction of two proteins is monitored by reconstitution of a protein fluorophore from its fragments attached to the interacting proteins. Similar to the FRET study, BiFC demonstrated homo-oligomerization of CYP2C2, but failed to detect CYP2E1 oligomers. In this case, however, some degree of the heterooligomerization of CYP2C2 and CYP2E1 was also detected [13]. These studies also demonstrated a pivotal role of the N-terminal transmembrane fragment of CYP2C2 in P450-P450 interactions [13].

FRET experiments have been used by Praporski and co-authors to study the oligomerization of human steroid-metabolizing P450 enzymes, aromatase (P450arom, CYP19) and cholesterol 17 α -hydroxylase (P450c17, CYP17). These studies demonstrate formation of the homooligomers of either P450arom or P450c17 in the ER membranes of living HEK293 cells expressing chimeras of these enzymes with YFP and CFP. However, the authors did not detect any heteroassociation between the two enzymes [12]. Atomic force microscopy

(AFM) also confirmed the formation of homodimers of either protein in the model membranes [12].

3. Functional consequences of oligomerization

3.1 Oligomerization as a cause of “persistent conformational heterogeneity” in microsomal cytochromes P450

There are numerous observations demonstrating a crucial effect of oligomerization of microsomal P450s on their functional properties. One of the most prominent consequences of oligomerization is given by “persistent heterogeneity” or apparent divergence of a pool of a single P450 species into non-interconverting and functionally separate fractions.

One of the first observations of this kind concerns the kinetics of reduction of purified CYP2B4 by sodium dithionite. The process obeys a bi-exponential kinetics with 60–70% of the heme protein reduced in the fast phase either in a solution of purified enzyme or in CYP2B4-containing proteoliposomes [31,84]. This distribution of the phases showed no dependence on the temperature [84] or addition of substrate (benzphetamine) and was similar to that observed in rat or rabbit liver microsomes [31]. The study of the temperature dependence of the reduction kinetics shows that the two phases are characterized by similar values of the enthalpy change (ΔH); the difference in the rate of reduction is due solely to the difference in the ΔS [31]. This observation is consistent with the major role of the accessibility of the heme pocket for the reducing agent ($\text{SO}_2^{\bullet-}$) as the rate-determining factor in the reduction. The fractions of the heme protein reduced in the slow- and the fast-phases are likely to differ in the accessibility of the heme pocket for dithionite anion-monomer.

Remarkably, the kinetics of reduction becomes monophasic upon dissolution of the enzyme oligomers, proteoliposomes or microsomes by detergent. It was hypothesized that the difference in the accessibility of the active site between the fast- and slow-reducible fractions is caused by a characteristic architecture of the oligomer that results in different conformation and/or orientation of the constituting subunits [31]. Subunit heterogeneity in CYP2B4 oligomers also explains the effect of monomerization of P450 on the kinetics of CO-recombination with P450(Fe^{2+}) [85], and reduction with eosin radical [86].

Oligomerization-related conformational heterogeneity was also demonstrated in the study of dithionite-dependent reduction of human CYP3A4 [20]. The kinetics of reduction in solution and in proteoliposomes with high surface density of CYP3A4 obeys a three-exponential equation. Notably, the fraction reduced in the fast phase is almost completely low-spin, while the high-spin state heavily predominates in the fraction reduced in the slowest phase. Similar to what is observed with CYP2B4, addition of a non-ionic detergent eliminates the enzyme oligomers and renders the kinetics monophasic. Conformational heterogeneity is also eliminated upon monomerization of CYP3A4 by its incorporation into lipoprotein Nanodiscs or into liposomes with high L/P ratio [20].

A sharp contrast in the kinetic behavior between the high- and low-spin states is difficult to explain in view of the rapid rate of P450 spin transitions [87–90], i.e. the high- and low-spin states must exist in rapid equilibrium during the reduction. This contradiction suggests that the spin equilibrium in the oligomers cannot be considered as being applied to the whole pool of the heme protein. The enzyme is rather distributed between sub-populations that do not interconvert, each characterized by a specific position of spin equilibrium.

Despite the parallels between the rate of the dithionite-dependent reduction and the spin state, the rate of reduction does not seem to be directly dictated by the spin state. The differences in the rate of reduction on one hand and the position of the spin equilibrium on

the other appear to reveal the differences between the P450 subpopulations in the solvent accessibility and the degree of hydration of the heme pocket. If the reduction rate is determined by the openness of the active site for $\text{SO}_2^{\cdot-}$ (and, thereafter, to the solvent), the spin state is largely determined by the heme pocket hydration [91–95]. The parallels between the spin state and the rate of dithionite-dependent reduction are therefore limited to the degree of interconnection between these two structural characteristics.

Differences in the kinetic behavior between the high- and low-spin fractions have also been observed in NADPH-dependent reduction. In contrast to the dithionite-dependent process, where the fast phase is characteristic by the preferential reduction of the low-spin state, the fast phase of the NADPH-dependent reduction is preferential for the high-spin heme protein. Selective reduction of the high-spin P450 in the fast phase of the NADPH-dependent process has been demonstrated with CYP2C11 [96], CYP2B4 [80], and most recently with CYP3A4 [78,97].

The difference between the dithionite-dependent and NADPH-dependent reduction is explainable by different rate-limiting steps in the two reduction processes. In contrast to dithionite-dependent reduction, where the rate is determined by the heme pocket accessibility, the rate of the NADPH-dependent process, is rather dictated by the rate of the electron transfer within the complexes of P450 with the flavoprotein [78,97]. Connection between this rate and the position of spin equilibrium is determined by the differences between the high- and the low-spin states in the redox potential and the reorganization energy associated with the reduction [98–101].

The relevance of the observations of kinetic heterogeneity in the reduction processes to conformational heterogeneity in P450 oligomers was addressed in the studies of the kinetics of electron transfer in the complexes of CYP3A4 with the flavin domain of bacterial P450BM-3 (BMR), which was used as a soluble substitute for the membrane-bound CPR [78,97]. The overall reducibility of oligomeric CYP3A4 in solution did not exceed 50% [97]. Similar incomplete reducibility was observed in the proteoliposomes with high surface density of the enzyme [78]. Notably, the reducibility of the high-spin fraction in both systems was always complete. Therefore, in contrast to the reducible fraction of the enzyme, which reveals substrate-dependent equilibrium between the high- and the low-spin states, the non-reducible fraction is represented by the low-spin heme protein only [78,97]. At the same time, monomerization of CYP3A4 by its incorporation into Nanodiscs or into the liposomes with low surface density of the heme protein renders the reducibility virtually complete [78]. An increase in reducibility of CYP3A4 in the liposomes with a high lipid-to-protein molar ratio (L/P ratio) was paralleled with a decrease in oligomerization of the enzyme evidenced by a FRET-based assay [78].

Further insight into the structural and functional consequences of P450 oligomerization may be derived from the studies of the pressure-induced transitions of microsomal cytochromes P450. The studies of pressure-induced inactivation of CYP2B4 demonstrated that only 65–70% of this oligomeric protein in solution is susceptible to a pressure-induced $\text{P450} \rightarrow \text{P420}$ transition [32,102–103]. At the same time, only the remaining 30–35% pressure-resistant fraction of the enzyme appears to be involved in pressure-sensitive spin equilibrium [32,103]. Since this heterogeneity disappears upon monomerization [32,102,104–105], it likely reflects an intrinsic property of the oligomers. Similar behavior was also demonstrated for human CYP3A4 in solution and in recombinant yeast microsomes [104]. Heterogeneity in the protein response to increasing hydrostatic pressure was also observed in pressure-perturbation studies with CYP2E1 [106] and mitochondrial P450_{scc} [93].

Overall, the oligomerization of microsomal cytochromes P450, such as CYP2B4 and CYP3A4, appears to give rise to their functional heterogeneity both in solution and in the membranes. This divergence of otherwise homogenous enzyme into two fractions with different spin states, accessibility of the heme pocket, ability to interact with substrates, sensitivity to pressure-induced inactivation and ability to form productive complexes with CPR, may reflect an important difference in the orientation and/or conformation of the subunits constituting the oligomer.

3.2. Mutual effects of two different cytochrome P450 species in mixed systems

As discussed above, the microsomal cytochromes P450 present in the membrane of ER are in high excess over the reductase. The competition of different P450s for interactions with CPR was demonstrated in microsomal membranes and in reconstituted systems containing rabbit CYP1A2 and CYP2B4 [107], human CYP2A6 and CYP2E1 [83], human CYP2D6 and CYP3A4 [108], and some other pairs of P450 species [108–110]. However, the observations of a decrease in activity of one P450 species in the presence of another often reveal relationships, which could not be fully explained in terms of a simple competition. In some cases such inhibition was observed even at equimolar or excessive concentrations of CPR in the membrane [33,111–112] where competition is negligible. Furthermore, such inhibition was observed only for some of the probed pairs of the P450 species [109]. For instance, human CYP3A4 exhibits no decrease in the rate of testosterone 6 β -hydroxylation in the presence of CYP2C10, CYP2D6, or CYP2E1 [109].

There are also numerous observations of an activation effect of one P450 species on the other, as well as the instances of asymmetric effects of two different P450 species, where one species is considerably more affected than the other. These effects cannot be accounted for by a simple competition; they may rather be explained by an involvement of direct physical interactions of the two P450 species. In particular, a considerable increase in the rate of the testosterone hydroxylation by CYP3A4 was observed in the presence of CYP1A2 [109]. Likewise, incorporation of CYP2C19 into a reconstituted system with CYP2C9 activates diclofenac 4'-hydroxylase activity of the latter, while the metabolism of methoxychlor and S-mephenytoin by CYP2C19 was inhibited in the presence of CYP2C9 [112].

Analyzing mutual effects of human CYP2C9 and CYP2D6 in a micellar reconstituted system, Tracy and co-authors found that addition of CYP2D6 inhibited CYP2C9-mediated S-flurbiprofen metabolism, even at high molar excess of CPR [111]. At the same time, CYP2C9 had no effect on CYP2D6-mediated dextromethorphan O-demethylation. This asymmetry suggests that CYP2C9 and CYP2D6 form mixed oligomers with altered catalytic properties of both enzymes.

Asymmetric reciprocal effects of the two P450 species were also observed in a CYP3A4-CYP2C9 pair [33]. While CYP2C9-mediated metabolism of S-naproxen and S-flurbiprofen was inhibited in the presence of CYP3A4, CYP2C9 had no effect on the CYP3A4-mediated metabolism of testosterone. These results, together with the results of co-immunoprecipitation experiments (see section 2.2), allowed the authors to postulate the formation of functionally important complexes between CYP2C9 and CYP3A4.

The most extensively studied P450-P450 interactions are those between rabbit liver microsomal CYP1A2 and CYP2B4 [11,26,34,107,113–116]. The presence of CYP2B4 in mixed reconstituted systems with CYP1A2 boosts 7-ethoxyresorufin O-deethylation (EROD), a CYP1A2-specific reaction [26,114]. In contrast, while showing a modest activation of CYP2B4-dependent N-demethylation of benzphetamine, CYP1A2 exerted a

profound inhibition of 7-pentoxoresorufin O-dealkylation (PROD) catalyzed by CYP2B4 [113].

Backes and co-authors explored the reciprocal effects of CYP1A2 and CYP2B4 in more detail and extended their studies from a micellar reconstituted system [114–116] to liver microsomes [107] and proteoliposomes [11]. They found that the activation of CYP1A2-dependent EROD by CYP2B4 is most pronounced at sub-saturating concentrations of CPR [114] while a simple additive effect is observed at excess CPR [114]. Furthermore, the study of the effect of ionic strength showed that the inhibition of CYP2B4-dependent PROD is better pronounced at low ionic strength and considerably diminished at high salt concentration [115]. Similar relationships were demonstrated between CYP1A2 and the rabbit liver cytochrome P450 2E1 (CYP2E1), which also intensifies CYP1A2-dependent EROD in the mixed reconstituted system. Similar to the case with CYP2B4, this effect was most pronounced at sub-saturating concentrations of CPR and low ionic strength [116]. The authors therefore conclude that the effects of CYP2B4 and CYP2E1 on CYP1A2-dependent EROD are exerted through the formation of the complexes of CYP1A2 with either CYP2B4 or CYP2E1, where electrostatic interactions between the proteins play an important role. In these complexes the interactions of CYP1A2 with CPR are promoted, while the CPR binding to its P450 counterpart is inhibited [11,114,117].

Compatible conclusions were made by Davydov and co-authors from their studies with the same pair of the P450 species [26]. These studies performed with a soluble reconstituted system in the presence of low concentrations of detergent also revealed a pronounced activation of CYP1A2-dependent EROD by CYP2B4, although no inhibition of CYP2B4-dependent PROD by CYP1A2 was detected [26]. The authors also studied the mutual effects of CYP1A2 and CYP2B4 on their interactions with CPR monitored by FRET. It was shown that CPR has similar affinities to either CYP1A2 or CYP2B4 alone ($K_D \approx 0.04 \mu\text{M}$). However, titration of the reductase with the mixtures of CYP1A2 and CYP2B4 in the presence of 7-ethoxyresorufin (7-ER) revealed a dependence of K_D on the CYP1A2:CYP2B4 ratio described by an asymmetric bell-shaped curve with the maximum at 4–5-fold molar excess of CYP2B4 [26]. This result allowed the authors to conclude that the association of CYP2B4 with CYP1A2 in the presence of 7-ER “hides” CYP2B4 (but not CYP1A2) from the interactions with CPR, so that the reductase interacts with CYP1A2 selectively [26].

4. Apparent mechanistic grounds and physiological role of the functional effects of P450-P450 interactions

4.1 Conformational heterogeneity in P450 oligomers: implications to the mutual effects of two P450 enzymes

Integrating the observations of functional interactions in pairs of P450 species reviewed above, in most cases one of the interacting species is inhibited by P450-P450 interactions, even at very high concentrations of CPR, while the response of the second enzyme ranges from virtually no effect to a considerable activation that increases at subsaturating reductase concentrations. This finding, combined with the observations of conformational heterogeneity and incomplete reducibility of microsomal cytochromes P450 (see Section 3.1), may be explained by an apparent shielding of some of the P450 subunits in the oligomer from the formation of productive complexes with CPR. According to the above discussion (Section 3.1), only a portion of the enzyme in homooligomers appears to be active since the productive interactions with the reductase (and, presumably, the substrate binding as well) in some subunits are hindered. Therefore, we hypothesize that due to oligomerization an important part of the P450 population in the membrane does not

participate in catalysis (*i.e.*, inactive) due to lack of ability of some of the subunits in the oligomer (“obstructed” subunits) to interact with substrates and to accept the electrons from redox partners.

In the case of the heterooligomers between two different P450 species, the distribution of the two enzymes between the “active” and “obstructed” populations may be uneven, so that one of the two enzymes (like CYP1A2 in the CYP2B4/CYP1A2 pair [26]) would preferentially occupy the active positions, while the second enzyme is more likely to take the inactive, “obstructed” positions. The resulting increase in the active population of the “preferred” enzyme in the heterooligomer causes its activation, while a decrease in the activity of the second P450 species results from a shrinkage of its active fraction. The degree of activation and inhibition is dependent on how strong the preference of the activated enzyme is for the active positions and how extensive the “shielding” is of the enzyme in “obstructed” positions.

The asymmetry of the distribution of the enzymes between the two types of positions in the oligomer may be caused by some inherent structural features of the “preferred” enzyme. Alternatively, it may be induced by P450 interactions with substrates, so that the distribution of the two enzymes between “active” and “obstructed” positions would depend on the presence of their specific substrates. This case is illustrated in Fig 1. The most likely situation, however, is when both possibilities are combined. In the absence of any substrates the distribution of the enzymes between the two types of positions may already be uneven, so that one of the enzymes preferentially occupies the active positions. Addition of its specific substrate may strengthen this preference, while addition of the substrate for the second enzyme may weaken or even reverse it, so that the “non-preferred” enzyme becomes activated. In this case a mutual activation of both enzymes might be observed when appropriate substrates are chosen. This situation may take place in the CYP2B4/CYP1A2 pair with 7-ER and benzphetamine as substrates – while the presence of CYP1A2 increases the activity of CYP2B4 with benzphetamine [113], addition of CYP2B4 increases CYP1A2-dependent EROD [114].

4.2. Possible structural basis of heterogeneity: what do we know about the architecture the oligomers?

The discussion above suggests that the interactions between several cytochrome P450 molecules brings forth an orientational or conformational difference between subunits. What then is the structural basis of this asymmetry in the oligomers’ architecture? Unfortunately, our current knowledge of the molecular mechanisms of P450-P450 interactions is very limited.

Several observations were interpreted as indications of the involvement of the N-terminal transmembrane anchors of the P450s in P450-P450 interactions in the membrane [33, Ozalp, #511, 77]. Indeed, the crosslinking of two CYP2C8 molecules at Cys-24 residue demonstrated in the study of Hu, Johnson and Kemper [77] provides convincing evidence of the closeness of the linkers between the catalytic and membrane-spanning segment in CYP2C8 oligomer. However, this does not necessary imply the role of the membrane-bound N-terminal anchors in the P450-P450 interactions.

Similarly, the homo-oligomerization of the N-terminal CYP2C1 anchor sequence and the interactions between this anchor and the full-length CYP2C2 [13] (see section 2.5) do not seem to provide ultimate proof of specific interactions of membrane anchors. These observations may reflect specific interactions of the fragments of yellow fluorescent protein (YFP), which were attached to the N-terminal membrane anchor sequence of CYP2C1 in these BiFC experiments. The lack of interactions of the N-terminal truncated CYP2C9 and

CYP3A4(t) with CYP2C9, in contrast to the association of the full-length proteins observed in coimmunoprecipitation experiments of Subramanian and Tracy [33], may be specific for the interactions in solution. Therefore, the role of the N-terminal hydrophobic segment in P450-P450 interactions in the membrane remains controversial.

According to the recent cross-linking study with CYP2C8 [77], the region of F-G loop also participates in P450-P450 interactions, at least in the case of CYP2C8 oligomers [77]. Involvement of this flexible region, which serves as a gateway of the substrate entry channel, is consistent with an important effect of P450-P450 interactions on substrate binding.

Studies from the laboratory of Wayne Backes suggest an important role of electrostatic interactions in the association of CYP1A2 with either CYP2B4 or CYP2E1 [115–116]. This type of interaction requires a complementarity of charges at the interacting surfaces so that the intermolecular interfaces of each subunit must therefore be different. This circumstance implies a difference in the orientation of the CYP2B4 or CYP2E1 and CYP1A2 subunits in the heterooligomer, which is also consistent with the functional data [11,26,34,114,116].

An example of different orientations of two P450 molecules in a dimer is given by a crystallization unit of the heme-containing domain of the cytochrome P450BM-3 (BMP). In the X-ray structure of BMP (PDB entry 2HPD [118]), the two BMP monomers have significantly different conformations, one with a more open substrate- and water-access to the heme moiety than the other. Here the loop at the C-terminal side of the α -helix K' of one molecule closely approaches the α -helix C of the other molecule. Possible parallels in the intermolecular interfaces in this and two other dimeric P450 structures, CYP154C1 (PDB entry 1GWI [119]) and CYP2C9 (1OG5 [120]), were discussed by Hazai, Kupfer and co-authors in relation to possible effect of P450 oligomerization on the interactions with the reductase [121].

Another important question concerns the size of the P450 oligomers. In most published reports the P450 heterooligomers are assumed to be dimers. However, this assumption is usually done for simplicity only. Rotational relaxation times of CYP2B4 in the membrane suggest that the enzymes are predominately hexameric [51,55]. As discussed in the section 2.2 electron microscopy of the oligomers of CYP2B4 and CYP1A2 in solution showed that both enzymes form hexamers, which are organized as a two-layer dimer of planar trimers [19,22]. The lateral interactions within these trimers may in principle take place in membrane-bound P450s as well, while the interface between the stacked trimers is supposedly formed by the hydrophobic membranous segments. Therefore, the trimeric unit of the hexamer in solution, supposedly correspond to an elementary unit of the P450 oligomer in the membrane. This assumption is consistent with the persistence of a 2:1 distribution of functionally different fractions in solution and in the membranes, which are revealed in the distribution of the phases in dithionite-dependent reduction [20,31] and partitioning of the heme protein between two fractions with different sensitivity to pressure-induced inactivation. [32,102–104]

4.3 P450 oligomers and cooperativity

Heterotropic cooperativity of cytochromes P450, in particular human CYP3A4, which results in the activation of the metabolism of (and/or increase in the affinity to) one substrate by the addition of another substrate has attracted detailed attention due to its potential importance in relation to adverse drug effects and drug-drug interactions (see [122–123] for review). Effector-induced redistribution of a pool of P450 conformers with different activity and substrate specificity was first brought forward as a possible mechanistic basis of CYP3A4 heterotropic cooperativity in the publications of Koley, Friedman and co-authors

[124–125]. This hypothesis is supported by the modulatory effect of allosteric activators, such as α -naphthoflavone (ANF), on the partitioning between the CYP3A4 conformers revealed in the kinetics of CO-rebinding after flash photolysis [124] or dithionite-dependent reduction [20] in the enzyme oligomers. The effector-induced changes in oligomerization of CYP3A4 are thought to be the most probable basis for this effect (see [122] for review).

With this relationship in mind, the observation of a peripheral ligand binding site at the interface between two subunits of the crystallographic dimer of CYP2C8 [126] is of high interest. Interactions of this kind are likely to affect the equilibrium of oligomerization and/or the architecture of the oligomers. A peripheral ligand binding site was also detected in the X-ray structure of CYP3A4 complexed with progesterone [127]. Recent studies of the interactions of ANF with CYP3A4 bearing a fluorescent probe (BADAN) attached to Cys-64 residue suggested the involvement of a remote ligand binding site in the mechanism of heterotropic cooperativity [128]. Therefore, modulation of homo- and hetero-oligomerization of cytochromes P450 by peripherally-bound allosteric effectors may be considered as a possible allosteric regulatory mechanism that underlies heterotropic cooperativity [122]. It was hypothesized that the physiological regulation of MMO through this mechanism may involve such endogenous effectors as reduced glutathione (GSH) [122,129].

4.4 Oligomerization of cytochromes P450 as a potential regulatory mechanism

According to our hypothesis, both “persistent heterogeneity” in homooligomers and asymmetric mutual effects of two cytochromes P450 reflect a difference in the conformation and/or orientation of the subunits inherent to the oligomers. This asymmetry is reflected in apparent “freezing” of the spin state during the reduction [20–21,80,96]. It also results in the heterogeneity of pressure-induced transitions [32,102,104], and an apparent inability of a fraction of the protein to bind substrates [20,32]. This asymmetry also appears to result in incomplete P450 reducibility (or very slow reducibility of some fraction of the P450 pool) by a flavoprotein partner [21,96–97,130], so that a substantial fraction of the enzyme appears to be unable to accept the electrons without dissociation of the enzyme oligomers.

At first glance, the biological advantage of hindering electron flow to a fraction of the enzyme and rendering it non-functional is unclear. However, physiological relevance of this mechanism becomes understandable when we take into account formation of mixed oligomers of several P450 species with different substrate specificity. If the distribution of these species between active and obstructed conformers in mixed oligomers is modulated by the interactions of the enzymes with their substrates, the suggested mechanism would allow a rapid redirection of the electron flow to a particular cytochrome P450 species in response to its specific substrate in the cell. This regulatory mechanism would block “idle” isoforms of P450 from unproductive electron flow and enable rapid adaptation of the cell in response to changing exposure to xenobiotics. The potential importance of this mechanism is justified by the poor coupling of electron flow to monooxygenation in drug-metabolizing cytochromes P450, leading to a significant production of reactive oxygen species (ROS) (see [131] for review). Therefore, the putative regulatory mechanism based on P450-P450 interactions may have evolved to optimize the functioning of MMO at limiting concentrations of CPR and a changing spectrum of P450 substrates as well as to maintain the balance between the monooxygenase activity and the production of ROS. This type of regulation can provide a rapid response of MMO to a changing spectrum of P450 substrates in the cell and other regulatory stimuli, while the modulation of synthesis and destruction of P450 may strengthen and extend the results achieved by this allosteric mechanism.

The suggestion that the uncoupling of MMO (and the resulting production of ROS) might be limited by hetero-oligomerization of different P450 species is supported by the observation

of Tan and co-workers [132] on the effect of CYP2E1 on H₂O₂ production by CYP2A6. While NADPH-dependent generation of H₂O₂ by CYP2A6 was drastically increased in the presence of coumarin, addition of this CYP2A6 substrate results in decreased H₂O₂ production by the reconstituted system composed of CYP2A6, CYP2E1 and CPR.

Conclusions

Recent advances in the studies of P450-P450 interactions, and studies of P450 oligomerization in living cells in particular, provided decisive support of a substantial degree of oligomerization of cytochromes P450 in the ER. Physical interactions between different P450 species resulting in the formation of mixed oligomers with altered activity and substrate specificity of the interacting enzymes has been clearly demonstrated at least for some pairs of interacting P450s. Furthermore, there are important indications that oligomerization of cytochromes P450 results in hindering electron flow to a fraction of the P450 population, which may render some P450 species non-functional. This feature, apparently resulting from conformational and/or orientational inequality of the subunits of the oligomer, is hypothesized to constitute a basis of an allosteric regulatory mechanism that regulates the catalytic properties of MMO as a whole.

Expert opinion

Recent research provides decisive support for the paradigm of homo- and heterooligomerization of cytochromes P450 in the membranes of the endoplasmic reticulum. The experimental results described here demonstrate that the composition of the pool of different P450 species coexisting in the membrane may affect the properties of the system in a very complex manner. On practical grounds, this complexity compromises considerably the predictive power of the current *in vitro* models of drug metabolism that do not consider the interactions between different P450 species. Oligomerization may account for numerous inconsistencies between the results of the studies of drug-drug interactions with recombinant cytochromes P450 and the data obtained in *in vivo* measurements or in the studies with human liver microsomes (HLM) and hepatocytes (see [133–137] for some examples). For instance, the interactions of CYP3A4 with CYP3A5 and/or other P450s in HLM may account for the lack of correlation between the proportions of CYP3A4 and CYP3A5 in HLM and the inhibitory potency of fluconazole (FKZ) towards the metabolism of such substrates as erythromycin or midazolam [134], although the inhibition with FKZ is much better pronounced with CYP3A4 than with CYP3A5 [134,138–139].

As it was rightfully noted by Wayne Backes, “P450s should not be tested alone, but need to be present in mixtures similar to those found in humans” [140]. To this, it must be added that the absolute concentrations (surface densities) of cytochromes P450 in the membrane are of equal importance, as they determine the degree of P450 (hetero)oligomerization; a decrease in P450 concentration in the membrane (taking place, for instance, during aging) may affect substrate specificity, activity and coupling of the P450 ensemble in a way which is difficult to predict. In particular, the effect of the differences in the surface density of P450 may contribute to enormous (up to several orders in magnitude) variability of the parameters of substrate metabolism (K_m and V_{max}) or inhibition (K_i or IC_{50}), which is frequently observed in the measurements with different preparations of model microsomes (e.g., Baculosomes[®] or Supersomes[®]) containing recombinant cytochromes P450 (see, for instance, [133,141]). In this context, the use of the experimentally measured lipid:protein ratio as one of the key parameters characterizing the preparations of microsomes (either HLM or model microsomes containing recombinant P450 enzymes) may considerably decrease uncertainties in the interpretation of the results of drug-metabolism studies. Unfortunately, lipid content is not routinely assessed by the commercial suppliers of the

microsomal preparations for drug metabolism studies (such as Baculosomes[®] or Supersomes[®], or HLM samples).

In my opinion, the complexities discussed above make the detailed studies of the molecular mechanism and functional consequences of homo- and heterooligomerization of P450s one of the highest priorities for further exploration of the mechanisms of function and regulation of the drug-metabolizing system. Delineating the architecture of the oligomers, mapping the oligomerization interfaces and understanding the relationship between P450-P450 interactions and binding of the P450 redox partners are ultimately required. The most important obstacle here is a lack of an appropriate model system. Most of the experimental results on P450-P450 interactions were obtained in solution, micellar reconstituted system, or proteoliposomes, and their extrapolation to the microsomal membranes or *in vivo* systems is complicated. Thus, there is an urgent need for new *in vitro* models, which would reproduce the composition of human hepatic MMO, and allow researchers to alter the ratio of its constituents in the membrane. Only a combination of these new models with advanced methods of detection of P450-P450 interactions in the membranes would allow us to fill the gap in our knowledge of the integral properties of MMO as a multienzyme system.

Acknowledgments

The author is grateful to Drs. James R. Halpert, Sean Gay, Elena V. Sineva and Jessica A.O. Rumfeldt for critical reading of the manuscript, useful comments and corrections.

References

1. Estabrook RW, Franklin MR, Cohen B, et al. Biochemical and genetic factors influencing drug metabolism. Influence of hepatic microsomal mixed function oxidation reactions on cellular metabolic control. *Metabolism*. 1971; 20:187–99. [PubMed: 4395592]
- 2*. Estabrook, RW.; Werringloer, J.; Masters, BSS., et al. The Microsomal Membrane: A Seraglio for Unique Electron Transport Carriers. In: Hatefi, Y.; Djavadi-Ohanian, L., editors. *The Structural Basis of Membrane Function*. New York: Academic Press; 1976. p. 429-45. One of the first attempts to integrate the knowledge about the microsomal redox carriers (cytochrome P450, cytochrome P450 reductase, cytochrome b5 and its reductase) and portrait MMO as a multienzyme system
3. Watanabe J, Asaka Y, Fujimoto S, et al. Densities of NADPH-ferrihemoprotein reductase and cytochrome P-450 molecules in the endoplasmic reticulum membrane of rat hepatocytes. *J Histochem Cytochem*. 1993; 41(1):43–9. [PubMed: 8417111]
4. Gomes AM, Winter S, Klein K, et al. Pharmacogenomics of human liver cytochrome P450 oxidoreductase: multifactorial analysis and impact on microsomal drug oxidation. *Pharmacogenomics*. 2009; 10(4):579–99. [PubMed: 19374516]
5. Locuson CW, Wienkers LC, Jones JP, et al. CYP2C9 protein interactions with cytochrome b(5): Effects on the coupling of catalysis. *Drug Metab Disp*. 2007; 35(7):1174–81.
6. Reed JR, Hollenberg PF. Examining the mechanism of stimulation of cytochrome P450 by cytochrome b(5): the effect of cytochrome b(5) on the interaction between cytochrome P4502B4 and P450 reductase. *J Inorg Biochem*. 2003; 97(3):265–75. [PubMed: 14511889]
7. Yamazaki H, Nakamura M, Komatsu T, et al. Roles of NADPH-P450 reductase and apo- and holo-cytochrome b(5) on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. *Protein Express Purif*. 2002; 24(3):329–37.
8. Murataliev MB, Guzov VM, Walker FA, et al. P450 reductase and cytochrome b(5) interactions with cytochrome P450: Effects. *Insect Biochem Mol Biol*. 2008; 38(11):1008–15. [PubMed: 18930820]
9. Yamazaki H, Shimada T, Martin MV, et al. Stimulation of cytochrome P450 reactions by apo-cytochrome b5: evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome b5 or heme oxygenase. *J Biol Chem*. 2001; 276(33):30885–91. [PubMed: 11413149]

10. Perret A, Pompon D. Electron shuttle between membrane-bound cytochrome P450 3A4 and b5 rules uncoupling mechanisms. *Biochemistry*. 1998; 37(33):11412–24. [PubMed: 9708976]
- 11**. Reed JR, Eyer M, Backes WL. Functional interactions between cytochromes P450 1A2 and 2B4 require both enzymes to reside in the same phospholipid vesicle. Evidence for physical complex formation. *J Biol Chem*. 2010; 285(12):8942–52. [PubMed: 20071338] A conclusive demonstration of the fact that the mutual effects of CYP1A2 and CYP2B4 on the activity of each other require the formation of the physical complexes between the two proteins
- 12**. Praporski S, Ng SM, Nguyen AD, et al. Organization of cytochrome P450 enzymes involved in sex steroid synthesis. Protein-protein interactions in lipid membranes. *J Biol Chem*. 2009; 284(48):33224–32. [PubMed: 19805543] A detailed investigation of P450-P450 interactions of P450arom (CYP19) and 7 alpha-hydroxylase (CYP17). FRET between YFP/CFP chimeric constructs was used to probe homo- and heterooligomerization of these enzymes. The study in living cells was complemented with the use of quartz crystal microbalances (QCM) and atomic force microscopy (AFM)
- 13**. Ozalp C, Szczesna-Skorupa E, Kemper B. Bimolecular fluorescence complementation analysis of cytochrome P450C2, 2E1, and NADPH-cytochrome P450 reductase molecular interactions in living cells. *Drug Metab Disp*. 2005(33):9–1382. An implementation of bimolecular fluorescence complementation (BiFC) to study homo- and heterooligomerization of CYP2C2, CYP2E1, as well as the interactions of the two P450s with CPR in the ER of the living cell
- 14*. Alston K, Robinson RC, Park SS, et al. Interactions among cytochromes P-450 in the endoplasmic reticulum. Detection of chemically cross-linked complexes with monoclonal antibodies. *J Biol Chem*. 1991; 266(2):735–9. [PubMed: 1985961] A study with a combination of chemical cross-linking with immunopurification that demonstrates the interactions between P450c (CYP1A1) with P450 2a (CYP3A2) in the membrane of rat liver microsomes
15. Guengerich FP, Holladay LA. Hydrodynamic characterization of highly purified and functionally active liver microsomal cytochrome P-450. *Biochemistry*. 1979; 18(24):5442–9. [PubMed: 117825]
16. French JS, Guengerich FP, Coon MJ. Interactions of cytochrome P-450, NADPH-cytochrome P-450 reductase, phospholipid, and substrate in the reconstituted liver microsomal enzyme system. *J Biol Chem*. 1980; 255(9):4112–9. [PubMed: 6768748]
17. Wendel I, Behlke J, Janig GR. Determination of the partial specific volume of cytochrome P-450 as a model. *Biomed Biochim Acta*. 1983; 42(6):623–31. [PubMed: 6314996]
18. Dean WL, Gray RD. Hydrodynamic properties of monomeric cytochrome P-450LM2 and cytochrome P-450LM4 in normal-octylglucoside solution. *Biochem Biophys Res Commun*. 1982; 107(1):265–71. [PubMed: 7126207]
- 19**. Tsuprun VL, Myasoedova KN, Berndt P, et al. Quaternary structure of the liver microsomal cytochrome P-450. *FEBS Lett*. 1986; 205(1):35–40. [PubMed: 3743769] Electron microscopy has been used to study the architecture of the oligomer of CYP2B4 in solution. The hexamers of the enzyme were shown to be organized as two-layered dimer of trimers
- 20*. Davydov DR, Fernando H, Baas BJ, et al. Kinetics of dithionite-dependent reduction of cytochrome P450 3A4: Heterogeneity of the enzyme caused by its oligomerization. *Biochemistry*. 2005; 44(42):13902–13. [PubMed: 16229479] Studies of the kinetics of CYP3A4 by dithionite were used to demonstrate of heterogeneity of CYP3A4 in oligomers in solution. The multiphasic character of the kinetics of reduction is compromised upon monomerization of the enzyme
21. Fernando H, Davydov DR, Chin CC, et al. Role of subunit interactions in P450 oligomers in the loss of homotropic cooperativity in the cytochrome P450 3A4 mutant L211F/D214E/F304W. *Arch Biochem Biophys*. 2007; 460(1):129–40. [PubMed: 17274942]
- 22*. Myasoedova KN, Tsuprun VL. Cytochrome P-450: hexameric structure of the purified LM4 form. *FEBS Lett*. 1993; 325(3):251–4. [PubMed: 8319808] Electron microscopy of the hexamer of CYP1A2 in solution
23. Dong MS, Yamazaki H, Guo Z, Guengerich FP. Recombinant human cytochrome P450 1A2 and an N-terminal-truncated form: construction, purification, aggregation properties, and interactions with flavodoxin, ferredoxin, and NADPH-cytochrome P450 reductase. *Arch Biochem Biophys*. 1996; 327(1):11–9. [PubMed: 8615680]

24. Kanaeva IP, Dedinskii IR, Skotselyas ED, et al. Comparative study of monomeric reconstituted and membrane microsomal monooxygenase systems of the rabbit liver. I. Properties of NADPH-cytochrome P450 reductase and cytochrome P450 LM2 (2B4) monomers. *Arch Biochem Biophys*. 1992; 298(2):395–402. [PubMed: 1416970]
25. Viner RI, Novikov KN, Ritov VB, et al. Effect of different solubilizing agents on the aggregation state and catalytic activity of two purified rabbit cytochrome P450 isozymes, CYP1A2 (LM4) and CYP2B4 (LM2). *Biochem Biophys Res Commun*. 1995; 217(3):886–91. [PubMed: 8554612]
- 26*. Davydov DR, Petushkova NA, Bobrovnikova EV, et al. Association of cytochromes P450 1A2 and 2B4: Are the interactions between different P450 species involved in the control of the monooxygenase activity and coupling? *Adv Exp Med Biol*. 2001; 500:335–8. [PubMed: 11764964] FRET-based study of protein-protein interactions between CYP1A2, CYP2B4 and CPR in soluble reconstituted system in the presence of 0.025% Emulgen-913 with FRET. The authors argue that P450-P450 interactions in CYP1A2/CYP2B4 pair results in “shielding” of CYP2B4 from the interactions with CPR
27. Kanaeva IP, Nikityuk OV, Davydov DR, et al. Comparative study of monomeric reconstituted and membrane microsomal monooxygenase systems of the rabbit liver. II. Kinetic parameters of reductase and monooxygenase reactions. *Arch Biochem Biophys*. 1992; 298(2):403–12. [PubMed: 1416971]
28. Pernecky SJ, Olken NM, Bestervelt LL, et al. Subcellular-localization, aggregation state, and catalytic activity of microsomal P450 cytochromes modified in the NH₂-terminal region and expressed in *Escherichia coli*. *Arch Biochem Biophys*. 1995; 318(2):446–56. [PubMed: 7733676]
29. Kempf AC, Zanger UM, Meyer UA. Truncated human P450 2D6: expression in *Escherichia coli*, Ni(2+)-chelate affinity purification, and characterization of solubility and aggregation. *Arch Biochem Biophys*. 1995; 321(2):277–88. [PubMed: 7646052]
30. Berndt P, Magretova NN, Myasoedova KN, et al. Cytochrome P-450 LM2 hexamer dissociation in the presence of a nonionic detergent Emulgene 913. *Biokhimiia*. 1989; 54(2):338–41. [PubMed: 2545283]
31. Davydov DR, Karyakin AV, Binas B, et al. Kinetic studies on reduction of cytochromes P-450 and b5 by dithionite. *Eur J Biochem*. 1985; 150(1):155–9. [PubMed: 4018075]
32. Davydov DR, Deprez E, Hui Bon Hoa G, et al. High-pressure-induced transitions in microsomal cytochrome P450 2B4 in solution - evidence for conformational inhomogeneity in the oligomers. *Arch Biochem Biophys*. 1995; 320(2):330–44. [PubMed: 7625841]
- 33*. Subramanian M, Tam H, Zheng H, et al. CYP2C9-CYP3A4 protein-protein interactions: Role of the hydrophobic N-terminus. *Drug Metab Disp*. 2010; 38(6):1003–9. Important demonstration of the mutual effects of human CYP2C9 and CYP3A4 in a reconstituted system. Physical interaction between the two enzymes is demonstrated by coimmunoprecipitation
34. Davydov DR, Petushkova NA, Archakov AI, et al. Stabilization of P450 2B4 by its association with P450 1A2 revealed by high-pressure spectroscopy. *Biochem Biophys Res Commun*. 2000; 276(3):1005–12. [PubMed: 11027582]
35. Scott EE, Spatzenegger M, Halpert JR. A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function. *Arch Biochem Biophys*. 2001; 395(1):57–68. [PubMed: 11673866]
36. von Wachenfeldt C, Richardson TH, Cosme J, et al. Microsomal P450 2C3 is expressed as a soluble dimer in *Escherichia coli* following modifications of its N-terminus. *Arch Biochem Biophys*. 1997; 339(1):107–14. [PubMed: 9056240]
37. Gillam EMJ. Engineering cytochrome P450 enzymes. *Chem Res Toxicol*. 2008; 21(1):220–31. [PubMed: 18067267]
38. Shukla A, Huang W, Depaz IM, et al. Membrane integration of recombinant human P450 forms. *Xenobiotica*. 2009; 39(7):495–507. [PubMed: 19534586]
39. Cosme J, Johnson EF. Engineering microsomal cytochrome P450C5 to be a soluble, monomeric enzyme - Mutations that alter aggregation, phospholipid dependence of catalysis, and membrane binding. *J Biol Chem*. 2000; 275(4):2545–53. [PubMed: 10644712]
40. Cosme J, Johnson EF. Analyzing binding of N-terminal truncated, microsomal cytochrome P450s to membranes. *Methods Enzymol*. 2002; 357:116–20. [PubMed: 12424903]

41. Mast N, Andersson U, Nakayama K, et al. Expression of human cytochrome P450 46A1 in *Escherichia coli*: effects of N- and C-terminal modifications. *Arch Biochem Biophys*. 2004; 428(1):99–108. [PubMed: 15234274]
42. Graham-Lorence S, Peterson JA. P450s: structural similarities and functional differences. *FASEB J*. 1996; 10(2):206–14. [PubMed: 8641554]
43. Williams PA, Cosme J, Sridhar V, et al. Mammalian microsomal cytochrome P450 monooxygenase: Structural adaptations for membrane binding and functional diversity. *Molecular Cell*. 2000; 5(1):121–31. [PubMed: 10678174]
44. Nakayama K, Puchkaev A, Pikuleva IA. Membrane binding and substrate access merge in cytochrome P4507A1, a key enzyme in degradation of cholesterol. *J Biol Chem*. 2001; 276(33): 31459–65. [PubMed: 11423554]
45. Muralidhara BK, Negi S, Chin CC, et al. Conformational flexibility of mammalian cytochrome P4502B4 in binding imidazole inhibitors with different ring chemistry and side chains - Solution thermodynamics and molecular modeling. *J Biol Chem*. 2006; 281(12):8051–61. [PubMed: 16439365]
46. Seliskar M, Kosir R, Roman D. Expression of microsomal lanosterol 14 alpha-demethylase (CYP51) in an engineered soluble monomeric form. *Biochem Biophys Res Commun*. 2008; 371(4):855–9. [PubMed: 18466759]
- 47*. McIntosh PR, Kawato S, Freedman RB, et al. Evidence from cross-linking and rotational diffusion studies that cytochrome P450 can form molecular aggregates in rabbit-liver microsomal membranes. *FEBS Lett*. 1980; 122(1):54–8. [PubMed: 7215544] Combination of the measurements of rotational mobility of microsomal P450 with protein-cross-linking in this study provided the first evidence for P450 oligomerization in the microsomal membranes
- 48*. Richter C, Winterhalter KH, Cherry RJ. Rotational diffusion of cytochrome P-450 in rat liver microsomes. *FEBS Lett*. 1979; 102(1):151–4. [PubMed: 456583] The first study of rotational mobility of microsomal P450 with the technique of decay in absorbance anisotropy after flash-photolysis
49. Gut J, Richter C, Cherry RJ, et al. Rotation of cytochrome P-450. Complex formation of cytochrome P-450 with NADPH-cytochrome P-450 reductase in liposomes demonstrated by combining protein rotation with antibody-induced cross-linking. *J Biol Chem*. 1983; 258(14): 8588–94. [PubMed: 6408090]
50. Gut J, Kawato S, Cherry RJ, et al. Lipid peroxidation decreases the rotational mobility of cytochrome P-450 in rat liver microsomes. *Biochim Biophys Acta*. 1985; 817(2):217–28. [PubMed: 3925992]
- 51*. Greinert R, Finch SA, Stier A. Cytochrome P-450 rotamers control mixed-function oxygenation in reconstituted membranes. Rotational diffusion studied by delayed fluorescence depolarization. *Xenobiotica*. 1982; 12(11):717–26. [PubMed: 7168192] An exemplary study of the rotational mobility of P450 in proteoliposomes with the technique based on depolarization of delayed fluorescence. The authors argue in favor of hexamers as the main oligomeric form of P450 in the membrane
52. Hildebrandt P, Garda H, Stier A, et al. Protein-protein interactions in microsomal cytochrome P-450 isozyme LM2 and their effect on substrate binding. *Eur J Biochem*. 1989; 186:383–8. [PubMed: 2598935]
53. Kawato S, Ashikawa I, Iwase T, et al. Drug-induction decreases the mobility of cytochrome P-450 in rat liver microsomes: protein rotation study. *J Biochem*. 1991; 109(4):587–93. [PubMed: 1907967]
54. Schwarz D, Pirwitz J, Ruckpaul K. Rotational diffusion of cytochrome P-450 in the microsomal membrane-evidence for a clusterlike organization from saturation transfer electron paramagnetic resonance spectroscopy. *Arch Biochem Biophys*. 1982; 216(1):322–8. [PubMed: 6285833]
- 55*. Schwarz D, Pirwitz J, Meyer HW, et al. Membrane topology of microsomal cytochrome P-450: Saturation transfer EPR and freeze-fracture electron microscopy studies. *Biochem Biophys Res Commun*. 1990; 171(1):175–81. [PubMed: 2168169] Study of rotational mobility of CYP2B4 in proteoliposomes with ST-EPR combined with electron microscopy. Hexamers are hypothesized to be the main oligomeric form of P450 in the membrane

56. Schwarz D, Chernogolov L, Kisselev P. Complex formation in vesicle-reconstituted mitochondrial cytochrome P450 systems (CYP11A1 and CYP11B1) as evidenced by rotational diffusion experiments using EPR and ST-EPR. *Biochemistry*. 1999; 38(29):9456–64. [PubMed: 10413522]
- 57*. Yamada M, Ohta Y, Bachmanova GI, et al. Effect of microsome-liposome fusion on the rotational mobility of cytochrome P450IIB4 in rabbit liver microsomes. *J Inorg Biochem*. 2001; 83(4):261–8. [PubMed: 11293546] The study of the dependence of the rotational mobility of P450 on surface density of the enzyme in rat liver microsomes. This work provides important arguments supporting P450 oligomerization in microsomal membrane
58. Yamada M, Ohta Y, Bachmanova GI, et al. Dynamic interactions of rabbit liver cytochromes P450IA2 and P450IIB4 with cytochrome b5 and NADPH-cytochrome P450 reductase in proteoliposomes. *Biochemistry*. 1995; 34(32):10113–9. [PubMed: 7640265]
59. Ohta Y, Sakaki T, Yabusaki Y, et al. Rotation and membrane topology of genetically expressed methylcholanthrene-inducible cytochrome P-450IA1 lacking the N-terminal hydrophobic segment in yeast microsomes. *J Biol Chem*. 1994; 269(22):15597–600. [PubMed: 8195206]
60. Ohta Y, Kawato S, Tagashira H, et al. Dynamic structures of adrenocortical cytochrome P-450 in proteoliposomes and microsomes: protein rotation study. *Biochemistry*. 1992; 31(50):12680–7. [PubMed: 1472505]
61. Iwase T, Sakaki T, Yabusaki Y, et al. Rotation and interactions of genetically expressed cytochrome P-450IA1 and NADPH-cytochrome P-450 reductase in yeast microsomes. *Biochemistry*. 1991; 30(34):8347–51. [PubMed: 1909175]
62. Ohta Y, Yanagibashi K, Hara T, et al. Protein rotation study of cytochrome P-450 in submitochondrial particles: effect of KCl and intermolecular interactions with redox partners. *J Biochem*. 1991; 109(4):594–9. [PubMed: 1869512]
63. Kawato S, Mitani F, Iizuka T, et al. Rotation and protein-protein interactions of cytochrome P-450 in the inner membrane of adrenocortical mitochondria. *J Biochem*. 1988; 104(2):188–91. [PubMed: 3182761]
64. Richter C. Biophysical consequences of lipid peroxidation in membranes. *Chem Phys Lipids*. 1987; 44:175–89. [PubMed: 3311416]
65. Taniguchi H, Imai Y, Sato R. Protein-protein and lipid-protein interactions in a reconstituted cytochrome P-450 dependent microsomal monooxygenase. *Biochemistry*. 1987; 26(22):7084–90. [PubMed: 3122827]
66. Kinoshita K, Kawato S, Ikegami A. Dynamic structure of biological and model membranes - analysis by optical anisotropy decay measurement. *Adv Biophys*. 1984; 17:147–203. [PubMed: 6399815]
67. Gut J, Richter C, Cherry RJ, et al. Rotation of cytochrome P-450. II. Specific interactions of cytochrome P-450 with NADPH-cytochrome P-450 reductase in phospholipid vesicles. *J Biol Chem*. 1982; 257(12):7030–6. [PubMed: 6806262]
- 68*. Kawato S, Gut J, Cherry RJ, et al. Rotation of cytochrome P-450. I. Investigations of protein-protein interactions of cytochrome P-450 in phospholipid vesicles and liver microsomes. *J Biol Chem*. 1982; 257(12):7023–9. [PubMed: 7085615] Detailed study of the effect of the surface density of CYP2B4 on its rotational mobility in the proteoliposomes that demonstrates a concentration-dependent oligomerization of the enzyme in the membrane
69. Schwarz D, Kruger V, Chernogolov AA, et al. Rotation of cytochrome P450SCC (CYP11A1) in proteoliposomes studied by delayed fluorescence depolarization. *Biochem Biophys Res Commun*. 1993; 195(2):889–96. [PubMed: 8373424]
70. Kiselev PA, Garda G, Finch SAE, et al. Rotational diffusion of cytochrome P-450 in the presence of NADPH-cytochrome P-450 reductase in liposomes of different phospholipid-composition. *Biol Membr*. 1990; 7(10):1026–36.
71. Greinert R, Finch SA, Stier A. Conformation and rotational diffusion of cytochrome P-450 changed by substrate binding. *Bioscience Reports*. 1982; 2(12):991–4. [PubMed: 7165794]
72. Greinert R, Staerk H, Stier A, et al. E-type delayed fluorescence depolarization, technique to probe rotational motion in the microsecond range. *J Biochem Biophys Methods*. 1979; 1(2):77–83. [PubMed: 95189]

- 73*. Ramsden JJ, Bachmanova GI, Archakov AI. Kinetetic Evidence for Protein Clustering at a Surface. *Phys Review, Ser E*. 1994; 50:5072–5. A study of oligomerization of CYP1A2 and CYP2B4 with an original technique based on the kinetics of P450 incorporation into a phospholipid bilayer studied with an optical biosensor. This interesting and original study got undeservedly little attention in P450 literature
74. Baskin LS, Yang CS. Identification of cross-linked cytochrome P-450 in rat liver microsomes by enzyme-immunoassay. *Biochem Biophys Res Commun*. 1982; 108(2):700–7. [PubMed: 6756404]
75. Myasoedova KN, Berndt P. Cytochrome P-450LM2 oligomers in proteoliposomes. *FEBS Lett*. 1990; 275(1–2):235–8. [PubMed: 2261995]
76. Myasoedova KN, Magretova NN. Cross-linking study of cytochrome P450 1A2 in proteoliposomes. *Biosci Rep*. 2001; 21(1):63–72. [PubMed: 11508695]
- 77*. Hu G, Johnson EF, Kemper B. Cytochrome P450 2C8 exists as a dimer in natural membranes. *Drug Metab Dispos*. 2010; 38(11):1976–83. [PubMed: 20699412] An elegant, state-of-the-art study demonstrating oligomerization of recombinant CYP2C8 in bacterial membranes and in ER of cultured human cells. This is the only study that allows mapping the molecular interfaces involved in P450-P450 interactions
- 78*. Davydov DR, Sineva EV, Sistla S, et al. Electron transfer in the complex of membrane-bound human cytochrome P450 3A4 with the flavin domain of P450BM-3: the effect of oligomerization of the heme protein and intermittent modulation of the spin equilibrium. *Biochim Biophys Acta*. 2010 Mar; 1797(3):378–90. [PubMed: 20026040] The study of the effect of the surface density of CYP3A4 in the liposomes on the reducibility of the enzyme by BMR, which was used as a model flavoprotein partner. Incomplete reducibility in the oligomer is rescued by monomerization. The paper also reports the first determination of the K_D of CYP3A4 oligomers in the membrane with a FRET-based technique.
79. Hardt SL. Rates of diffusion controlled reactions in one, 2 and 3 dimensions. *Biophys Chem*. 1979; 10:239–43. [PubMed: 16997220]
80. Karyakin AV, Davydov DR. Kinetics of electron-transfer reactions in monooxygenase system. *Vestn Akad Med Nauk SSSR*. 1988; 1988(1):53–62.
81. Davydov, DR.; Sineva, EV.; Davydova, NY., et al. Oligomerization of cytochrome P450 3A4 in phospholipid bilayer and its role in heterotropic cooperativity of the enzyme. 18-th International Symposium on Microsomes and Drug Oxidations; Beijing. 2010. p. 124
82. Watanabe J, Asaka Y, Kanai K, et al. Relation between cytochrome P-450 increase and endoplasmic-reticulum proliferation in hepatocytes of mice treated with phenobarbital - a microphotometric and morphometric study. *J Histochem Cytochem*. 1992; 40(3):353–7. [PubMed: 1552174]
- 83*. Szczesna-Skorupa E, Mallah B, Kemper B. Fluorescence resonance energy transfer analysis of cytochromes P450 2C2 and 2E1 molecular interactions in living cells. *J Biol Chem*. 2003; 278(33):31269–76. [PubMed: 12766165] Study homo- and heterooligomerization of CYP2C2 and CYP2E1 in the ER of the living cell with the use of FRET in the pair of CFP/YFP chimeric constructs. This landmark study presents the first demonstration of P450 oligomerization in the membranes of living cells
84. Davydov DR, Kurganov BI. Comparative Study of the Reaction Kinetics of Cytochrome P-450 Reduction by NADPH-Cytochrome P-450 Reductase and Dithionite. *Biokhimiia*. 1982; 47(9): 1476–82. [PubMed: 6814537]
85. Davydov RM, Khanina OY, Iagofarov S, et al. Effect of lipids and substrates on the kinetics of interactions of ferrocycytochrome P-450 with CO. *Biokhimiia*. 1986; 51(1):125–9. [PubMed: 3955103]
86. Ledenev AN, Tverdokhlebov EN, Davydov RM. Reduction of Ferricytochrome P-450 with Eosin Photoreductant. *Biofizika*. 1984; 29(5):730–2. [PubMed: 6210112]
87. Tsong TY, Yang CS. Rapid conformational changes of cytochrome P-450: effect of dimyristoyl lecithin. *Proc Nat Acad Sci*. 1978; 75:5955–9. [PubMed: 282617]
88. Fisher MT, Sligar SG. Temperature jump relaxation kinetics of the P-450cam spin equilibrium. *Biochemistry*. 1987; 26(15):4797–803. [PubMed: 3663627]

89. Brenner S, Hay S, Girvan HM, et al. Conformational dynamics of the cytochrome P450BM3/N-palmitoylglycine complex: The proposed “proximal-distal” transition probed by temperature-jump spectroscopy. *J Phys Chem B*. 2007; 111(27):7879–86. [PubMed: 17571881]
90. Ziegler M, Blanck J, Ruckpaul K. Spin equilibrium relaxation kinetics of cytochrome P450 LM2. *FEBS Lett*. 1982; 150(1):219–22.
91. Makris TM, von Koenig K, Schlichting I, et al. Alteration of P450 distal pocket solvent leads to impaired proton delivery and changes in heme geometry. *Biochemistry*. 2007; 46(49):14129–40. [PubMed: 18001135]
92. Haines DC, Tomchick DR, Machius M, et al. Pivotal role of water in the mechanism of P450BM-3. *Biochemistry*. 2001; 40(45):13456–65. [PubMed: 11695892]
93. Bancel F, Bec N, Ebel C, et al. A central role for water in the control of the spin state of cytochrome P-450(SCC). *Eur J Biochem*. 1997; 250(2):276–85. [PubMed: 9428674]
94. Schulze H, Hui Bon Hoa G, Helms V, et al. Structural changes in cytochrome P-450cam effected by the binding of the enantiomers (1R)-camphor and (1S)-camphor. *Biochemistry*. 1996; 35(45):14127–38. [PubMed: 8916898]
95. Di Primo C, Deprez E, Hui Bon Hoa G, et al. Antagonistic effects of hydrostatic pressure and osmotic pressure on cytochrome P-450(cam) spin transition. *Biophys J*. 1995; 68(5):2056–61. [PubMed: 7612848]
96. Backes WL, Tamburini PP, Jansson I, et al. Kinetics of cytochrome P-450 reduction: evidence for faster reduction of the high-spin ferric state. *Biochemistry*. 1985; 24(19):5130–6. [PubMed: 3935158]
97. Fernando H, Halpert JR, Davydov DR. Kinetics of electron transfer in the complex of cytochrome P450 3A4 with the flavin domain of cytochrome P450BM-3 as evidence of functional heterogeneity of the heme protein. *Arch Biochem Biophys*. 2008; 471(1):20–31. [PubMed: 18086551]
98. Honeychurch MJ, Hill HAO, Wong LL. The thermodynamics and kinetics of electron transfer in the cytochrome P450(cam) enzyme system. *FEBS Lett*. 1999; 451(3):351–3. [PubMed: 10371219]
99. Denisov IG, Makris TM, Sligar SG, et al. Structure and chemistry of cytochrome P450. *Chem Reviews*. 2005; 105(6):2253–77.
100. Sligar SG, Cinti DL, Gibson GG, et al. Spin state control of the hepatic cytochrome P450 redox potential. *Biochem Biophys Res Commun*. 1979; 90(3):925–32. [PubMed: 228675]
101. Das A, Grinkova YV, Sligar SG. Redox potential control by drug binding to cytochrome p450 3A4. *J Am Chem Soc*. 2007; 129(45):13778–9. [PubMed: 17948999]
102. Davydov DR, Knyushko TV, Hui Bon Hoa G. High pressure induced inactivation of ferrous cytochrome P-450 LM2 (2B4) CO complex: evidence for the presence of two conformers in the oligomer. *Biochem Biophys Res Commun*. 1992; 188:216–21. [PubMed: 1417844]
103. Davydov, DR.; Hui Bon Hoa, G. Pressure-induced transitions in cytochrome P450 2B4 as an evidence of its functional inhomogeneity in the proteoliposomal membrane. In: Heremans, K., editor. *High Pressure Research in the Biosciences and Biotechnology*. Leuven: Leuven University Press; 1997. p. 111–4.
104. Davydov DR, Halpert JR, Renaud JP, et al. Conformational heterogeneity of cytochrome P450 3A4 revealed by high pressure spectroscopy. *Biochem Biophys Res Commun*. 2003; 312(1):121–30. [PubMed: 14630029]
105. Davydov DR, Baas BJ, Sligar SG, et al. Allosteric mechanisms in cytochrome P450 3A4 studied by high-pressure spectroscopy: pivotal role of substrate-induced changes in the accessibility and degree of hydration of the heme pocket. *Biochemistry*. 2007; 46(26):7852–64. [PubMed: 17555301]
106. Anzenbacherova E, Hudecek J, Murgida D, et al. Active sites of two orthologous cytochromes P450 2E1: Differences revealed by spectroscopic methods. *Biochem Biophys Res Commun*. 2005; 338(1):477–82. [PubMed: 16129410]
107. Cawley GF, Zhang SX, Kelley RW, et al. Evidence supporting the interaction of CYP2B4 and CYP1A2 in microsomal preparations. *Drug Metab Disp*. 2001; 29(12):1529–34.

108. Kaminsky LS, Guengerich FP. Cytochrome P-450 isozyme/isozyme functional interactions and NADPH-cytochrome P-450 reductase concentrations as factors in microsomal metabolism of warfarin. *Eur J Biochem.* 1985; 149(3):479–89. [PubMed: 3924614]
109. Yamazaki H, Gillam EM, Dong MS, et al. Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with cytochrome P450 3A4 and other forms: effects of cytochrome P450- P450 and cytochrome P450-b5 interactions. *Arch Biochem Biophys.* 1997; 342(2):329–37. [PubMed: 9186495]
110. Li DN, Pritchard MP, Hanlon SP, et al. Competition between cytochrome P-450 isozymes for NADPH-cytochrome P-450 oxidoreductase affects drug metabolism. *J Pharmacol Exp Ther.* 1999; 289(2):661–7. [PubMed: 10215637]
- 111*. Subramanian M, Low M, Locuson CW, et al. CYP2D6-CYP2C9 protein-protein interactions and isoform-selective effects on substrate binding and catalysis. *Drug Metab Disp.* 2009; 37(8): 1682–9. An interesting paper that demonstrates the mutual effects of human CYP2C9 and CYP2D6 in a reconstituted system
112. Hazai E, Kupfer D. Interactions between CYP2C9 and CYP2C19 in reconstituted binary systems influence their catalytic activity: Possible rationale for the inability of CYP2C19 to catalyze methoxychlor demethylation in human liver microsomes. *Drug Metab Disp.* 2005; 33(1):157–64.
113. Cawley GF, Batie CJ, Backes WL. Substrate-dependent competition of different P450 isozymes for limiting NADPH-cytochrome P450 reductase. *Biochemistry.* 1995; 34(4):1244–7. [PubMed: 7827074]
- 114**. Backes WL, Batie CJ, Cawley GF. Interactions among P450 enzymes when combined in reconstituted systems: formation of a 2B4-1A2 complex with a high affinity for NADPH-cytochrome P450 reductase. *Biochemistry.* 1998; 37(37):12852–9. [PubMed: 9737863] A landmark paper presented the first example of a considerable activation of one P450 (CYP1A2 in this case) by the presence of another (CYP2B4). This work led off the cycle of studies on “asymmetric” effects of CYP1A2-CYP2B4 interactions and the mutual relationships in the CPR-CYP1A2-CYP2B4 triangle
115. Kelley WK, Reed JR, Backes WL. Effect of ionic strength on the functional interactions between CYP2B4 and CYP1A2. *Biochemistry.* 2005; 44:2632–41. [PubMed: 15709776]
116. Kelley RW, Cheng DM, Backes WL. Heteromeric complex formation between CYP2E1 and CYP1A2: Evidence for the involvement of electrostatic interactions. *Biochemistry.* 2006; 45(51): 15807–16. [PubMed: 17176103]
- 117*. Backes WL, Kelley RW. Organization of multiple cytochrome P450s with NADPH-cytochrome P450 reductase in membranes. *Pharm Ther.* 2003; 98(2):221–33. The first review devoted to the interactions between different P450 species and their functional effects
118. Ravichandran KG, Boddupalli SS, Hasermann CA, et al. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science.* 1993; 261(5122):731–6. [PubMed: 8342039]
119. Williams PA, Cosme J, Ward A, et al. Crystal structure of human cytochrome P4502C9 with bound warfarin. *Nature.* 2003; 424(6947):464–8. [PubMed: 12861225]
120. Podust LM, Kim Y, Arase M, et al. The 1.92-angstrom structure of *Streptomyces coelicolor* A3(2) CYP154C1 - A new monooxygenase that functionalizes macrolide ring systems. *J Biol Chem.* 2003; 278(14):12214–21. [PubMed: 12519772]
121. Hazai E, Bika Z, Simonyi M, et al. Association of cytochrome P450 enzymes is a determining factor in their catalytic activity. *J Comp-Aid Mol Des.* 2005; 19:271–85.
122. Davydov DR, Halpert JR. Allosteric P450 mechanisms: multiple binding sites, multiple conformers or both? *Expert Opinion On Drug Metabolism & Toxicology.* 2008; 4(12):1523–35. [PubMed: 19040328]
123. Denisov IG, Frank DJ, Sligar SG. Cooperative properties of cytochromes P450. *Pharmacol Ther.* 2009; 124(2):151–67. [PubMed: 19555717]
124. Koley AP, Buters JTM, Robinson RC, et al. Differential mechanisms of cytochrome P450 inhibition and activation by alpha-naphthoflavone. *J Biol Chem.* 1997; 272(6):3149–52. [PubMed: 9013547]

125. Koley AP, Robinson RC, Markowitz A, et al. Drug-drug interactions: effect of quinidine on nifedipine binding to human cytochrome P450 3A4. *Biochem Pharm.* 1997; 53(4):455–60. [PubMed: 9105395]
126. Schoch GA, Yano JK, Wester MR, et al. Structure of human microsomal cytochrome P4502C8 - Evidence for a peripheral fatty acid binding site. *J Biol Chem.* 2004; 279(10):9497–503. [PubMed: 14676196]
127. Williams PA, Cosme J, Vinkovic DM, et al. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science.* 2004; 305(5684):683–6. [PubMed: 15256616]
128. Tsalkova TN, Davydova NE, Halpert JR, et al. Mechanism of interactions of alpha-naphthoflavone with cytochrome P450 3A4 explored with an engineered enzyme bearing a fluorescent probe. *Biochemistry.* 2007; 46:106–119. [PubMed: 17198380]
129. Davydov DR, Davydova NY, Tsalkova TN, et al. Effect of glutathione on homo- and heterotropic cooperativity in cytochrome P450 3A4. *Arch Biochem Biophys.* 2008; 471(2):134–45. [PubMed: 18206979]
130. Backes WL, Eyer CS. Cytochrome P-450 LM2 reduction. Substrate effects on the rate of reductase-LM2 association. *J Biol Chem.* 1989; 264(11):6252–9. [PubMed: 2495281]
131. Zangar RC, Davydov DR, Verma S. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicol Appl Pharm.* 2004; 199(3):316–31.
132. Tan Y, Patten CJ, Smith T, Yang CS. Competitive interactions between cytochromes P450 2A6 and 2E1 for NADPH-cytochrome P450 oxidoreductase in the microsomal membranes produced by a baculovirus expression system. *Arch Biochem Biophys.* 1997; 342(1):82–91. [PubMed: 9185616]
133. Greenblatt DJ, Venkatakrishnan K, Harmatz JS, et al. Sources of variability in ketoconazole inhibition of human cytochrome P450 3A in vitro. *Xenobiotica.* 2010; 40(10):713–20. [PubMed: 20712450]
134. Isoherranen N, Ludington SR, Givens RC, et al. The influence of CYP3A5 expression on the extent of hepatic CYP3A inhibition is substrate-dependent: An in vitro-in vivo evaluation. *Drug Metab Disp.* 2008; 36(1):146–54.
135. Ito K, Brown HS, Houston JB. Database analyses for the prediction of in vivo drug-drug interactions from in vitro data. *Brit J Clin Pharmacol.* 2004; 57(4):473–86. [PubMed: 15025746]
136. Henshall J, Galetin A, Harrison A, et al. Comparative analysis of CYP3A heteroactivation by steroid hormones and flavonoids in different in vitro systems and potential in vivo implications. *Drug Metab Disp.* 2008; 36(7):1332–40.
137. Hallifax D, Houston JB. Methodological Uncertainty in Quantitative Prediction of Human Hepatic Clearance from In Vitro Experimental Systems. *Curr Drug Metab.* 2009; 10(3):307–21. [PubMed: 19442091]
138. Gibbs MA, Thummel KE, Shen DD, et al. Inhibition of cytochrome P-450 3A (CYP3A) in human intestinal and liver microsomes: Comparison of K-I values and impact of CYP3A5 expression. *Drug Metab Disp.* 1999; 27(2):180–7.
139. Niwa T, Murayama N, Yamazaki H. Comparison of the Contributions of Cytochromes P450 3A4 and 3A5 in Drug Oxidation Rates and Substrate Inhibition. *J Health Sci.* 2010; 56(3):239–56.
140. Reed JR, Eyer M, Backes WL. Drug-metabolizing enzymes: if they work together, they should be tested together. *Pharmacogenomics.* 2010; 11(5):672.
141. Niwa T, Murayama N, Emoto C, et al. Comparison of kinetic parameters for drug oxidation rates and substrate inhibition potential mediated by cytochrome P450 3A4 and 3A5. *Curr Drug Metab.* 2008; 9(1):20–33. [PubMed: 18220568]

Fig. 1.

A cartoon illustrating the hypothetical mechanism of substrate-dependent activation in the oligomers of two different P450 species. The figure shows the complexes of CPR (gray) with a dimer of two different P450 species shown in black and white, respectively. The subunits in the dimer differ in their orientation and/or conformation in such a way that CPR may form a productive complex only with the rightmost subunit of each dimer. The leftmost subunits are therefore “obstructed”. In the absence of substrate the distribution of the P450 species between the active and the obstructed positions is random. Addition of the substrate for the “black” P450 species results in reorganization of the complexes, so that this enzyme now preferentially occupy the active positions, while the “white” P450 is displaced to the “obstructed” positions and is therefore inactive.