Cytochrome-P450-Induced Ordering of Microsomal Membranes Modulates Affinity for Drugs

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Abstract: Although membrane environment is known to boost drug metabolism by mammalian cytochrome P450s, the factors that stabilize the structural folding and enhance protein function are unclear. In this study, we use peptide-based lipid nanodiscs to “trap” the lipid boundaries of microsomal cytochrome P450 2B4. We report the first evidence that CYP2B4 is able to induce the formation of raft domains in a biomimetic compound of the endoplasmic reticulum. NMR experiments were used to identify and quantitatively determine the lipids present in nanodiscs. A combination of biophysical experiments and molecular simulations revealed a sphingomyelin binding region in CYP2B4. The protein-induced lipid raft formation increased the thermal stability of P450 and dramatically altered ligand binding kinetics of the hydrophilic ligand BHT. These results unveil membrane/protein dynamics that contribute to the delicate mechanism of redox catalysis in lipid membrane.

Structure, function and regulation of membrane proteins are elicited through interaction with cell membrane.[1] A systematic “mapping” of lipid–protein interactions is one of the current challenges of molecular and cell biology,[1] and could represent a determinant advancement for understanding biological processes at membrane level. Indeed, more than 60% of drug targets are located at the cell surface and other membrane subcellular interfaces.[2] Drugs and xenobiotics detox functions are also regulated by membrane-anchored cytochrome P450s[3] located in the cytosolic side of the endoplasmic reticulum (ER): they have a broad catalytic activity which includes mono-oxygenation and dealkylation of a variety of ligands, including >70% of commercially available drugs, as well as xenobiotics;[4] several P450 isoforms are also involved in hormone synthesis and the arachidonic acid cascade.[5] The biophysical challenges posed by the lipid membrane, as well as its molecular intricacies, have impeded a molecular characterization of P450-lipid dynamics. There is consensus about the significance of the lipid membrane in the catalytic activity of P450:[5] it provides the landscape for electron transfer to occur, through physical interaction with the electron transferase CPR.[6] Membrane anchoring is one of the speculated mechanisms that prevents P450 trafficking towards other subcellular compartments.[7] Also, lipid membrane is the main access pathway for hydrophobic substrates, playing a critical role in P450 drug metabolism and pharmacokinetics.[8–12] There is mounting evidence that protein–protein interactions are also driven by cross-talking between transmembrane domains (TMD),[13–15] which further amplifies the role of lipids on the structural stability and function of P450. This, there is a need for new biophysical tools that can enlighten such presently precluded dynamics, and provide critical knowledge on the function of P450.

For membrane proteins, the information regarding subcellular organization in different membrane compartments is obtained through several methods, including indirect detergent-based assay (DRM), single-molecule imaging and spectroscopy,[16] and mass spectrometry.[17] Notwithstanding the critical role of membrane in P450 catalysis, only a few groups have addressed the fundamental questions regarding the organization of P450 in ER. DRM experiments reported that P450 isoforms and CPR likely to co-exist in ER microdomains.[18] Nonetheless, given the controversies associated with DRM,[19] as well as the more recent definition of lipid rafts as small (<10 nm) and transient SM-rich domains,[20] new strategies are needed, particularly to reveal the underestimated role played by proteins in driving lipid segregation. Since P450 strongly interacts with lipid membrane through its TMD and FG-loop,[13,21] we hypothesized that any protein-driven change in lipid composition could be trapped at the nanometric scale of a nanodisc.

The 4F peptide was able to form nanodiscs (see Figure S1 in the Supporting Information) that retained the ER membrane composition (Table S1) after SEC purification (Figure S1). Peptide-based nanodiscs have several advantages over membrane scaffolding protein nanodiscs (MSP) for studying membrane proteins. First, they allow detergent-free protein incorporation, which avoids post-reconstitution purification steps,[22,23] as shown by both size-exclusion chromatography (SEC, Figure S3a) and dynamic light scattering...
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CYP2B4-induced liquid-ordered domain modulates drug affinity. a) $^{31}$P NMR on detergent-treated nanodiscs was used to assess the composition of the protein-containing fraction after overnight lipid exchange. b) Lipids and cholesterol contents in empty, CYP2B4, and cytb$_{6}$, 4F-ER nanodiscs after lipid exchange, as measured by $^{31}$P NMR and GC-MS. Data are expressed as average ± standard deviation (n = 3). $^p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. c) Fluorescence autocorrelation functions for nanodiscs. Scatter plot represent average ± standard deviation of two independent experiments; solid lines represent numerical fitting using a 3D correlation diffusion model. The lipid probe DiI C12 differently partitioned in P450-containing nanodiscs, causing changes in the autocorrelation function. d) DSC curves. e) Protein-induced changes in lipid boundaries alter the affinity for BHT. f) Schematic of lipid-induced modulation of ligand affinity for BHT.

Figure 2. CYP2B4-induced liquid-ordered domain modulates drug affinity. a) $^{31}$P NMR on detergent-treated nanodiscs was used to assess the composition of the protein-containing fraction after overnight lipid exchange. b) Lipids and cholesterol contents in empty, CYP2B4, and cytb$_{6}$, 4F-ER nanodiscs after lipid exchange, as measured by $^{31}$P NMR and GC-MS. Data are expressed as average ± standard deviation (n = 3). $^p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. c) Fluorescence autocorrelation functions for nanodiscs. Scatter plot represent average ± standard deviation of two independent experiments; solid lines represent numerical fitting using a 3D correlation diffusion model. The lipid probe DiI C12 differently partitioned in P450-containing nanodiscs, causing changes in the autocorrelation function. d) DSC curves. e) Protein-induced changes in lipid boundaries alter the affinity for BHT. f) Schematic of lipid-induced modulation of ligand affinity for BHT.

nanodiscs. We compared the effect of ligands, BHT and 4-CPI, which have different partitioning between membrane and bulk solution (their logP values are 5.54 and 1.99, respectively). UV/Vis titration with BHT led to several observations. First, in ER$_{c}$ nanodiscs, the initial high-spin population ([BHT] = 0) was higher than in solution (in ER +15%, p < 0.05). Second, the equilibrium affinity $K_{D}$ increased by 4-fold from solution (12 μM) to ER$_{c}$ nanodiscs (3 μM), whereas in ER it was midway (8 μM). Third, the maximum high-spin fraction extrapolated by the sigmoidal binding curve showed a significant increase from solution (40%) to ER (60%); however, in ER$_{c}$, the l$_{c}$ domain surrounding the protein increased the population to 100% high-spin. Similar experiments were performed with the more hydrophilic 4-CPI (Figure S7), a tight type II binder, which coordinates to the heme iron through its N-1 atom in the imidazole ring. When in solution, CYP2B4 showed lower affinity for 4-CPI ($K_{D}$ = 0.2 μM, p < 0.05), with a sigmoidal binding curve indicative of multiple binding events, as reported. The reconstitution of CYP2B4 into nanodiscs attenuated the sigmoidal shape of the binding curve and increased the affinity by 5-fold. No significant differences were observed in the affinities among the lipid nature, with the $K_{D}$ values in the 0.04–0.07 μM range (Figure S7). Hydrophobic ligands, such as BHT, are thought to partition into the membrane and reach the prosthetic group site as revealed from its backbone RSMD. The CYP2B4 soluble domain depicted a constrained conformation with an average RMSD of 2.3 Å (ER), 2.0 Å (ER$_{c}$) and 2.9 Å (POPC) in comparison to its full-length conformation. In contrast, the RMSD of C$_{l}$ atoms of the full-length CYP2B4 were higher than 2.5 Å and the average RMSD were 3.1 Å (ER), 4.2 Å (ER$_{c}$) and 3.6 Å (POPC). This suggested that TMD and lipid composition greatly modulate the overall CYP2B4 dynamics, and the lipid composition modulates the dynamics of both TMD and soluble domain.

To gain further insight, we performed CG-MD at microsecond time scale. As anticipated from the all-atom MD, a comparatively high TMD dynamics was observed in POPC (Figure 3b). The perpendicularly oriented TMD-to-bilayer surface ended with a tilt of ca. 45° during 10 μs MD simulations for both ER and POPC membrane nanodiscs. However, the translational motion of TMD (from center) was greater in POPC, whereas in ER the translational motion was restricted by the 4F-peptide belt. In contrast, the translational motion was well-restrained in ER membrane nanodiscs during multi-microsecond MD calculation and was not found in close proximity to the belt (Figure 3b). Transient interaction between CYP2B4’s soluble domain and lipid membrane was consistently observed in all simulations. Simulations showed that in SM-enriched ER membrane (ER$_{c}$), the M1-F20 is an α-helix along the entire
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Figure 3. Atomistic insights into the full-length CYP2B4 interaction with ER membrane. a) All-atom (left) and coarse-grained (right) models of CYP2B4. Protein and lipids are shown as cartoon and stick, respectively, in the all-atom model, whereas as spheres and dotted spheres in the CG-MD models. b) CG-MD snapshots showing the TMD orientation in POPC and ER nanodiscs at 0 (cyan) and 10 (orange) μs. c) Percentage of helicity rise per residue in CYP2B4-TMD with simulation time. d) Root mean square fluctuation of CYP2B4. TMD and linker connecting TMD and soluble domain are highlighted in blue and cyan, respectively. e) MD snapshot illustrating the interaction between CYP2B4 and ER, and f) SM and cholesterol-rich ERex. SM and POPS are shown in black and purple, respectively.

course of simulation, whereas in both POPC and ER the M1-L5 residues were mostly disordered (Figure 3c). Monitoring the RMS fluctuation of backbone atoms of CYP2B4 in POPC, ER and ERex membranes (Figure 3d), it was found that RMS > 2.0 Å were observed for TMD, whereas the mobility of the proline-rich (P36-P49) loop was significant only in POPC. Loops connecting helix motifs in the soluble domain showed similar fluctuations over time. In the ER membrane, the FG-loop (bearing the F- and G’ helices), as well as β2, β5 and β6 sheets (also known as β-finger) close to the C-terminus presented higher rigidity, due to an increased interaction with the membrane hydrophobic core (Figure 3d).

All-atom MD simulations unveiled interactions between specific residues and lipids (Figure 3). In ER, the soluble domain was found to interact with lipid head groups, in particular the FG-loops and the β-finger loop (Figure 3e). Results also showed SM clustering around the TMD-connecting loop and the β-finger, corresponding to the region spanning the T375 to I382 chain. Further structure analysis showed H-bonding interaction between SM and Y380 (Figure 3e, insert). Simulations on ERex showed a dramatic increase of SM clustering, with additional specific interactions with the Pro-rich TM connecting loop (G28-L40). The TM e-helical character of the domain was calculated over the 100-ns simulation time (Figure S8). Compared to ER, the enrichment of SM and cholesterol in ERex caused the helix to have an ideal angle (100°) along the entire simulation time (Figure S8a). Finally, we calculated the number of H-bonds over simulations in both ER systems, as a tool to evaluate the interaction with polar groups of the lipids (Figure S8b). As expected, the number of H-bonds was found to be substantially higher in SM-enriched ERex, indicating more interaction with the membrane surface. Sequence alignment shows that both TMD connecting region and β-finger are largely conserved among microsomal P450s (Figure S9). Particularly, the β-finger has several conserved hydrophobic residues, including the 32β-Ile-Pro-Lys34 sequence, as well as the proline-rich connector domain. This leads us to hypothesize that other microsomal P450s can possess similar ability of specific interaction with lipid components.

In conclusion, this study reveals that P450s is highly dynamic, and able to interact and modify its surrounding lipid environment to enhance both function and stability. Protein-induced formation of SM-rich domain could facilitate P450 monomerization and stability. Recent experimental and computational evidences on the lateral organization of microsomal cytochrome P450 pointed to a strong interaction of both TMD and soluble domain with the lipid bilayer.13,15,21,29,33 The results presented here also provide significant molecular insights into the factors governing P450 ligand affinity for hydrophobic molecules. Further, the innovative combination of peptide-based nanodiscs and 31P NMR experiments to quantitatively determine the different type of lipids constituting nanodiscs will be useful to study a variety of membrane proteins (like GPCR and pore-forming proteins30) and membrane-assisted amyloid aggregation.

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Conflict of interest

The authors declare no conflict of interest.

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Redox catalysis in the lipid membrane: A novel application of peptide nanodiscs shows that cytochrome P450 2B4 is able to induce the formation of lipid raft domains in a biomimetic compound of the endoplasmic reticulum (ER). The protein-induced lipid rafts increase the thermal stability cytochrome P450 and dramatically alter the ligand-binding kinetics of the hydrophilic ligand BHT.