Identification of the contact region responsible for the formation of the homomeric CYP1A2•CYP1A2 complex

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Abstract

Previous studies showed that cytochrome P450 1A2 (CYP1A2) forms a homomeric complex that influences its metabolic characteristics. Specifically, CYP1A2 activity exhibits a sigmoidal response as a function of NADPH-cytochrome P450 reductase (POR) concentration and is consistent with an inhibitory CYP1A2•CYP1A2 complex that is disrupted by increasing [POR] (Reed et al., (2012) Biochem. J. 446, 489-497). The goal of this study was to identify the CYP1A2 contact regions involved in homomeric complex formation. Examination of X-ray structure of CYP1A2 implicated the proximal face in homomeric complex formation. Consequently, involvement of residues L91-K106 (P₁ region) located on the proximal face of CYP1A2 was investigated. This region was replaced with the homologous region of CYP2B4 (T81-S96) and the protein was expressed in HEK293T/17 cells. Complex formation and its disruption was observed using bioluminescence resonance energy transfer (BRET). The P₁-CYP1A2 (CYP1A2 with the modified P₁ region) exhibited a decreased BRET signal as compared to wild-type CYP1A2 (WT-CYP1A2). On further examination, P₁-CYP1A2 was much less effective at disrupting the CYP1A2•CYP1A2 homomeric complex, when compared to WT-CYP1A2, thereby demonstrating impaired binding of P₁-CYP1A2 to WT-CYP1A2 protein. In contrast, the P₁ substitution did not affect its ability to form a heteromeric complex with CYP2B4. P₁-CYP1A2 also showed decreased activity as compared to WT-CYP1A2, which was consistent with a decrease in the ability of P₁-CYP1A2 to associate with WT-POR, again implicating the P₁ region in POR binding. These results indicate that the contact region responsible for the CYP1A2•CYP1A2 homomeric complex resides in the proximal region of the protein.

Keywords

Cytochrome P450; protein-protein interaction; bioluminescence resonance energy transfer (BRET); membrane protein; structure-function; electron transfer; NADPH-cytochrome P450 reductase; CYP1A2

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AUTHOR CONTRIBUTIONS

A.S., J.P.C., J.R.R. and C.S.L. conducted the experiments. A.S wrote the initial draft of the manuscript. J.R.R. and W.L.B. conceptualized the study and supervised its progress. J.R.R., J.P.C. and W.L.B. read and edited the manuscript. W.L.B. obtained funding for the study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest with regard to this manuscript.
INTRODUCTION

The cytochrome P450s (P450s) involved in drug and xenobiotic metabolism are heme-containing, membrane-bound enzymes that reside primarily in the endoplasmic reticulum of cells in the liver, spleen and small intestine. These enzymes act as monooxygenases and obtain electrons from an interaction with NADPH-cytochrome P450 reductase (POR) to oxidize both exogenous and endogenous compounds [1]. P450s have been shown to form homomeric and heteromeric complexes that modulate their metabolic function [2-9]. Initial evidence for these complexes and their effect on function were observed in reconstituted systems [2-4, 10-14]. In reconstituted systems containing POR and CYP1A2 with and without CYP2B4, CYP1A2-mediated dealkylation of 7-ethoxyresorufin (EROD) was synergistically stimulated by CYP2B4 [15, 16]. These functional changes were shown to be the result of the formation of a stable CYP1A2•CYP2B4 complex [14, 17]. Evidence for these interactions were also reported in liver microsomes [11].

P450 enzymes not only are capable of heteromeric association but can also form homomeric complexes. This was shown with some, but not all, P450 enzymes. Whereas CYP2B4 exhibited expected Michaelis-Menten (hyperbolic) kinetics as a function of the POR concentration, CYP1A2 and CYP2E1 exhibited non-Michaelis-Menten behavior [13]. CYP1A2 and CYP2E1 produced sigmoidal curves as a function of the POR concentration that became more evident at higher P450 levels (where protein crowding was more likely). Data from chemical cross-linking and bioluminescence resonance energy transfer (BRET) provided support for the idea that the sigmoidal kinetic response was the result of the formation of a homomeric complex, particularly with CYP1A2 [17]. Furthermore, the results showed that the CYP1A2•CYP1A2 complex could be disrupted by the cotransfection of either POR or CYP2B4 [17].

These data raised questions regarding the contact regions involved in the formation of the CYP1A2•CYP1A2 complex, and the sigmoidal relationship between POR concentration and CYP1A2-mediated activities. Examination of the structure of CYP1A2 crystals suggested that the CYP1A2 molecules may interact through their proximal regions (PDB: 2HI4) [2].

The goal of this study was to identify the contact regions responsible for the CYP1A2•CYP1A2 homomeric complex. These studies are based on our previous observations that CYP2B4 does not appear to be able to form homomeric complexes in HEK293T/17 cells [17]. We decided to take advantage of this difference in complex formation by substituting surface regions of the proximal face of CYP1A2 (P1 region: L91-K106) with the homologous regions from CYP2B4 (T81-S96) to generate chimeric CYP1A2 proteins (P1-CYP1A2). The rationale for this approach was that substitution of the 15 surface residues of CYP2B4 into CYP1A2 would make the P1-CYP1A2 unable to form homomeric complexes. To test this, the chimeric (P1-CYP1A2) and wild-type (WT-CYP1A2) proteins were then expressed in HEK293T/17 cells, and complex formation was examined using BRET. The results indicate that residues in the proximal region of CYP1A2 were responsible for homomeric complex formation and that the CYP1A2•CYP1A2 complex
interferes with POR binding. Again, modified P₁-CYP1A2 was incapable of disrupting POR binding.

**EXPERIMENTAL**

**Materials**

HEK293T/17 cells were obtained from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM), PBS, EDTA, SDS, NuPAGE 10% Bis-Tris gel and Lipofectamine 2000 were purchased from Invitrogen (Eugene, OR). Coelenterazine 400A, kanamycin and DTT were purchased from Gold Biotechnology (St. Louis, MO), and coelenterazine h was purchased from Promega (Madison, WI). The BRET vectors were purchased from BioSignal Packard (Waltham, WA). Mutagenesis primers were purchased from Integrated DNA Technologies (Coralville, IA). Zeocin was purchased from Invivogen (San Diego, CA). NADPH, cumene hydroperoxide (CHP), 5-aminolevulinic acid (ALA) magnesium chloride (MgCl₂), phenylimethylsulfonyl fluoride (PMSF), catalase, calcium chloride (CaCl₂), dilaurylphosphatidylcholine (DLPC) and sodium chloride (NaCl) were purchased from Sigma (St. Louis, MO). 7-Ethoxyresorufin (7ER) was purchased from AnaSpec (Fremont, CA). HEPES buffer and glycine were purchased from Biomatik Corporation (Ontario, Canada). Potassium chloride (KCl), sodium bicarbonate (NaHCO₃) and monobasic potassium phosphate (KH₂PO₄) were purchased from Mallinckrodt Pharmaceuticals (Hazelwood, MO). Dibasic potassium phosphate (K₂HPO₄) was purchased from EMD chemicals (Gibbstown, NJ). Acetonitrile was purchased from Fisher Scientific (Waltham, MA). Complete EDTA-free PI tablets were purchased from Roche (Indianapolis, IN). 0.25% trypsin and bis(sulfosuccinimidyl)suberate (BS3) were purchased from Gibco-Thermo Fisher (Waltham, MA). Sucrose and Tris-HCl were purchased from DOT Scientific Inc. (Burton, MI). Bromophenol blue and β-mercaptoethanol were purchased from Bio-Rad (Hercules, CA). Anti-CYP1A2 antibody 56073 was purchased from Abcam (Cambridge, MA). Mouse anti-green fluorescent protein (GFP) monoclonal antibody was purchased from Millipore (Burlington, MA). Cytochrome P450 reductase, pAb was purchased from Enzo (Farmingdale, NY). Anti-rabbit IgG (whole molecule) peroxidase conjugate and anti-mouse IgG (whole molecule) peroxidase conjugate were purchased from Sigma (St. Louis, MO). Anti-CYP1A2 antibody (Proteintech 19936-1-AP) was purchased from Proteintech (Rosemont, IL). Anti-CYP1A2 antibody (Abcam 4227) was purchased from Abcam (Cambridge, MA).

**Generation of Fusion Proteins**

The GFP² and Rluc BRET vectors were generated according to previous methods [17]. Briefly, for generation of the chimeric CYP1A2 proteins, the specific surface region of CYP1A2 was identified from the crystal structure (P₁: L91-K106) and substituted with the homologous residues of CYP2B4 (P₁: T81-S96) using molecular cloning. Primers were designed to insert and amplify the homologous surface region of CYP2B4 into the multiple cloning site (MCS) of the CYP1A2 molecule using polymerase chain reaction (PCR). The forward primer 5’-CCC GTG GTG GTG CTC AGC GGC ACG ATC GCC GCC GCC-3’ and the reverse primer 5’- GCT GTA GTC AGG CCG GCC AGA AAA GGC CTC AGC TTG GTC-3’ were used to insert the P₁ region of CYP2B4 into the CYP1A2
molecule. The first round of PCR was performed to insert the CYP2B4 site into the MCS of CYP1A2. The PCR product was then verified by gel electrophoresis (1.5% agarose) and purified. The second round of PCR was performed to extend the CYP1A2 sequence, generating the complete CYP1A2 transcript, according to previous protocols [18-20]. This was followed by Dpn-1 digestion (New England Biolabs Inc., Ipswich, MA) for 2 hours at 37°C, followed by transformation of the GFP2 and Rluc-tagged vectors in DH5α cells (New England Biolabs Inc., Ipswich, MA) on zeocin and kanamycin plates, respectively. DNA from individual colonies was then isolated and sequenced. The positive clones were expressed in the HEK293T/17 cell line.

**Generation of unlabeled proteins using site-directed mutagenesis**

The QuikChange mutagenesis kit was purchased from Agilent (Santa Clara, CA). A base pair (T1550) on the fusion protein sequence was deleted to create a stop codon to generate the unlabeled WT-CYP1A2 and unlabeled P1-CYP1A2. The primers were designed using the primer design tool on the QuikChange Primer Design website. Forward primer 5′-CTTCTCCGACCAGTGATCCCACCGGTCG-3′ and reverse primer 5′-CGACCGGTGGGATCACTGGTCGGAGAAG-3′ were used with the GFP2 tagged fusion protein as templates. PCR was followed by Dpn-1 digestion at 37°C for 1 hour. This was then transformed into XL-Gold Ultracompetent cells, followed by transformation on zeocin plates. DNA from the colonies was isolated and sequenced. The positive clones were then transfected and expressed in HEK293T/17 cells.

**Cell Culture**

HEK293T/17 cells were maintained at 37°C with 5% CO2 in a humidified atmosphere. Dulbecco’s modified Eagle’s medium (DMEM) was supplemented with 10% fetal bovine serum and 1X antibiotic-antimycotic solution (250ng/ml amphotericin B, 100μg/ml streptomycin and 100U/ml penicillin) and was used as the cell growth media. The cells were grown in 10 cm plates.

**Transfection for BRET**

Six well plates were seeded from the 10 cm plates. Transfection was carried out at >75% confluence the next day. Transfections were carried out with preformed complexes of Lipofectamine 2000 and DNA. Cells were transfected with different ratios for the GFP2 and Rluc constructs, and DNA amounts adjusted to maintain similar amounts of total protein expression. This was done to create a series of conditions (cells) expressing approximately the same amount of total protein at a range of GFP to Rluc ratios to ensure that the BRET complex formation was specific and that the disruptions in the signal were not due to protein crowding. Each transfection was performed with three types of accompanying controls: (1) un-transfected cells, (2) cells expressing the GFP2-Rluc fusion protein and (3) cells transfected with the Rluc-tagged protein from the experimental BRET pair alone (in the absence of GFP2-tagged protein).
**BRET Assays**

The goal of these studies was to determine whether two proteins of interest formed physical complexes. The idea is to label one protein with a Renilla luciferase tag, and the other with a GFP tag. These fusion proteins are then co-transfected into HEK293T/17 cells at increasing GFP:Rluc ratios, keeping the total protein expression constant. The substrate Coelenterazine 400A, is then added to the transfected cells. Coelenterazine 400A causes activation of Rluc, leading to luminescence at 410 nm. If the proteins of interest form a complex, then the Rluc luminescence is absorbed by the GFP, leading to fluorescence at 510 nm. The BRET signal was measured as a ratio of the GFP and Rluc signals. As the GFP:Rluc ratio in the cells is increased, there is an increase in the BRET signal which saturates at a maximum value. In the event that the proteins do not form a complex, the 510 nm fluorescence is not expected. BRET measurements were conducted using modifications of existing protocols [21-24].

GFP fluorescence was checked 24 hours post transfection using fluorescence microscopy to ensure expression of the GFP2-tagged fusion proteins. Cells were harvested with 1 ml of 1X PBS, centrifuged and resuspended in 650 μl of 1X PBS. The suspension (100 μl) was added in quadruplicate to an opaque white 96-well plate. For the BRET measurement, a TriStar LB 941 microplate reader (Berthold Technologies, Bad Wildbad, Germany) was used. Coelenterazine 400A substrate was diluted in PBS and was used to measure the BRET signal for each well. The instrument was programmed to inject a 100 μl of the substrate solution into each the well, shake for 1 second to mix and then read the Rluc emission at 410 nm for 3 seconds followed by the GFP emission at 515 nm for 3 seconds. The average GFP and Rluc values from the untransfected cells were used to zero out each individual measurement. Individual measurements were used to calculate the raw BRET signal by dividing the GFP signal by the Rluc signal. The baseline BRET ratio generated by the cells transfected with the Rluc-tagged protein alone was then subtracted from the raw BRET signal to obtain the net BRET value.

**Determination of Relative Protein Expression**

Relative protein expression levels were measured using a spectrofluorometer. The photometric measurements of the GFP2- and Rluc-tagged proteins were compared to the measurements of a cell transfected with the GFP2-Rluc fusion protein. The Rluc expression was measured by adding coelenterazine h in 1X PBS to a final concentration of 5 μM in the opaque white 96-well plate. GFP expression was determined by measuring fluorescence at 410 nm excitation and 515 nm emission. The cell suspension was added to each well of a black 96-well clear bottom plate and the plate was read on the SpectraMax M5 plate reader. The GFP and Rluc signals of each sample were normalized against that of the GFP2-Rluc fusion protein. The fusion protein was assumed to have a 1:1 GFP: Rluc expression ratio and dividing the normalized GFP value by the normalized Rluc value yielded an approximation of the actual GFP: Rluc expression ratio.

**Immune blotting to quantify levels of transfected protein**

For protein quantification, 15 μl of sample was loaded into individual lanes of an SDS/PAGE (10% gel). The gel was run at 140 V for 65 min. Gels were then transferred on to
nitrocellulose membranes and immunoblotted using anti-CYP1A2 antibodies. Proteins were quantified by comparison to a standard curve generated with the purified proteins.

**Competition Experiments**

In order to establish more completely a direct interaction between two proteins, the BRET complexes were disrupted by transfection of varying amounts of unlabeled protein. The rationale for these studies is that the BRET complex formed by two labeled proteins can be disrupted by co-transfection of an unlabeled protein that competes for the protein binding site. A BRET pair of the two fusion proteins that form a complex are transfected at a high GFP:Rluc ratio, which will lead to a BRET$_{\text{max}}$ response. We then co-transfect different amounts of an unlabeled protein that may compete for the same binding site. If the unlabeled protein competes with the BRET pair, a decrease in the BRET signal would be expected; however, if the unlabeled protein does not compete, the signal from the BRET pair will be unchanged. The BRET signal was plotted as a function of concentration of the unlabeled protein (determined by immune blotting). The levels of the labeled proteins were not affected by the addition of unlabeled proteins at the levels used in these experiments. The relative concentrations of the GFP-labeled proteins were always in excess of the unlabeled competitors.

**Transfection for activity measurements**

Cells were seeded in 10 cm plates and transfections were carried out at >75% confluence using preformed complexes of Lipofectamine 2000 and DNA. DMEM media was supplemented with 1mM ALA. P$_1$-CYP1A2-GFP and WT-CYP1A2-GFP, were transfected into HEK293T/17 cells and co-transfected with and without POR. One 10cm plate was used for each condition. Each condition was measured as triplicate transfections with the data reported as the Mean ± SD.

**Cell Lysis and extraction**

Forty hours after transfection, media were replaced with DMEM supplemented with 10% fetal bovine serum, 1X antibiotic-antimycotic solution (250ng/ml amphotericin B, 100μg/ml streptomycin and 100U/ml penicillin), and 1M ALA. The cells were harvested 48 hours post transfection with 1X PBS, and the samples centrifuged at 201 x g at 4°C for 5 minutes. The media was aspirated, and the pellet was resuspended in 1 ml of hypotonic buffer (10 mM HEPES at pH of 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 0.5 mM DTT, and EDTA-free Protease Inhibitor tablet (1 tablet per 5 ml buffer)) and placed on ice for 30 minutes. The suspension was then passed 7 times through a 27½-gauge needle. This was followed by centrifugation at 560 x g for 6 minutes, and the supernatant collected and kept on ice.

**Activity measurement**

7 Ethoxyresorufin - deethylation was determined by monitoring the formation of the fluorescent product resorufin as described [14, 25]. The final concentrations of reaction components were: 355 μl cell lysate, 15 mM MgCl$_2$, and 10,000 units/ml catalase, in 50 mM HEPES buffer (pH 7.5). The reaction was initiated by the addition of either 4 μM 7-ethoxyresorufin (7-ER) and 250 μM NADPH or 0.02 mM 7-ethoxyresorufin (7-ER) and
4 mM cumene hydroperoxide (CHP). Fluorescence was measured at 535 nm (excitation) and 585 nm (emission) at 25°C. Initial rates were calculated using a standard curve generated from known quantities of resorufin. The activities were normalized to P450 protein expression from western blot.

As the constructs used for activity measurements contained the C-terminal GFP tags, we were interested in determining whether there were any major changes in activity resulting from the presence of the GFP. In separate experiments, NADPH-supported EROD activity was measured using both the WT-CYP1A2-GFP and unlabeled WT-CYP1A2 constructs. Although EROD activity for WT-CYP1A2-GFP was about 50% lower than that of the unlabeled construct, the activities of both the unlabeled and GFP constructs were responsive to changes in POR levels.

Data analysis

The BRET data in Figs. 2-5 are from a single experiment with each point being measured in triplicate. Generally, the error bars were smaller than the size of the data points. Because there is day-to-day variability in transfection levels, both the x- and y-axis values varied with repeated transfections. However, each experiment was repeated on separate days with newly transfected proteins to ensure reproducibility. Similar results were obtained with each of the repeated experiments. The 7-ethoxyresorufin activity data are represented as the mean ± SD for three transfections conducted on separate days. GraphPad Prism 5 was used to analyze and plot the data.

RESULTS

This study was prompted by previous data suggesting that the sigmoidal non-Michaelis-Menten CYP1A2 response, when measured as a function of POR concentration, was the result of a CYP1A2•CYP1A2 complex that impaired the binding of POR. This hypothesis was corroborated by the CYP1A2 crystal structure [2], which showed CYP1A2 monomers oriented with their proximal regions in contact with one another (Fig. 1). As the proximal region is the known location of the POR•P450 redox complex, the putative CYP1A2•CYP1A2 complex could inhibit its ability to interact with POR.

In an effort to identify the contact region responsible for formation of the CYP1A2•CYP1A2 complex, chimeric proteins were generated with the proximal region (L91-K106) of CYP1A2, identified as P1 (Fig. 1). As explained in our previous review [2], the rationale for focusing on this region was due to the observation of a proximal-proximal CYP1A2•CYP1A2 complex based on its crystal structure, and the sigmoidal kinetic relationship seen when CYP1A2-mediated activities were measured as a function of the POR [26]. These results suggested that the CYP1A2•CYP1A2 complex was inhibitory and needed to dissociate in order to unmask its activity. In a previous study, we were able to show that unlike CYP1A2, CYP2B4 did not appear to be capable of forming inhibitory BRET-detectable homomeric complexes [13, 17]. Taking advantage of this difference, we replaced a region of CYP1A2 with the homologous region of CYP2B4. The ability of this chimera to form and disrupt complexes was detected using BRET.
In the first experiments, the ability of P1-CYP1A2 (CYP1A2 with the modified P1 region) to form a binary complex was compared to that of the naturally occurring wild-type-CYP1A2 (WT-CYP1A2) binary complex. Both the wild type and chimeric proteins were expressed with either Renilla luciferase (Rluc) or GFP, with the labels attached to the C-termini of the proteins to avoid affecting CYP1A2 membrane binding. These constructs were transfected into HEK293T/17 cells at different GFP:Rluc ratios with the goal of maintaining constant total tagged protein expression [27]. When the two labeled proteins form a complex, there is an increase in the BRET signal that saturates at a high GFP:Rluc ratio. This can readily be observed in Fig. 2A where WT-CYP1A2-GFP forms a complex with WT-CYP1A2-Rluc (blue). Interestingly, the P1-CYP1A2-GFP\textsuperscript{2}/P1-CYP1A2-Rluc pair generated a lower BRET\textsubscript{max} (red) suggesting a decrease in complex formation. The decrease in the BRET\textsubscript{max} signal suggests that the P1 region may influence CYP1A2 homomeric complex formation. Fig. 2B shows that the total protein levels were similar for both WT-CYP1A2 and P1-CYP1A2 chimeras, precluding the potential difference in the BRET signals in Fig. 2A being due to differences in total protein concentration [27]. The fluorescence levels of the GFP\textsuperscript{2} (Fig. S1A) and luminescence levels of Rluc-constructs (Fig. S1B) for each data point are shown in Supporting Information.

To establish that the P1 substitution affected complex formation, a competition study was performed to determine if unlabeled P1-CYP1A2 could disrupt the WT-CYP1A2•WT-CYP1A2 complex (Fig. 2C). WT-CYP1A2-GFP\textsuperscript{2} and WT-CYP1A2-Rluc were transfected at a high GFP:Rluc ratio (about 30:1), approximating BRET\textsubscript{max}. The BRET pair was co-transfected with either unlabeled WT-CYP1A2 or unlabeled P1-CYP1A2. If unlabeled protein (not containing either GFP\textsuperscript{2} or Rluc tags) disrupted the BRET complex (i.e., by competing with GFP\textsuperscript{2}-tagged protein for Rluc-tagged protein binding), a decrease in BRET signal would be observed, with the magnitude of decrease being proportional to the unlabeled protein expression level. As expected, co-transfection of unlabeled WT-CYP1A2 led to a concentration-dependent disruption of the BRET signal. In contrast, co-transfection of unlabeled P1-CYP1A2 did not affect the WT-CYP1A2•WT-CYP1A2 complex formation. These results indicate that the binding site of CYP1A2 was affected by modification of the proximal region (L91-K106) in the chimera.

In the next set of experiments, the ability of P1-CYP1A2 to form a physical complex with CYP2B4 was compared to that of WT-CYP1A2 (Fig. 3). The rationale for this experiment is based on the idea that the contact regions for the CYP1A2•CYP2B4 complex differed from those of the homomeric CYP1A2 complex. WT-CYP2B4-GFP\textsuperscript{2} and WT-CYP1A2-Rluc were co-transfected into HEK293T/17 cells (blue). This was repeated with WT-CYP2B4-GFP\textsuperscript{2} and P1-CYP1A2-Rluc (red). Both WT-CYP1A2 and P1-CYP1A2 were capable of forming a complex with CYP2B4. In fact, the BRET\textsubscript{max} value for P1-CYP1A2 was slightly higher, but clearly not diminished. Fig. 3B shows that the lower signal for the WT/WT curve was not due to lower total protein expression. Again, the fluorescence of the GFP\textsuperscript{2} (Fig. S2A) and luminescence of Rluc-constructs (Fig. S2B) for each data point are shown in Supporting Information.

In an effort to determine if the P1 substitution affected complex formation between CYP1A2 and CYP2B4, the ability of unlabeled WT- and P1-CYP1A2 to disrupt the WT-
CYP1A2•WT-CYP2B4 BRET complex was examined (Fig. 3C). WT-CYP2B4-GFP\(^2\) and WT-CYP1A2-Rluc were transfected at a high GFP:Rluc ratio (about 50:1), approximating BRET\(_{\text{max}}\). The BRET pair was co-transfected with either unlabeled WT-CYP1A2 or unlabeled P\(_1\)-CYP1A2. Both unlabeled WT-CYP1A2 and the P\(_1\)-CYP1A2 were equally effective at disruption of the WT-CYP1A2•WT-CYP2B4 complex. The data in Fig. 3C indicate that P\(_1\)-CYP1A2 still had an intact binding site that was capable of associating with CYP2B4.

The activity of P\(_1\)-CYP1A2 was measured using the substrate 7-ethoxyresorufin. P\(_1\)-CYP1A2-GFP\(^2\) and WT-CYP1A2-GFP\(^2\) were separately transfected, and as an additional experiment, WT-CYP1A2 and P\(_1\)-CYP1A2 were co-transfected with POR. After transfection, the cells were lysed and EROD was measured to determine whether P\(_1\)-CYP1A2 was functional (Table 1) using either CHP or NADPH. P\(_1\)-CYP1A2 was functional in the CHP-supported reaction, although its activity was lower than that of WT-CYP1A2, suggesting that the modification did have some effect on catalysis. As the CHP-supported reaction does not require POR, its activity was not stimulated by POR co-transfection. However, a different response was observed when NADPH-supported activity was examined. When co-transfected with POR (POR:CYP1A2 ~ 1:1), the NADPH-supported activity of WT-CYP1A2 exhibited a 12.5-fold increase (when comparing low vs high POR). In contrast, P\(_1\)-CYP1A2 only exhibited a 2-fold increase in activity by the co-transfection of POR. These results indicate that the P\(_1\) substitution has a marked effect on its ability to be catalyzed by NADPH, and suggest that the lower activity of the P\(_1\)-CYP1A2 was due, primarily, to an impaired ability to form a functional complex with POR.

With the CYP1A2•CYP1A2 (Fig. 2) and POR•CYP1A2 complexes [2-4, 28] reported to involve the proximal face of the P450 enzyme, as well as the decreased ability of P\(_1\)-CYP1A2 to be stimulated by POR in the NADPH-supported reaction (Table 1), we expected both CYP1A2•CYP1A2 (Fig. 2) and POR•CYP1A2 interactions to be differentially inhibited by the P\(_1\)-substitution. In order to test this hypothesis, the ability of P\(_1\)-CYP1A2 to form a complex with POR was examined using BRET (Fig. 4). WT-CYP1A2-GFP\(^2\) and WT-POR-Rluc were co-transfected into HEK293T/17 cells (blue). This was repeated with P\(_1\)-CYP1A2-GFP\(^2\) and WT-POR-Rluc (red). The decrease in BRET\(_{\text{max}}\) of the P\(_1\)-CYP1A2 curve (red) is consistent with diminished binding of the chimera with POR (Fig. 4A), not a surprising result as the proximal region of CYP1A2 is involved in complexation with POR [2, 13, 17]. Fig. 4B shows that total protein expression was not significantly different for the WT- and P\(_1\)-constructs. Supporting Information shows the fluorescence (Fig. S3A) and luminescence (Fig. S3B) levels of each of the constructs.

In order to establish that the binding of P\(_1\)-CYP1A2 to POR was impaired, the ability of unlabeled WT-CYP1A2 and unlabeled P\(_1\)-CYP1A2 to disrupt the WT-POR•WT-CYP1A2 BRET complex were compared (Fig. 4C). WT-CYP1A2-GFP\(^2\) and WT-POR-Rluc were transfected at an elevated GFP:Rluc ratio (about 50:1), approximating BRET\(_{\text{max}}\). The BRET pair was co-transfected with either unlabeled WT-CYP1A2 or unlabeled P\(_1\)-CYP1A2. As expected, co-transfection of unlabeled WT-CYP1A2 led to a concentration-dependent disruption of the BRET signal. In contrast, co-transfection of unlabeled P\(_1\)-CYP1A2 was
unable to disrupt POR•CYP1A2 complex formation. These results indicate that the POR binding site was affected by the P1-substitution into CYP1A2.

Fig. 3 demonstrated that the P1 substitution did not affect the ability of CYP1A2 to interact with CYP2B4, however, Fig. 4 showed that the P1 modification impaired the ability of CYP1A2 to interact with POR. Therefore, we also would expect P1-CYP1A2 and WT-CYP1A2 to affect POR binding to CYP2B4 differently. Previous experiments established that CYP1A2 and CYP2B4, when co-expressed, are able to form a heteromeric CYP1A2•CYP2B4 complex [17]. Although POR is able to bind to this complex thereby forming a ternary complex, POR was shown to selectively associate with the CYP1A2 moiety of this complex at sub saturating POR. POR could only bind to the CYP2B4 moiety once all the CYP1A2-binding sites were occupied [10, 14, 15, 17]. As the P1-substitution affected POR binding to CYP1A2, we expected that its ability to disrupt the POR•CYP2B4 complex would also be affected. In an effort to test this, the ability of unlabeled WT- and P1-CYP1A2 to disrupt the POR•CYP2B4 complex was examined. WT-CYP2B4-GFP2 and WT-POR-Rluc were transfected at a high GFP:Rluc ratio (about 44:1), approximating BRETmax. The BRET pair was co-transfected with either unlabeled WT-CYP1A2 or unlabeled P1-CYP1A2. Fig. 5 shows that the presence of unlabeled WT-CYP1A2 caused a significant disruption of the WT-POR•WT-CYP2B4 complex; however, the P1-substitution was less disruptive. Taken together, these results show that despite the ability of P1-CYP1A2 to form a heteromeric CYP1A2•CYP2B4 complex (Fig. 3C), its ability to disrupt the POR•CYP2B4 complex, due to its proximal substitution, was diminished.

DISCUSSION

Interactions among the P450s in the endoplasmic reticulum affect their catalytic efficiency [29]. Previous studies have shown that the presence of one P450 enzyme can influence the function of another P450 when combined in a reconstituted system [30, 31]. Various heteromeric P450•P450 interactions and their effect on metabolic function have been reported for CYP2C9•CYP2D6, CYP2C9•CYP3A4, CYP1A2•CYP2B4, CYP1A2•CYP2E1, CYP1A1•CYP3A2, CYP2E1•CYP2B4, CYP2D6•CYP2E1, CYP3A4•CYP2E1, and CYP2C9•CYP3A4 [4, 5, 7, 9, 11, 14-17, 29, 32, 33].

Evidence of homomeric interactions amongst the P450s have also been observed. Studies have established the impact of these complexes on metabolic function, and in some cases, identified the regions within these structures that are involved in complex formation. Studies using cysteine scanning and cross-linking, or oxidation of sulfhydryl groups to disulfides have found that Cys-24 of the linker region and specific residues in the F-G loop of CYP2C8 promote dimer formation both in mammalian endoplasmic reticulum and as a recombinant form in bacterial membranes. Furthermore, the CYP2C8 dimer was shown to be similar (but not identical) to the CYP2C8 X-ray crystal structure [34]. Both fluorescence resonance energy transfer [35] and bimolecular fluorescence complementation [36] were used to show that CYP2C2 also formed homo-oligomeric structures when tagged forms were expressed in Cos1 and HEK cells and that the N-terminal anchor was probably involved in the contact region of the quaternary structure.
Studies by Davydov have used many approaches to demonstrate homomeric P450 interactions. Rapid scanning stopped flow spectrophotometry revealed multi-phasic reduction of CYP2B4 [37] and CYP3A4 [38-40] by both dithionite and the reductase domain of BM3, providing evidence for homomeric oligomerization of the P450s. Evidence for homomeric interactions of these enzymes was also provided by hyperbaric induced changes in the P450 spin states [41, 42]. His work also identified regions responsible for homomeric CYP3A4 interactions using cysteine scanning combined with chemical cross-linking, and both luminescence and fluorescence resonance energy transfer. As we have proposed in this study for the homomeric CYP1A2 complex, several of the Davydov studies have provided evidence for a homomeric CYP3A4 quaternary structure in purified, reconstituted systems that is similar to an arrangement in its X-ray crystal structure [7, 43, 44].

Further evidence of homomeric complex formation was found with P450c17 (CYP17A1) and P450arom (CYP19A1) [45]. According to the authors, the P450c17 and P450arom form homomeric but not heteromeric complexes. It was proposed that this binding selectivity may play a functional role in segregating different pathways of steroid metabolism [45]. In addition, homomeric complexes could also allow for sequential metabolism of intermediates in the multi-step pathway required for generation of the final steroid products. This would be consistent with the study performed by Yamazaki et al [46].

**CYP1A2•CYP1A2 Complex –**

In the current study, CYP1A2 was shown to form a homomeric complex involving surface residues in the proximal region. An initial examination showed that a stable complex was observed in the crystal structure with the proper orientation to reside in the membrane [2, 47]. The crystal structure suggested the formation of a proximal-proximal complex. The potential for such a complex was therefore examined by substituting a region of the proximal face of CYP1A2 (P₁ (L91-K106)), with the homologous region of CYP2B4 (T81-S96). The BRET signal of P₁-CYP1A2 was diminished when compared to the WT-CYP1A2, suggesting that the homomeric interaction was affected. This was confirmed in the experiment showing that unlabeled WT-CYP1A2 was able to disrupt the WT-CYP1A2•WT-CYP1A2 BRET complex, but the unlabeled P₁-CYP1A2 was less effective (Fig. 2C). The inability of unlabeled P₁-CYP1A2 to disrupt the WT-CYP1A2•WT-CYP1A2 complex demonstrated that the P₁-region was responsible for the formation of this complex.

Although P₁-CYP1A2 had a lower catalytic activity than the wild-type protein with both NADPH and CHP assay, it was functional, thereby supporting the idea that protein folding was not significantly affected. There are at least two possible ways that the P₁ substitution could affect protein function, (a) the substitution could affect the P450 active site, leading to diminished activity, and/or (b) the substitution could affect its ability to associate with POR. There is clear precedent for the modification of distant sites to affect enzyme activity, most recently in a report on the naturally occurring CYP2C9*2 mutant [48]. However, the major reason for the diminished activity of P₁-CYP1A2 is due to impaired POR binding. The ability of CHP to support EROD for P₁-CYP1A2 showed that the modified protein was functional, albeit at a diminished rate, and the CHP-supported activity was not stimulated
by co-transfection with POR. However, the substantial increase in NADPH-supported EROD for WT-CYP1A2 in the presence of POR was not observed with P1-CYP1A2. This is consistent with the P1 substitution disrupting both the CYP1A2•CYP1A2 homomer (Fig. 2) as well as the POR•CYP1A2 complex (Fig. 4C). This is consistent with molecular modeling data that indicates that K94 and K105 of CYP1A2 (rat isoform) are involved in the CYP1A2•POR heteromeric complex formation [49]. The homologous amino acids (K95 and K106 of rabbit CYP1A2) fall within the P1 region.

These results clearly demonstrate that the proximal region of CYP1A2 is involved in the formation of the homomeric CYP1A2•CYP1A2 complex and the heteromeric POR•CYP1A2 complex. They also provide a mechanistic explanation for the sigmoidal kinetic responses that were previously reported for CYP1A2 when measured as a function of [POR] [13]. The results point to the idea that the region responsible for this complex is distinct from the contact residues that form the CYP1A2•CYP2B4 complex, and clearly demonstrate that P450•P450 complexes are unique for the different proteins involved and can be affected by small structural differences in the proteins.

**Interactions with CYP2B4 –**

As previously reported [10, 14, 15, 17], CYP1A2 forms a stable complex when in the presence of CYP2B4, and formation of this complex leads to the selective binding of POR to the CYP1A2 moiety of this complex. The fact that the CYP1A2•CYP2B4 complex does not mask CYP1A2-mediated activities (as does the CYP1A2•CYP1A2 complex) suggests that CYP2B4 associates with a different surface region of CYP1A2 than POR, and that formation of the CYP1A2•CYP2B4 complex causes dissociation of homomeric CYP1A2•CYP1A2. In this report, we have shown that CYP1A2 interacts with POR on the proximal face and that the P1 substitution affects the ability of CYP1A2 to bind to POR. This led us to the hypothesis that the proximal region (P1) is not involved in the CYP1A2•CYP2B4 complex. This is borne out by these studies showing that the heteromeric CYP1A2•CYP2B4 BRET pair was equally disrupted by both WT-CYP1A2 and the P1-CYP1A2 (Fig. 3C). These results show that both P1- and WT-CYP1A2 are capable of associating with CYP2B4, and that the proximal region of CYP1A2 is not involved in formation of the CYP1A2•CYP2B4 complex.

Based on the data in this report, we would anticipate that the P1 modification will allow for the formation of a stable CYP1A2•CYP2B4 complex. However, because P1-CYP1A2 has an altered POR binding site, it would not be expected to disrupt the POR•CYP2B4 complex as effectively as WT-CYP1A2. This prediction is supported by the data in Fig. 5.

Taking this data together with that of our previous publications [17, 26], the following scheme can be envisioned (Fig. 6). The WT-CYP1A2 is readily able to form a homomeric complex (Fig. 6A). This CYP1A2•CYP1A2 complex cannot be disrupted by the presence of P1-CYP1A2, which is consistent with the involvement of the proximal region as essential for homomeric complex formation. This complex can be readily disrupted in the presence of POR, which will permit CYP1A2 to function. CYP2B4 also readily combines with CYP1A2, which disrupts formation of the homomeric CYP1A2•CYP1A2 complex and exposes the CYP1A2 proximal surface to allow POR binding. Formation of the
CYP1A2•CYP2B4 complex increases the affinity of the CYP1A2 moiety of the complex to bind to POR. POR selectively binds to the CYP1A2 moiety of the CYP1A2•CYP2B4 complex at subsaturating POR.

Figure 6B shows the potential interactions with the P₁ construct. Because of the substitution of proximal residues, the P₁ construct is much less capable of forming a homomeric complex. As expected, this proximal substitution also disrupts its ability to interact with POR. In contrast, P₁-CYP1A2 is perfectly capable of interacting with CYP2B4 and thus demonstrates that this interaction involves another surface region of CYP1A2. However, because of its proximal substitution, P₁-CYP1A2 cannot bind to POR. This leaves the POR available to bind to CYP2B4.

Although much has been learned about the effect of P450-P450 interactions on their function, much more information is needed before we can more completely understand how the presence of multiple P450s can affect drug disposition. Based on our current knowledge of CYP1A2, we know that CYP1A2 exists with many other P450s within the endoplasmic reticulum. We also know that the POR concentration is limiting in vivo [50], and that CYP1A2 must compete with all of the other P450s in order to function. The presence of other P450s and consequently the competition for POR is expected to effectively decrease the relative amount of POR that would be available to bind to CYP1A2. Our current data show that CYP1A2 tends to exist as a proximal-proximal dimer, and that the CYP1A2•CYP1A2 complex will limit its ability to bind with POR and consequently mask its activity [17, 26].

Of course, this scheme will depend on which other P450s are present. We know that CYP1A2 forms complexes with other P450s such as CYP2B4 [15] and CYP2E1 [16], which can be induced by numerous drugs and alcohol. Interestingly, in cases where CYP2B4 is induced, the formation of a CYP1A2•CYP2B4 complex leads to the dissociation of the CYP1A2•CYP1A2 homomers and unmasks CYP1A2-mediated activities [17]. This may also be occurring after induction of CYP2E1 by alcohol and similar inducers where CYP1A2•CYP2E1 complexes can affect monoxygenase function [16, 51]. Whether interactions between CYP1A2 and other P450 enzymes occur, have yet to be determined. Taken together, the function of the P450 system is not simply dependent on the P450s competing for the available POR but is also dependent on the multiple complexes that may form, and the specific P450s that are induced within an organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

All data are contained within the manuscript and supporting information.

Abbreviations:

- (WT-POR) Wild-type NADPH-Cytochrome P450 reductase
- (Rluc) Renilla luciferase
- (WT-CYP1A2) wild-type CYP1A2
- (P1-CYP1A2) (CYP1A2 with the modified P1 region)
- (P450) cytochrome P450
- (7ER) 7-ethoxyresorufin
- (EROD) 7-ethoxyresorufin-O-dealkylation
- (CYP1A2) cytochrome P450 1A2
- (WT-CYP2B4) wild-type cytochrome P450 2B4
- (CYP2E1) cytochrome P450 2E1
- (BRET) bioluminescence resonance energy transfer
- (PAGE) polyacrylamide gel electrophoresis
- (SDS) sodium dodecyl sulfate
- (ALA) 5-aminolevulinic acid
- (CHP) cumene hydroperoxide
- (BS3) bis(sulfosuccinimidyl)suberate
- (DLPC) dilaurylphosphatidylcholine
- (NaCl) sodium chloride
- (KCl) potassium chloride
- (MgCl2) magnesium chloride
- (CaCl2) calcium chloride
- (NaHCO3) sodium bicarbonate
- (PMSF) phenylmethylsulfonyl fluoride
- (KH2PO4) monobasic potassium phosphate
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Figure 1:
Crystal structure depicting homomeric CYP1A2 dimer bound to the inhibitor, ANF (PDB:2HI4). The heme groups are shown as red spheres, F-G helices region in yellow on the distal face, the N-terminal region in blue. This figure shows putative homomeric dimerization of CYP1A2 at the proximal regions of both moieties of the dimer. The P₁ region is shown in magenta. Modified from Reed and Backes [2].
Figure 2:
A. Effect of replacement of a proximal segment P₁ of CYP1A2 with the homologous region from CYP2B4 on CYP1A2 homomeric complex formation. CYP1A2 fusion proteins were generated and transfected into HEK293T/17 cells at different ratios of the GFP:Rluc tags. Complex formation was assessed by measuring the BRET signals. WT-CYP1A2-GFP² and WT-CYP1A2-Rluc (WT/WT pair) produced a significant BRET signal demonstrating formation of a homomeric complex (blue). The P₁ region (L91-K106) of the CYP1A2-GFP² and CYP1A2-Rluc fusion proteins were replaced with the homologous region from CYP2B4 and co-transfected, followed by measurement of the BRET signals (red). B. Total labeled protein expression (sum of fluorescence of GFP and luminescence of Rluc). C. Disruption of the CYP1A2 homomeric complex by unlabeled WT-CYP1A2 and unlabeled P₁-CYP1A2. WT-CYP1A2-GFP² and WT-CYP1A2-Rluc were co-transfected into HEK293T/17 cells in the absence and presence of different amounts of unlabeled WT-CYP1A2 (blue) and P₁-CYP1A2 (red). A constant high ratio of WT-CYP1A2-GFP² to WT-CYP1A2-Rluc (30:1) was chosen and the effect of increasing unlabeled WT-CYP1A2 and P₁-CYP1A2 was measured. For all figures, X stands for WT-CYP1A2 or P₁-CYP1A2. The data points represent the mean ± S.D. for three determinations with the error bars being smaller than
the size of the data points. Each experiment was repeated by a separate transfection showing similar results.
Figure 3:
A. Effect of replacement of a proximal segment P₁ of CYP1A2 with the homologous region from CYP2B4 on CYP1A2•CYP2B4 heteromeric complex formation. CYP1A2 and CYP2B4 fusion proteins were generated and transfected into HEK293T/17 cells at different ratios of the GFP:Rluc tags. Complex formation was assessed by measuring the BRET signals. WT-CYP2B4-GFP² and WT-CYP1A2-Rluc (WT/WT pair) produced a significant BRET signal demonstrating formation of a heteromeric complex (blue). The P₁ region (L91-K106) of the CYP1A2-Rluc fusion protein was replaced with the homologous region from CYP2B4 and co-transfected with WT-CYP2B4-GFP², followed by measurement of the BRET signals (red).
B. Total labeled protein expression (sum of fluorescence of GFP and luminescence of Rluc).
C. Disruption of the CYP1A2•CYP2B4 heteromeric complex by unlabeled WT-CYP1A2 and unlabeled P₁-CYP1A2. WT-CYP2B4-GFP² and WT-CYP1A2-Rluc were co-transfected into HEK293T/17 cells in the absence and presence of different amounts of unlabeled WT-CYP1A2 (blue) and P₁-CYP1A2 (red). A constant high ratio of WT-CYP2B4-GFP² to WT-CYP1A2-Rluc (50:1) was chosen and the effect of increasing unlabeled WT-CYP1A2 and P₁-CYP1A2 was measured. For all figures, X stands for WT-CYP1A2 or P₁-CYP1A2. The data points represent the mean ± S.D. for three
determinations. In most cases, the error bars were smaller than the size of the data points. Each experiment was repeated by a separate transfection, showing similar results.
Figure 4:
A. Effect of the P₁ substitution on POR•CYP1A2 complex formation. The POR fusion protein (POR-Rluc) was generated and co-transfected with either the WT-CYP1A2-GFP fusion protein (blue) or P₁-CYP1A2-GFP (red) to measure the ability of the constructs to associate with POR. Complex formation was estimated using BRET. B. Total labeled protein expression (sum of fluorescence of GFP and luminescence of Rluc). C. Disruption of the CYP1A2•POR heteromeric complex by unlabeled WT-CYP1A2 and unlabeled P₁-CYP1A2. WT-CYP1A2-GFP² and WT-POR-Rluc were co-transfected into HEK293T/17 cells in the absence and presence of different amounts of unlabeled WT-CYP1A2 (blue) and P₁-CYP1A2 (red). A constant high ratio of WT-CYP1A2-GFP² to WT-POR-Rluc (50:1) was chosen and the effect of increasing unlabeled WT-CYP1A2 and P₁-CYP1A2 was measured. For all figures, X stands for WT-CYP1A2 or P₁-CYP1A2. The data points represent the mean ± S.D. for three determinations. In most cases, the error bars were smaller than the size of the data points. Each experiment was repeated by a separate transfection, producing similar results.
Figure 5:
Disruption of the POR•CYP2B4 complex by unlabeled WT-CYP1A2 and unlabeled P1-CYP1A2. WT-CYP2B4-GFP and WT-POR-Rluc were co-transfected into HEK293T/17 cells in the absence and presence of different amounts of unlabeled WT-CYP1A2 (blue) and P1-CYP1A2 (red). A constant high ratio of WT-CYP1A2-GFP to WT-POR-Rluc (44:1) was used and the effect of increasing unlabeled WT-CYP1A2 and P1-CYP1A2 was measured. The data points represent the mean ± S.D. for three determinations. In most cases, the error bars were smaller than the size of the data points. Each experiment was repeated by a separate transfection, producing similar results.
Figure 6:
Schematic describing the potential interactions among POR, CYP1A2, and CYP2B4, and the effect of the P₁ substitution on these complexes. A. Interactions with WT-CYP1A2. B. Interactions with P₁-CYP1A2.
Table 1:
Effect of P<sub>1</sub> substitution on CHP- and NADPH-supported EROD. HEK293T/17 cells were transfected with either WT-CYP1A2 or P<sub>1</sub>-CYP1A2 (low POR). Both proteins were also co-transfected with POR (high POR). The cells were lysed 48 h post-transfection and the EROD activities of the post-nuclear supernatants were determined. Both CHP- and NADPH-supported activities were determined and expressed as a function of the CYP1A2 concentration. The results represent the mean ± SD for 3 separate transfections. The POR and CYP1A2 levels were determined by immune blotting.

| POR:P450  | Cumene Hydroperoxide | Fold difference | NADPH       | Fold difference |
|-----------|----------------------|-----------------|--------------|-----------------|
| Low POR   | 0.2:1 ± 0.08         | 2.1±1           | 0.66         | 0.67±0.56       | 2.09 |
| High POR  | 1.7:1 ± 0.22         | 1.4±0.16        | 1.4±0.099    |                 |

| P<sub>1</sub>-CYP1A2-GFP<sup>2</sup> |
|-------------------------------------|
| POR:P450                           |
| Low POR                            | 0.2:1 ± 0.08     | 2.1±1           | 0.66         | 0.67±0.56       | 2.09 |
| High POR                           | 1.7:1 ± 0.22     | 1.4±0.16        | 1.4±0.099    |                 |

| WT CYP1A2-GFP<sup>2</sup> |
|--------------------------|
| POR:P450                 |
| Low POR                  | 0.1:1 ± 0.08     | 9±6             | 0.88         | 2±1.3          | 12.5 |
| High POR                 | 0.8:1 ± 0.03     | 8±0.6           |              | 25±0.003       |     |

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