Supplemental Materials:

Materials and Methods

Materials. *E. coli* C41 cells for protein overexpression were purchased from Lucigen (Middleton, WI). Yeast extract, tryptone, for unlabeled growth media was purchased from Sigma-Aldrich. [15N] ammonium chloride, [15N] CELTONE rich medium powder and D2O were purchased from Cambridge Isotope Laboratories (Andover, MA). Resins, buffer components, and all the other chemicals were purchased from Sigma-Aldrich. Glycerol used in NMR experiments was purchased from Sigma-Aldrich.

Expression and purification of the soluble FMN binding domain of rat CPR. The unlabeled and 15N labeled FBD as well as the full-length FBD was individually expressed and purified as described previously.[1] The U-15N-labeled FBD was expressed with unlabeled glucose and [15N] CELTONE rich medium powder. For expression of unlabeled FBD, the adapted cells were inoculated to 1 L LB medium at a starting OD600 value of 0.03 and induced at OD600 = 2. The purified FBD appeared as a single band on the SDS-polyacrylamide gel. The concentration of the oxidized FBD was determined using the extinction coefficients 12.2 mM\(^{-1}\)cm\(^{-1}\) at 454 nm.[2]

Full-length wild-type rabbit cytochrome P450 2B4 (cyt P450 2B4) and U-15N labeled full-length wild-type rabbit cyt b5 were expressed and purified individually as described previously.[1,3-5]

Solution NMR experiments. All solution NMR experiments were carried out at 298 K in NMR buffer (40 mM potassium phosphate, 10% D2O, pH 7.4) using a 600 MHz Bruker NMR spectrometer and a cryo-probe. All NMR data was processed by Topspin 2.1 (Bruker) and analyzed in Sparky.[6]

Substrate modulation on the interaction between cyt b5 and cytP450. To investigate the effects of a range of different substrates on the interaction between cyt b5 and cyt P450 2B4, similar approaches were applied as detailed in Zhang et al., 2015[7]. Briefly, each substrate, including methoxyflurane, benzphetamine and cyclohexane, was added to a 1:1 15N-cyt b5 : unlabeled cyt P450 2B4 complex sample. 2D 1H/15N TROSY HSQC spectra with 64 scans and 256 t1 increments.
Substrate modulation on competitive binding between cytb5 and trFBD in the trFBD-cytP450 -cytb5 tertiary protein system. 2D $^1$H/$^{15}$N TROSY HSQC NMR spectrum was first recorded for a sample of $^{15}$N-trFBD with one molar equivalence unlabeled cyt P450 2B4 in the absence or presence of three molar equivalence substrate methoxyflurane, benzphetamine, or cyclohexane. Then one molar equivalence of unlabeled cyt $b_5$ was added to the sample, followed by acquisition of 2D $^1$H/$^{15}$N TROSY HSQC NMR spectrum with 32 scans and 256 t1 increments.

References:


Supplemental Figure 1: Substrate effect on trFBD – cytP450 interaction. Comparison of the average relative intensity of $^{15}$N-labeled trFBD in complex with cytP450 in the absence and presence of different substrates.

Supplemental Figure 2: 2D $^1$H/$^{15}$N-TROSY HSQC NMR spectrum of $^{15}$N-cytb$_5$. 
**Supplemental Figure 3:** 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled cyt$b_5$ after titration with one molar equivalent of cytP450 2B4.

**Supplemental Figure 4:** 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled cyt$b_5$ after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of FBD.
Supplemental Figure 5: 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled cyt$b_5$ after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of flFBD.

Supplemental Figure 6: 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-cyt$b_5$ after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of CPR.
Supplemental Figure 7: 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled cyt$b_5$ after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of BHT.

Supplemental Figure 8: 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled cyt$b_5$ after titration with one molar equivalent of cytP450 2B4, one molar equivalent of BHT, and one molar equivalent of flFBD.
Supplemental Figure 9: 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled FBD.

Supplemental Figure 10: 2D $^1$H/$^{15}$N-TROSY-HSQC NMR spectrum of $^{15}$N-labeled FBD after titration with one molar equivalent of cytP450 2B4.
**Supplemental Figure 11:** 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-FBD after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of BHT. Peaks marked by ‘x’ and without contour color significantly broadened to the noise level due to FBD interaction with P450.

**Supplemental Figure 12:** 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-FBD after titration with one molar equivalent of cytP450 2B4, one molar equivalent of BHT, and one molar equivalent of cyt$b$s.
Supplemental Figure 13: 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-FBD after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of BZ.

Supplemental Figure 14: 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-FBD after titration with one molar equivalent of cytP450 2B4, one molar equivalent of BZ, and one molar equivalent of cyt$b5$. 
Supplemental Figure 15: 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-FBD after titration with one molar equivalent of cytP450 2B4 and one molar equivalent of MF.

Supplemental Figure 16: 2D $^{15}$N/$^1$H TROSY HSQC NMR spectrum of $^{15}$N-FBD after titration with one molar equivalent of cytP450 2B4, one molar equivalent of MF, and one molar equivalent of cytb5.