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Substrate Mediated Redox Partner Selectivity of Cytochrome P450

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Abstract

Investigating the interplay between cytochrome-P450 and its redox partners (CPR and cytochrome-*b*₅) is vital for understanding the metabolism of most hydrophobic drugs. Dynamic structural interactions with the ternary complex, with and without substrates, captured by NMR reveal a gating mechanism for redox partners to promote P450 function.

Graphical Abstract

Cytochrome P450s (P450s) are a ubiquitous superfamily of enzymes responsible for metabolizing a dazzling array of exogenous and endogenous compounds, including carcinogens, hormones, and over 75% of the drugs on the current market.^[1–5] For each turn of the catalytic cycle, P450 requires two electrons which are delivered by P450's two redox partners, cytochrome P450 reductase (CPR) and cytochrome *b*₅ (cyt*b*₅).^[6] CPR and cyt*b*₅ have different capabilities of interacting with P450 and their unbalanced stoichiometry within the endoplasmic reticulum, make it an interesting and challenging question to understand the interplay between this ternary complex.

CPR is a 78 kDa protein comprised of four major domains: a NADPH/FAD binding domain, a linker domain, a FMN binding domain (FBD), and a N-terminal transmembrane domain.^[5,7] After NADPH binding, electrons are shuttled through the FAD cofactor to the FMN cofactor to the heme group of the electron acceptor P450, a process involving both intra- and inter-protein electron transfers. The inter-protein electron transfer step depends on

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association and interaction between FBD and P450. Studies have demonstrated the feasibility of using the FBD alone to study the interaction between CPR and P450.^[8,9]

The third protein of this ternary complex is *cyt_b5*, a 15.7 kDa protein, which is only capable of donating the second electron to P450 due to the large difference in redox potential between *cyt_b5* and ferric P450.^[5,10,11] Although P450 metabolism can function with only CPR as the sole electron donor, deletion of *cyt_b5* causes extreme effects of P450 catalysis both sizeable increases and decreases in metabolism rates.^[12, 13] Through kinetic experiments, *cyt_b5* has been shown to affect the catalytic activity of over twenty P450 isoforms, including 3A4, 2B6, 2C9, and 2E1.^[2,14,15] *Cyt_b5* seems to have some level of substrate or reaction preference, best seen through its influence of the 17,20-lyase reaction in P450 17α1.^[16,17] Various recent studies with microsomal P450 have helped to elucidate that *cyt_b5* can stimulate a reaction through increasing the rate of formation of the catalytically active oxidizing species and product formation.^[6,18] Overall, the role of *cyt_b5* in P450 metabolism is very perplexing, since it has been reported to stimulate, inhibit, or not affect P450 activities depending on the substrates involved, the particular isoform of P450, and the experimental conditions. In this study, we investigate the effect of substrates on the complex formation between *cyt_b5* and P450, FBD and P450, and the ternary complex, demonstrating the differential regulation potential of substrates. Using NMR, we probe the relationship between rabbit full-length P450 2B4, FBD, and full-length *cyt_b5* at residue specific resolution.

Cyt_b5's effect on P450 metabolism is both isoform and substrate-dependent. Differences in rates of metabolism of various drugs with the addition or absence of *cyt_b5* have been noted for a variety of isoforms and substrates^[14,19–22], but differences in complex formation strength has not been investigated which this study provides for the first time. We use NMR to monitor changes in complex formation between P450 2B4 and *cyt_b5*. As previously described,^[23] the substrate butylated hydroxytoluene (BHT) enhances the complex formation between *cyt_b5* and P450, as measured by the increasing ¹⁵N-NMR line width. A similar strategy was applied in this study to test a variety of substrates: benzphetamine (BZ), methoxyflurane (MF), and cyclohexane (CH). ¹⁵N-labeled rabbit *cyt_b5* was expressed, purified, and used to form a 1:1 complex with P450. 2D-TROSY-HSQC NMR experiments were used to monitor changes in complex formation as each substrate was titrated into the protein-protein complex and the line-broadening of *cyt_b5* residues was monitored. One measure of the complex stability (or the strength of complex formation) is through understanding the line-broadening of residues which can be measured by the overall average signal intensity change. *Cyt_b5* alone would have 100% signal intensity, whereas *cyt_b5* in complex with P450 has a signal intensity of ~70%^[24]. In general, all substrates used (BHT, BZ, MF, and CH) led to an increase in line-widths of *cyt_b5* resonances (Figure 1A). Substrates modulate this interaction to varying degrees with BHT strengthening the complex formation followed by BZ, and lastly CH and MF. Residues with relative intensity loss more than one standard deviation below average were considered to be significantly broadened upon binding to P450 and highly implicated to be part of the binding interface. These residues are mapped onto *cyt_b5*: H44, F63, E64, D65, T70, and D71 in the presence of BZ (Figure 1C); H44, D65, V66, T70, and D71 in the presence of cyclohexane (Figure 1D); and I17, F40, R52, E64, D65, L75 for methoxyflurane (Figure 1E). All the substrate-titrated

samples demonstrated overlapping regions affected by the binding to P450, mainly, the front face of *cyt_{b5}* specifically on the lower cleft which is supported by the literature.^[23–27]

Next, to investigate the effect of substrates on the interaction between FBD and P450, substrates (BHT, BZ, MF) were added to 1:1 complex of ¹⁵N-uniformly labelled FBD and P450. 2D ¹H-¹⁵N TROSY-HSQC NMR spectra were compared before and after the addition of a substrate. For all three substrates, chemical shift perturbations were negligible indicating the proteins are under fast exchange in the NMR time scale. All of the substrate additions led to very slight enhancement in line-broadening – seen as a decrease in signal intensity (Supplemental Figure 1). In general, substrates do not affect FBD-P450 2B4 interaction much implying that, within the scope of this study, complex formation between these two redox partners are not modulated by substrates.

Not much is known about the interplay between the three proteins. Structurally, we know the FBD of CPR and *cyt_{b5}* share an overlapping binding surface on P450.^[2,6,8,16,17] As CPR/FBD has a higher affinity for P450 than does *cyt_{b5}*^[28], CPR/FBD has the potential to bind preferentially to P450 in the presence of *cyt_{b5}*. The rate constants for the interprotein electron transfer reactions are of the same magnitude for CPR-P450 and *cyt_{b5}*-P450 which eliminates the ability to transfer an electron to P450 from being the driving force behind the choice of redox partner. One hypothesis is that concentration is the major factor behind P450's choice of redox partner where the poorer binding redox partner (*cyt_{b5}* in this case) would have to be present at a higher concentration in order to be successful in outcompeting the stronger binding redox partner (CPR).^[28] As these two proteins have unique, but overlapping binding sites on P450^[2,6,22], this means they are in direct competition and these two enzymes cannot bind to P450 at the same time.

Due to the higher affinity favoring the complex formation of CPR/FBD and P450, we tested the ability of CPR/FBD to dislodge *cyt_{b5}* from its complex with P450. We first formed a 1:1 complex between ¹⁵N-labeled *cyt_{b5}* and P450, then titrated in CPR or FBD while 2D-TROSY-HSQC NMR spectra were utilized to monitor signal intensity (Figure 2, SI Figures 2–8). As CPR is titrated into the 1:1 *cyt_{b5}*:P450 complex, we should see an increase in overall signal intensity of *cyt_{b5}* if CPR is able to disrupt the complex. Surprisingly, this effect was not seen. The *cyt_{b5}*:P450 complex was stable and unaffected even in the excess of CPR, meaning that CPR is unable to interrupt or hinder the *cyt_{b5}*-P450 interaction over the concentrations of 0.5 to 1.5 molar equivalents (Figure 2E, black). This trend holds true for looking at the overall signal intensity as well as the intensity of *cyt_{b5}* residues (i.e., 60–70) implicated in binding with P450 (Figure 2E, red). This experiment was performed with CPR, FBD, and full-length FBD (flFBD); flFBD is FBD with the transmembrane domain which was used to rule out the influence of the transmembrane domain in this interaction. Very similar results were obtained for flFBD and FBD demonstrating that no form of CPR is capable of disrupting the *cyt_{b5}*-P450 complex, and also proving the feasibility of working with FBD to simplify this question. This experiment was repeated with a substrate, BHT, and similar results were seen with FBD unable to disrupt the *cyt_{b5}*-P450 complex. Figure 2A–D demonstrates residue-specific change in signal intensity when binding to P450 or P450+BHT, and upon titration with FBD. No significant change was observed for residues

D65 and E74; while D65 has been identified in the binding interface with P450^[23–27], E74 has not.

As FBD was shown to be unable to disrupt the *cytb*₅-P450 complex, we carried out the “opposite” experiment by testing the ability of *cytb*₅ to destabilize the FBD-P450 complex. Uniformly ¹⁵N-labeled FBD was expressed, purified, and used to form a 1:1 complex with P450. When *cytb*₅ was titrated into this complex, the average signal intensity of FBD residues observed from TROSY-HSQC spectra greatly increased. Our results clearly show (Figure 3) that *cytb*₅ is capable of dislodging P450-bound FBD into free solution. It is important to clarify that the ratio of *cytb*₅ added was not in excess meaning that this phenomenon is not (solely at least) a concentration based effect.

As we have shown, substrates can greatly enhance the interaction between *cytb*₅ and P450 while only minimally affecting the complex between FBD and P450. *Cytb*₅ has been shown to exert substrate-dependent effects on P450 catalysis. We performed the same experiment but we added various substrates (BHT, BZ, and MF) to the 1:1 FBD:P450 complex to ascertain if ligands affect *cytb*₅'s ability to dislodge FBD from P450 (SI Figures 9–16). Then *cytb*₅ was added and the resulting change in FBD average signal intensity was quantified. Our results (Figure 3) demonstrate a substrate dependent modulation of *cytb*₅'s ability to disrupt complex formation. Figure 3B–I displays residue G89, which has been implicated in FBD binding to P450^[28], as well as S123 which is not involved. The significant loss of G89's peak intensity after titration with P450 or P450+substrate and restoration after *cytb*₅ addition confirms our conclusion that *cytb*₅ is capable of dislodging FBD from the P450-FBD complex. The strongest effect was observed with BHT, followed by BZ and MF. With BHT and BZ, ~100% FBD was freed from the P450-bound state, while in the presence of MF, FBD still remained partially bound to P450. This difference between various substrates suggests some sort of mechanism that is regulated by different substrates. It has been reported in the literature that *cytb*₅ stimulates P450 activity to a much higher extent for MF than BZ: 8.5-fold increase in activity versus 1.3-fold increase^[6,29,30]. This has been related to an increased coupling efficiency due to electron transfer, as well as allosteric activation mediated by *cytb*₅.^[6] In our experiment, we see an inverse of this relation – that the interaction strength of the complex is much higher for BZ over MF. Based on these results, we propose a competitive binding mechanism where *cytb*₅ and CPR are competing to bind to an overlapping, but unique binding site on P450 and substrates help to modulate this effect (Figure 4).

One explanation for this inverse relationship of metabolism increase and complex formation is that the first electron transfer to P450, which can only be fulfilled by CPR, may be inhibited due to *cytb*₅ occupying the binding interface on P450. The addition of a substrate to P450 could increase *cytb*₅'s affinity for P450 which allows it to outcompete CPR, which gives newfound insight into past findings where *cytb*₅ was found to inhibit BZ demethylation.^[31] A previous study demonstrated that these proteins have an overlapping but unique binding site on P450, which indicates that the two enzymes cannot bind simultaneously. This can explain why *cytb*₅'s stimulation effect on P450 activity changes for different substrates; each substrate could alter *cytb*₅'s affinity for P450 in a different way. Other P450 isoforms have shown this substrate-dependent increase in binding affinity, most

recently, with CYP101D1's binding affinity for its redox partner, Adx, after the addition of camphor.^[32] Another explanation is the concept of P450's structural plasticity whereby after ligand binding P450 undergoes a conformational change.^[33] This conformational change could favor one redox partner binding over the other. The larger of the substrates that we tested, BHT and BZ, are bulky compounds which could have more of a rigidifying effect on the distal region on P450, leading to allosteric changes in the proximal, redox partner binding site of P450 whereas a smaller compound like MF has a lesser effect. While P450s are known to be highly homologous in their proximal site, their distal region is more varied which could explain the differences amongst isoforms of P450. Thus, the results presented in this study shed light on substrate regulation on the tertiary FBD-P450-cyt_b₅ system, providing insights into the structural basis of the interplay of the three redox partners.

In conclusion, for the first time, we have shown that substrates play very important roles in the dynamic interplay between P450 2B4 and its redox partners. Cyt_b₅ interaction with P450 is greatly increased by the addition of substrates to varying degrees whereas FBD-P450 interaction is not significantly affected. Although FBD and CPR have higher affinity for P450, they are unable to dislodge cyt_b₅ from binding to P450. Cyt_b₅, on the other hand, is capable of disrupting the FBD-P450 complex interaction in a substrate dependent manner. Since all three proteins are membrane-anchored and membrane has been shown to play important roles on P450 function,^[2,5,34] probing the roles of membrane on the ternary complex is in progress in our lab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Substrate effect on *cytb*₅-P450 interaction

(A) 2D ^1H - ^{15}N TROSY-HSQC NMR spectrum of ^{15}N -labeled *cytb*₅. (B) Comparison of the average intensity of *cytb*₅ in complex with P450 in the absence and presence of different substrates. Highlighted (in blue) residues are part of the P450-*cytb*₅ binding interface are mapped on *cytb*₅ (PDB: 2M33) with the heme group displayed in red sticks for (C) benzphetamine (BZ), (C) methoxyflurane (MF), and (D) cyclohexane (CH).

Figure 2. Cyt b_5 -P450 complex is unperturbed by interaction with CPR and its variants
 2D ^1H - ^{15}N TROSY-HSQC NMR spectral regions of ^{15}N -labeled cyt b_5 reveal changes due to interactions with P450, BHT, and CPR variants. (A) Free ^{15}N -cyt b_5 (green), (B) ^{15}N -cyt b_5 titrated with 1 molar equivalent of P450 (red), (C) ^{15}N -cyt b_5 +P450 titrated with BHT (blue), and (D) ^{15}N -cyt b_5 +P450+BHT after FBD titration (black). (E) The average signal intensity of cyt b_5 residues (total residues in black; residues in the lower cleft (60–70) in red) over the course of a titration with CPR, f1FBD, or trFBD at the indicated molar ratios (0.5, 1, and 1.5). Negligible change in the observed signal intensity indicates cyt b_5 is not replaced from the cyt b_5 -P450 complex by CPR or its variants.

Figure 3. Cyt_b5 disrupts complex between FBD and P450 in a substrate dependent manner
 2D ¹H-¹⁵N TROSY-HSQC NMR spectra of ¹⁵N-labeled FBD reveal changes due to interaction with P450, substrates, and cytb₅. (A) ¹H-¹⁵N TROSY-HSQC NMR spectrum of ¹⁵N-FBD. Example residues are zoomed in (B) ¹⁵N-FBD in solution (black), (C) ¹⁵N-FBD titrated with 1 molar equivalent of P450 (red), ¹⁵N-FBD+P450 titrated with a substrate BHT (blue, D), BZ (magenta, F), or MF (purple, H), and ¹⁵N-FBD+P450+substrate titrated with cytb₅ (green) for BHT (E), BZ (G), and MF (I). (J) Average signal intensity of ¹⁵N-FBD quantified after the addition of P450, P450+substrate (BHT, BZ, or MF), and P450+substrate (BHT, BZ, or MF) +cytb₅.

Figure 4. Schematic of the ternary interplay

When *cytb₅* is added to the BHT-P450 complex and FBD, *cytb₅* is able to replace FBD to form a *cytb₅*-P450 complex. On the other hand, FBD is unable to replace *cytb₅* from the *cytb₅*-P450 complex.