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Membrane Environment Drives Cytochrome P450's Spin Transition and its Interaction with Cytochrome b5

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Abstract

Heme's spin-multiplicity is key in determining the enzymatic function of cytochrome P450 (cytP450). The origin of the low-spin state in ferric P450 is still under debate. Here, we report the first experimental demonstration of P450's membrane interaction altering its spin equilibrium which is accompanied by a stronger affinity for cytochrome *b*₅. These results highlight the importance of lipid membrane for the function of P450.

Cytochrome P450s are heme monooxygenases responsible for the functionalization of drugs and xenobiotics, as well as steroids biosynthesis.¹ The importance of the spin state equilibrium of cytochrome P450 (cytP450) lies in its link to enzymatic catalysis.² In the resting state, cytP450 is predominantly in low-spin state, having a water molecule as the sixth ligand.² Substrates that bind in the distal pocket and displace water result in a high-spin pentacoordinate heme, which has a higher redox potential;³ this allows for one-electron reduction by NADPH-dependent protein redox partners cytochrome P450-reductase (CPR) and cytochrome *b*₅ (cyt*b*₅).^{4, 5} Early studies indicated that free fatty acids (particularly oleic acid) were responsible for the increased high-spin population observed in a microsomal P450, since the addition of neutral lipid was not able to shift the spin equilibrium.^{6, 7} Since ferric heme complexes weakly bound to a ligand such as water typically have a high-spin ground state, the driving forces for spin multiplicity in P450 have been the subject of intense debate in the last decades.^{4, 5, 8, 9}

Initial experimental findings on the soluble P450cam and comparisons to other hemeproteins such as globins could not be explained based on the observed spin multiplicity to heme exclusively. Indeed, *ab initio* studies reported that both the electronic structure as well as the

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electric field surrounding the heme needed to be included in order to match the experimental evidence.^{4, 9} Green's group further explored the role of the thiolate axial ligand through *ab initio* calculations, showing that sulfur spin density also plays a role in determining the low-spin characteristic of ferric cytochrome P450.⁸ Recent findings on the effect of negatively charged phospholipids on both coupling activity of cytochrome P450 3A4 (CYP3A4),¹⁰ and redox potential of CPR¹¹ strongly evokes an additional role played by membrane in modulating P450 heme chemistry. Indeed, here we report experimental evidences that the membrane is also responsible for altering the spin multiplicity in the microsomal CYP2B4, with direct implications in the spin-state of the resulting P450-cyt b_5 redox complex.

For P450 and its redox counterparts CPR and cytb $_5$, choosing different membrane biomimetics can lead to determinant consequences in the protein-protein complex interfaces.^{12, 13} As a matter of fact, cytochrome P450s have been reconstituted in a variety of membrane mimetics, from simple detergent-based micelles,¹⁴ to homogeneous¹⁵ and heterogeneous¹⁶ liposomes. Nanodiscs have provided a more sophisticated tool, since they are able to monomerize P450 in a controlled and size-determined environment.¹⁷ However, studies have been limited to homogeneous DMPC or POPC nanodiscs, mainly because research efforts have been focused on catalytic reconstitution and electron transfer mechanisms.¹⁸

In this study, for the first time we used 4F-peptide based nanodiscs¹⁹ to reconstitute the cytochromes P450 and cytb $_5$ complex (Figure 1a) in lipid compositions varying from pure DMPC and POPC, to POPC-PS at several molar ratios (from 20 to 100% POPS). Compared to membrane scaffold proteins,^[16] peptide-based nanodiscs allow detergent-free protein incorporation. Disc-shaped nanodiscs were obtained (Figure S1a), which were able to monomerize CYP2B4, as shown by dynamic light scattering (DLS) and size-exclusion chromatography (SEC) (Figures 1b, S1 and S2). DLS revealed the overall diameter of the nanodisc (~ 8 nm for all preparations) and the changes induced by protein incorporation. Reconstituted CYP2B4 was active (CO-bound reduced protein showed a maximum at 450 nm, Figure S3) and stable at room temperature for several days (Figure S4).

In cytochromes, the Soret absorption band represents a mixture of high-spin (HS) and low-spin (LS) heme (Figure 1a);² depending on the protein preparation and storage conditions, a P420 fraction derived from the irreversible loss of the thiolate bond is also present.^{20, 21} From our experiments, the first observation is that changing the fatty acid chain from the fully saturated DMPC to the unsaturated (and more physiological) POPC caused an increase of the high-spin content (Table S1). This cannot be attributed to ligand-type interactions of POPC, since neutral phospholipids have been shown to not alter the spin multiplicity.⁶ The differences in the hydrophobic thickness and dynamics between DMPC and POPC membranes²² and the subsequent alteration of the protein tertiary structure probably cause changes in the heme environment to be more exposed to the bulk solvent. A second observation is that the addition of negatively charged POPS shifted the equilibrium to high-spin population from 26% (at 0% POPS) to about 33% (at 100% POPS); DMPC nanodiscs had the lowest high-spin content (17%). POPS also stabilized the protein: the percentage of P420 slightly lower (7%) compared to DMPC (9%) or POPC (13%) nanodiscs (Table S1). More interestingly, the measured affinity for the substrate benzphetamine was higher in

POPC (~40%), and decreased with the addition of POPS. This again suggest an increased structural flexibility in POPS-containing membrane, which can modulate the association/dissociation dynamics of small molecules, as recently reported for CYP3A4.²²

Figure 2 shows the changes in absorption spectra of CYP2B4 in DMPC (Figure 2a), POPC-PS (1:1) (Figure 2b), and POPC-PS (8:2) (Figure 2c), when titrated with the full-length *cytb₅*. In all the titrations, a type I spin-shift was observed, with an isosbestic point at 405 nm. Addition of *cytb₅* dramatically shifted the spin equilibrium, until reaching saturation (Figure 2, inserts). A blue-shift in the maximum absorbance peak for the high-spin population was also observed (Table S2), which was 11 nm at POPC-PS (1:1) molar ratio. Fitting the respective sigmoidal curves showed that the maximum achievable high-spin (HS_{max}) was considerably higher in POPC-PS containing nanodiscs (Table S2). In DMPC the initial spin state mixture of CYP2B4 (~17% high-spin, ~74% low-spin) was only partially converted to high-spin (~41%), whereas the presence of POPS induced a high-spin shift from ~26–33% to about 60–80% (Tables S1 and S2). In 1:1 POPC-PS, a lower affinity was also observed, being the $K_d = 1.4$ equivalents of *cytb₅* (Table S2). The sigmoidal shape of the binding curve suggests multiple binding events, a result which was further supported by NMR experiments (Figures S5 and S6).

The effectiveness of membrane composition towards the alteration of P450 affinity for its partner *cytb₅*, was corroborated using 2D $^{15}N/^1H$ HSQC spectrum. The ^{15}N -labeled ferric (rabbit) *cytb₅* was incorporated in DMPC or 8:2 POPC-PS nanodiscs and titrated with 0, 0.4, 0.8 and 1.2 equivalents of unlabeled ferric CYP2B4. Chemical shift perturbations (δ) in the amide-NH chemical shifts and peak intensity were measured to identify the binding *cytb₅* epitope for CYP2B4 during complex formation. A weak δ for most of the *cytb₅* backbone amide resonances was observed (Figure S5), due to the formation of an ensemble of dynamic encounter complexes, which are in the fast-to-intermediate NMR exchange time scale. The observed differential line broadening and the disappearance of peaks clearly confirm a tighter protein-protein complex formation (Figure 3). Decrease in signal intensity is plotted as relative intensity changes in the presence of different amounts of CYP2B4 (Figure 3). Compared to DMPC (Figure 3a), line broadening observed from POPC-PS nanodiscs was more pronounced even for 0.8 equivalent of P450 (Figure 3b). *Cyt_{b5}* residues that exhibited significant differential line broadening were mapped onto its structure (Figure S6). The widespread line broadening is concentrated on solvent exposed residues, which can be attributed to encounter complexes that form in redox partner pairs before the productive binding site is found.²³ At *cytb₅*:CYP2B4 1:0.8 ratio, two sites of interactions can be identified. On the front side of *cytb₅*, residues in both upper and lower clefts showed marked line broadening (Figure S6), with residual signal intensity <20% compared to free *cytb₅*. Three residues are in the lower cleft (His-31, His-32, and Thr-70), whereas Gly-47 and Glu-48 are found in the upper cleft above the heme.

Next, we used the line-broadening information measured from $^{15}N/^1H$ HSQC spectra obtained from complex containing 0.8 equivalents of P450, and the results from previous mutational studies,²³ to build a putative model for the full-length *cytb₅*-CYP450 complex and enable a direct visualization of the POPS induced changes. This was achieved using HADDOCK 2.2 (Figure 3c and d).²⁴ The ambiguous line-broadening restraints obtained

from NMR experiments on different lipid nanodiscs (Table S3) and unambiguous restraints from site directed mutagenesis data were used in HADDOCK simulations.²³ Low energy structural cluster from docking simulations shows that basic residues on the proximal side of CYP450 are directly involved in interaction with convex surface of *cytb₅* (Figure 3c and d). The overlapped complex structural models of the binary complex obtained from DMPC and POPC-PS (8:2) are shown in Figure S7a. In agreement with the UV-Vis observation (Figure 2), both ligand-free complexes generated through HADDOCK also expose the heme to the solvent (Figure S8). Since it is known that the protein stability is altered by changing nanodiscs lipid composition,¹⁸ the observed changes in spin equilibrium could be attributable to heme solvent exposure, which is possibly modulated by the membrane environment, particularly the presence of anionic lipid POPS.

In *cytP450*, ligand binding increases the rigidity of the enzyme architecture and also its thermal stability.^{18, 22} In CYP2B4, binding of benzphetamine caused a shift in electronic configuration to about 60–65% high-spin, regardless of the nature of phospholipidic mixture used (Figure 4(a and b)). However, titrations of the CYP2B4-benzphetamine complex with *cytb₅* caused a near complete shift to high-spin state (~95%) (Figure S9). A complete high-spin shift was not observed in other reconstituted systems: when incorporated in DLPC/DHPC isotropic bicelles, CYP2B4 spin equilibrium was only modestly altered by the addition of *cytb₅*.¹² In order to prove the physiological relevance of our reconstituted system, we tested the ability of *cytb₅* to deliver the second electron to a fully reduced CYP2B4 complexed with benzphetamine. For both proteins, autoxidation – i.e. without a redox counterpart – is slower than oxidation in the presence of the enzymatic partner, suggesting electron transfer between the two proteins (Figure 4c and d). Rates were computed through non-linear fitting of the kinetic traces (Table S5). In solution, the kinetics for CYP2B4 are triphasic in the presence of benzphetamine. The presence of lipids reduced the kinetics to biphasic. However, no significant differences were observed between solution and nanodiscs.

Important conclusions from this study is that membrane acts on cytochrome P450 in two folds: 1) it alters the spin multiplicity of the heme, and 2) provides the energetics and structural requirements for protein-protein interactions to occur. A more negatively charged membrane (POPC:PS 8:2) caused a significant shift of the spin equilibrium to high-spin state (from 17 to 33%). In POPC-PS membrane, CYP2B4 commitment to interact with its redox counterpart *cytb₅* was increased, as indicated by line broadening of NMR resonances. The similar kinetics associated with the second electron transfer between *cytb₅* and CYP2B4 is consistent with the negligible differences between the structural models generated for the P450-*cytb₅* complexes in DMPC and POPC-PS using a ligand-bound P450. Questions about the role played by lipids on the catalytic efficiency and uncoupling of the *cytP450*-*cytb₅* complex are under investigation.²⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. De Montellano, PRO. Cytochrome P450: structure, mechanism, and biochemistry. Springer; New York: 2005.
2. Denisov IG, Makris TM, Sligar SG, Schlichting I. Chem Rev. 2005; 105:2253–2277. [PubMed: 15941214]
3. Gibson GG, Sligar SG, Cinti DL, Schenkman JB. Biochem Soc Trans. 1980; 8:101–102. [PubMed: 6245949]
4. Harris D, Loew G. J Am Chem Soc. 1993; 115:8775–8779.
5. Conner KP, Schimpf AM, Cruce AA, McLean KJ, Munro AW, Frank DJ, Krzyaniak MD, de Montellano PO, Bowman MK, Atkins WM. Biochemistry. 2014; 53:1428–1434. [PubMed: 24576089]
6. Gibson GG, Cinti DL, Sligar SG, Schenkman JB. J Biol Chem. 1980; 255:1867–1873. [PubMed: 6766456]
7. Gibson GG, Cinti DL, Sligar SG, Schenkman JB. Journal. 1979; 7:1289–1290.
8. Green MT. J Am Chem Soc. 1998; 120:10772–10773.
9. Loew GH, Harris DL. Chem Rev. 2000; 100:407–420. [PubMed: 11749241]
10. Grinkova YV, Denisov IG, McLean MA, Sligar SG. Biochem Biophys Res Commun. 2013; 430:1223–1227. [PubMed: 23266608]
11. Das A, Sligar SG. Biochemistry. 2009; 48:12104–12112. [PubMed: 19908820]
12. Zhang M, Huang R, Im SC, Waskell L, Ramamoorthy A. J Biol Chem. 2015; 290:12705–12718. [PubMed: 25795780]
13. Yamamoto K, Gildenberg M, Ahuja S, Im SC, Pearcy P, Waskell L, Ramamoorthy A. Sci Rep-Uk. 2013; 3:2556.
14. Ingelman-Sundberg M, Hagbjork AL, Ueng YF, Yamazaki H, Guengerich FP. Biochem Biophys Res Commun. 1996; 221:318–322. [PubMed: 8619853]
15. Ingelman-sundberg M. FEBS letters. 1977; 78:72–76. [PubMed: 872940]
16. Shaw PM, Hosea NA, Thompson DV, Lenius JM, Guengerich FP. Arch Biochem Biophys. 1997; 348:107–115. [PubMed: 9390180]
17. Denisov IG, Sligar SG. Biochim Biophys Acta. 2011; 1814:223–229. [PubMed: 20685623]
18. Treuheit NA, Redhair M, Kwon H, McClary WD, Guttman M, Sumida JP, Atkins WM. Biochemistry. 2016; 55:1058–1069. [PubMed: 26814638]
19. Kariyazono H, Nadai R, Miyajima R, Takechi-Haraya Y, Baba T, Shigenaga A, Okuhira K, Otaka A, Saito H. J Pept Sci. 2016; 22:116–122. [PubMed: 26780967]
20. Guengerich FP, Martin MV, Sohl CD, Cheng Q. Nat Protoc. 2009; 4:1245–1251. [PubMed: 19661994]
21. Renaud JP, Davydov DR, Heirwegh KP, Mansuy D, Hui Bon Hoa GH. Biochem J. 1996; 319:675–681. [PubMed: 8920966]
22. McClary WD, Sumida JP, Scian M, Paco L, Atkins WM. Biochemistry. 2016; 55:6258–6268. [PubMed: 27782404]
23. Ahuja S, Jahr N, Im SC, Vivekanandan S, Popovych N, Le Clair SV, Huang R, Soong R, Xu J, Yamamoto K, Nanga RP, Bridges A, Waskell L, Ramamoorthy A. J Biol Chem. 2013; 288:22080–22095. [PubMed: 23709268]
24. Volkov AN, Ferrari D, Worrall JA, Bonvin AM, Ubbink M. Protein Sci. 2005; 14:799–811. [PubMed: 15689516]
25. Barnaba C, Gentry K, Sumangala N, Ramamoorthy A. F1000Res. 2017; 6:662. [PubMed: 28529725]

Figure 1.

P450 heme prosthetic group and preparation and characterization of 4F-nanodiscs containing CYP2B4 and the cytb₅-CYP2B4 complex. a) Heme coordination in low-spin (left) and high-spin (right) states, as induced by water and the type I coordinating drug benzphetamine, respectively. b) Scheme depicting the two-step protocol for the complex formation. DLS (c) and SEC (d) traces of empty (black), cytb₅ (green) and cytb₅-CYP2B4 (red) POPC-PS nanodiscs; SEC and DLS traces for DMPC nanodiscs can be found in Figure S1. SEC traces for nanodiscs with varying lipid composition and with and without CYP2B4 are given in Figure S2.

Figure 2.

Monitoring the binding of cytb₅ to ligand-free CYP2B4. Spectral titrations of ferric-CYP2B4 with cytb₅ in DMPC (a), POPC-PS 1:1 (b) and POPC-PS 8:2 (c) nanodiscs. Insets show the binding profile, fitted with eq. 1 (see the Experimental Section in Supplemental Information).

Figure 3. Effect of P450 binding on Cyt b_5 reconstituted in peptide-based nanodiscs from $^{15}\text{N}/^1\text{H}$ HSQC

Left: histograms representing the differential line broadening NMR data for the cytb_5 -CYP2B4 complex in DMPC (a) and 8:2 POPC:PS (b) 4F-nanodiscs at different cytb_5 :CYP2B4 molar ratios. All peak intensities were normalized to the Cterminal residue Asp-134 in the free membrane-bound cytb_5 spectrum to account for the change in intensity upon complex formation. On the right side, the space filling representations show the complex (CYP450- cytb_5) in DMPC (c) and POPC-PS (d) with ligand free CYP2B4. HADDOCK generated model was used to visualize the protein-protein complex formation with the listed cytb_5 residues (marked green). In panels c and d, the black horizontal solid lines represent the lipid membrane.

Figure 4.

Monitoring the binding of cytb₅ to CYP2B4 in the presence of substrate (benzphetamine) and electron transfer kinetics. UV-vis spectral titrations of CYP2B4 incorporated in DMPC (a) and 8:2 POPC:PS (b) 4F-nanodiscs. Stopped-flow kinetics for the electron transfer between cytb₅ (red trace) and cytP450 (black trace) measured in the presence of excess benzphetamine for DMPC (c) and POPC-PS 8:2 nanodiscs (d). Fitting parameters are shown in Table S5.