Effect of Detergent Binding on Cytochrome P450 2B4 Structure as Analyzed by X-ray Crystallography and Deuterium-Exchange Mass Spectrometry

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Graphical Abstract

ABSTRACT

Multiple crystal structures of CYP2B4 have demonstrated the binding of the detergent 5-cyclohexyl-1-pentyl- β -D-maltoside (CYMAL-5) in a peripheral pocket located adjacent to the active site. To explore the consequences of detergent binding, X-ray crystal structures of the peripheral pocket mutant CYP2B4 F202W were solved in the presence of hexaethylene glycol mono-octyl ether (C_8E_6) and CYMAL-5. The structure in the presence of CYMAL-5 illustrated a closed conformation indistinguishable from the previously solved wild-type. In contrast, the F202W structure in the presence of C_8E_6 revealed a detergent molecule that coordinated the heme-iron and extended to the protein surface through the substrate access channel 2f. Despite the overall structural similarity of these detergent complexes, remarkable differences were observed in the A, A', and H helices, the F-G cassette, the C-D and β_4 loop region. Hydrogen-deuterium exchange mass spectrometry (DXMS) was employed to probe these differences and to test the effect of detergents in solution. The presence of either detergent increased the H/D exchange rate across the plastic regions, and the results obtained by DXMS in solution were consistent in general with the relevant structural snapshots. The study provides insight into effect of detergent binding and the interpretation of associated conformational dynamics of CYP2B4.

Keywords: Cytochrome P450, X-ray Crystallography, Hydrogen-Deuterium-Exchange Mass Spectrometry

INTRODUCTION

The role of detergents is critical in the purification and crystallization of membrane proteins, which represent about one third of the proteins in mammalian genomes. Detergents provide the much needed solution stability to these membrane-associated macromolecules, thereby facilitating successful extraction and isolation of high quality preparations. Progress in the last ten years in crystallization and structure determination of membrane proteins can be attributed largely to effective protein purification methods that involve the use of appropriate detergents [1-4]. In particular, the growing list of crystal structures of mammalian cytochrome P450 (CYP)-dependent monooxygenases, which metabolize a vast array of drugs and endogenous chemicals, have used one or multiple detergents for purification and crystallization of these enzymes. However, in many of the structures analyzed no electron density of the detergents was observed in the actual crystal packing. One exception is CYP24A1, where the two available crystal structures demonstrated the presence of trapped detergent molecules in the hydrophobic access channel and binding pocket [5]. Four molecules of the detergent [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate] (CHAPS) were observed in one of the CYP24A1 crystal structures, with two molecules associated with membrane binding regions and structural lattice contacts, one in the access channel, and another in the active site above the heme. In a second structure, two molecules of 5-cyclohexyl-1-pentyl- β -D-maltoside (CYMAL-5) were located in the substrate access channels [5].

A majority of the crystal structures of mammalian CYP enzymes available in the Protein Data Bank (PDB) that illustrate the binding of detergents to cytochrome P450s are from the CYP2B subfamily. Over 25 crystal structures of N-terminally truncated and modified CYP2B4 and CYP2B6, combined with solution biophysical studies have provided a wealth of knowledge

on conformational dynamics of the CYP2B subfamily of enzymes. In several of these structures, the cyclohexyl group of the detergent CYMAL-5 occupies a peripheral binding pocket between the F- and G-helices [6-9]. Residues surrounding this peripheral pocket in CYP2B4 are S176, C180, F188, F195, L198, L199, F202, I241, F244, I245, F296, and T300. More recently, the functional importance of this peripheral pocket was studied via site directed mutagenesis of F202 in CYP2B4 and CYP2B6 [10, 11]. The results indicated that the identity of residues within but not at the surface of the pocket is crucial in governing substrate turnover and redox coupling.

In order to further investigate the role of CYMAL-5 binding at this peripheral site in CYP2B4 using structural and biophysical techniques, we crystallized and determined the structure of the previously characterized CYP2B4 F202W mutant in the presence of CYMAL-5. In addition, we also determined the structure in the presence of an alternate detergent hexaethylene glycol monoethyl ether (C_8E_6), which does not bind in the peripheral pocket. Furthermore, deuterium-exchange mass spectrometry (DXMS) was employed for the detection of detergent-induced conformational changes and identification of specific regions of the CYP2B4 affected by C_8E_6 binding. The results obtained were analyzed in light of the previously demonstrated solution behavior of CYP2B4 in the presence of CYMAL-5. Evidence from the already published structure of the ligand-free CYP2B4 with CYMAL-5 bound in the peripheral pocket, and the novel structure of CYP2B4 (F202W) in the presence of an alternate detergent C_8E_6 allows interpretation of the role various detergents play in site-specific interactions and facilitating crystallization. More importantly, the results presented here using structural and biophysical techniques provide insight into the potential effects of detergents on protein conformation and dynamics, which may otherwise not be easily revealed in ligand binding studies carried out in solution.

EXPERIMENTAL PROCEDURES

Materials. Nickel-nitrilotriacetic acid affinity resin was purchased from Qiagen (Valencia, CA), and Macro-Prep CM cation exchange resin was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). The TOPP3 cells were obtained from Agilent Technologies (Santa Clara, CA). The Index Screen HR2-144 and the Wizard 3 crystallization screens were obtained from Hampton Research (Aliso Viejo, CA) and Emerald Biosciences (Seattle, WA), respectively. CYMAL-5, C₈E₆ and IPTG (β -D-1-thiogalactopyranoside) were from Anatrace (Maumee, OH). 3 α ,7 α ,12 α -Tris[(β -D-maltopyranosyl) ethyloxy]cholane (FA-4) is a custom made facial amphiphile [1]. Ampicillin, δ -aminolevulinic acid (ALA), phenylmethanesulfonyl fluoride (PMSF), lysozyme, dithiothreitol (DTT), 2-mercaptoethanol (β -ME), potassium phosphate, sucrose, and yeast extract required to prepare Terrific broth medium were purchased from Sigma-Aldrich (St. Louis, MO). CHAPS was from Calbiochem (EMD Chemicals, San Diego, CA). Sodium chloride (NaCl), Ethylenediaminetetraacetic acid (EDTA), and glycerol were from Fisher Scientific (Waltham, MA), and L-histidine was from Spectrum Chemical (New Brunswick, NJ). All protein model figures were created using MacPyMOL [12]. CYP2B4 F202W was generated by PCR using the pKK2B4dH (H226Y) plasmid as described previously [10, 13] using Agilent's QuikChange XL site-directed mutagenesis kit. The initial structural studies of CYP2B4, including the truncation of the protein and the open ligand-free structure [14], was completed using protein with the native His²²⁶. However, due to the formation of a dimer involving coordination of His²²⁶ of each monomer with the heme iron of the other monomer, subsequent solution biophysical work and crystallography efforts have utilized the internal mutant H226Y. In this manuscript, CYP2B4 wild-type will refer to CYP2B4dH H226Y unless otherwise indicated. This is also the background in which all mutations were made.

Protein Expression and Purification. CYP2B4 enzymes were expressed in *Escherichia coli* TOPP3 cells as previously described [13]. Protein expression was induced by adding IPTG, 0.5 mM) and ALA (1 mM) to Terrific broth medium ($A_{600} \sim 0.7$ at 37 °C) in the presence of ampicillin (100 mg/ml). The cells were grown for 68-72 h at 30 °C (190 rpm) and were harvested by centrifugation (4,000×g). Protein purification was carried out at 4°C according to a protocol described previously [15]. The pellet was resuspended in the buffer containing 20 mM potassium phosphate (pH 7.4 at 4 °C), 20% (v/v) glycerol, 10 mM β -ME, and 0.5 mM PMSF. The resuspended cells were then treated with 0.3 mg/ml lysozyme, stirred for 30 min, and centrifuged for 30 min at 7500×g. The supernatant was decanted and spheroplasts were resuspended in buffer containing 500 mM potassium phosphate (pH 7.4 at 4 °C), 20% (v/v) glycerol, 10 mM β -ME, and 0.5 mM PMSF, followed by sonication for 3 × 45 s on ice. Detergent CHAPS was added at a final concentration of 0.8%, and this solution was allowed to stir for 30 min at 4 °C. The supernatant was collected after ultracentrifugation for 1 h at 245,000×g. The CYP enzyme concentration was determined by measuring a difference spectrum of the ferrous carbonyl complex of the heme protein as described previously [16, 17].

The supernatant was applied to equilibrated nickel-nitrilotriacetic acid resin, and the buffer containing 100 mM potassium phosphate (pH 7.4 at 4°C), 100 mM NaCl, 20% (v/v) glycerol, 10 mM β -ME, 0.5 mM PMSF, 0.5% CHAPS, and 1 mM histidine was used to wash the column. The same buffer with 40 mM histidine was used to elute the protein off the column. The pooled P450-containing fractions of highest quality as measured by the A_{417}/A_{280} ratios were collected and diluted 10-fold with buffer containing 5 mM potassium phosphate (pH 7.4 at 4°C), 20% (v/v) glycerol, 1 mM EDTA, 0.2 mM DTT, 0.5 mM PMSF, and 0.5% CHAPS and applied to a Macro-Prep CM cation exchange column. The Macro-Prep CM column was washed using 5

mM potassium phosphate (pH 7.4 at 4°C), 20 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 0.2 mM DTT, and the protein was eluted with high-salt buffer containing 50 mM potassium phosphate (pH 7.4 at 4°C), 500 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 0.2 mM DTT. Protein fractions with the highest A_{417}/A_{280} ratios (~2) were collected, and the P450 concentration was determined using the reduced CO-difference spectra.

Protein Crystallization and Data collection. Following ion-exchange chromatography, pooled F202W protein was diluted to a final concentration of 18 μ M in a buffer containing 50 mM potassium phosphate (pH 7.4 at 4 °C), 500 mM sucrose, 500 mM NaCl, 1 mM EDTA, and 0.2 mM DTT, and incubated overnight at 4 °C on ice. The protein was concentrated to 200 μ M and supplemented with 4.8 mM CYMAL-5 and 0.028% (w/v) FA-4 before crystallization. The sample was then screened via the sitting-drop vapor diffusion method using the Hampton Research Index screen. Crystals of F202W with CYMAL-5 grew over a week after the protein had been incubated at 18 °C in a 1:1 ratio with the precipitant containing 5% (w/v) tacsimate (pH 7.0), 0.1 M HEPES (pH 7.0), and 10% (w/v) Polyethylene glycol monomethyl ether 5,000. Crystals of F202W with C₈E₆ were obtained from the Wizard 3 screen (Emerald Biosciences) after incubating the protein in a 1:1 ratio with the precipitant containing 20% (w/v) PEG4000, 0.1 M citrate (pH 5.5), and 10% (w/v) 2-propanol at 18 °C.

Crystals were soaked in the mother liquor containing 20% (v/v) sucrose as a cryoprotectant before being flash-frozen in liquid nitrogen. Crystallographic data for F202W in the presence of CYMAL-5 were collected remotely at Advanced Light Source beamline (BL) 5.0.3 in Berkeley, CA, using 1° oscillations over 240 frames and 20 s exposure with a ADSC Q315R detector at 100 K. The data for F202W in the presence of C₈E₆ were collected remotely at Stanford Synchrotron Radiation Lightsource (SSRL) BL 11-1 [18] using 1° oscillations over 240 frames

and 10 s exposures with a Quantum 315 CCD detector at 100 K. Data were integrated to 2.73 Å for F202W with CYMAL-5 using iMOSFLM [19] and to 2.34 Å for F202W with C₈E₆ using the autoxds script at SSRL; both datasets were scaled using SCALA in CCP4i [20, 21].

Structure Determination and Refinement. Structures of F202W in the presence of CYMAL-5 or C₈E₆ were determined by molecular replacement in Phaser [22] and using an ensemble of CYP2B4 structures consisting of those with PDB entries 3MVR, 3TMZ, 4JLT, and 1PO5 [8, 9, 14, 23]. For F202W in the presence of CYMAL-5, Matthews coefficient analysis suggested the presence of two molecules per asymmetric unit with 51 % solvent content and a solution in space group P3₁. For the F202W-C₈E₆ complex, similar analysis indicated one molecule per asymmetric unit with 65 % solvent content in space group P3₁21. The F202W-CYMAL-5 complex data set processed using the P3₁21 space group yielded a similar structure to that in P3₁, however, the R-values were superior in the space group P3₁. The output model from Phaser was subjected to rigid body and restrained refinement in REFMAC [24] before models were built in COOT [25] using both F_o-F_c and 2F_o-F_c maps contoured at 3-σ and 1-σ, respectively. The crystallographic information library description file for C₈E₆ was created using PRODRG server [26]. Iterative model building and refinement was performed until the R-factor and R_{free} stopped improving. The clash score of all atoms was in the 98th percentile and the 99th percentile for the F202W-CYMAL-5 complex and the F202W-C₈E₆ complex, respectively, as obtained from MOLPROBITY [27], which ranked the F202W-CYMAL-5 structure in the 94th percentile and the F202W-C₈E₆ structure in the 99th percentile in terms of overall geometry when compared with other structures at similar resolution. There were 280 water molecules in the F202W-CYMAL-5 complex and 140 in the F202W-C₈E₆ complex. In the F202W-CYMAL-5 structure, two CYMAL-5 molecules were observed per molecule in the asymmetric unit, with

one being present in the peripheral pocket near residues F202 and F296. In the F202W-C₈E₆ structure, the electron density for the part of C₈E₆ molecule was disordered at the previously defined substrate access channel region and near the surface of the protein, located at the interface of the A'- and F'-helices (Supplemental Figure 1). The difficulty in modelling complete detergent molecules or improving B-factors in membrane protein structures determined at a resolution in the 2.3 to 2.7 Å range has been noted in several previous cases [1, 5, 28]. The oxygen atom of the C₈E₆ molecule was facing the heme iron at a distance of ~2.9 Å. Residues 28 to 492 were present in the final model of both of the structures, and one C-terminal histidine was located as part of the His-tag in each of the structures. Electron density for residues 473 and 474 was poorly defined, so they were not modeled in the F202W complex with C₈E₆. Structure refinement statistics are summarized in Table 1.

Deuterium Exchange Analysis. Optimization of the fragmentation conditions for H/D exchange experiments was accomplished previously, and the preparation of deuterated samples and the following DXMS analysis were performed as previously described [8]. In brief, CYP2B4 samples in the presence of detergent were prepared by mixing 6.2 µl of 25 mM CYMAL-5 or C₈E₆ (final concentration of 1 mM) with 150 µl of 226 µM CYP2B4 and incubated at 0 °C for 30 min. Detergent-free samples were prepared by mixing 150 µl of CYP2B4 with 6.2 µl of CM elution buffer (50 mM potassium phosphate (pH7.4 at 4 °C), 500 mM NaCl, 20% glycerol (v/v), 1 mM EDTA, 0.2 mM DTT), making the final volume equivalent to the ligand-bound samples.

H/D exchange samples were prepared by mixing 3 µl of protein samples incubated in the absence or presence of detergent with 9 µl of deuterated CM elution buffer containing the detergent present in the incubated sample or no detergent and incubated at 0 °C for 10, 100, 1000, 10,000, or 100,000 s. The exchange reaction was then quenched with 18 µl of ice-cold

quench solution (1.6 M guanidine hydrochloride in 0.8 % (v/v) formic acid, pH 2.2-2.5). The samples were subsequently transferred to ice-cold autosampler vials, frozen on dry ice, and stored at -80 °C. Frozen samples were transferred to the dry ice-containing sample basin of the cryogenic autosampler module of the DXMS apparatus. Samples were thawed on ice and immediately passed over a protease column (16 μ l bed volume) filled with porcine pepsin at a flow rate of 20 μ l min⁻¹ with 0.05% (v/v) trifluoroacetic acid (TFA) for a 40 s digestion. Proteolytic products were directly collected by a C18 column (Michrom MAGIC C18 AQ 0.2 x 50 mm) and then eluted with a linear gradient of 0.046% (v/v) TFA, 6.4% (v/v) acetonitrile to 0.03% (v/v) TFA, 38.4% (v/v) acetonitrile for 30 min. The column effluent was analyzed on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA) and data acquisition in either data-dependent tandem mass spectrometric mode or MS1 profile mode. Determination of pepsin-generated peptides from the resulting MS/MS data sets was facilitated through the use of Proteom Discoverer (Thermo Fisher Scientific, San Jose, CA). This set of peptides was then further examined by specialized software, DXMS Explorer (Sierra Analytics Inc., Modesto, CA), and all data processing was the same as previously described [29]. Corrections for back-exchange were determined via the methods of Zhang and Smith [30].

RESULTS AND DISCUSSION

Structure of CYP2B4 F202W in the Presence of CYMAL-5 or C₈E₆. We hypothesized that the increased bulk introduced by the F202W mutation would prevent CYMAL-5 binding in the peripheral pocket of CYP2B4 and allow us to elucidate the role of the detergent binding at this site observed in multiple prior CYP2B crystal structures. However, despite the mutation the cyclohexyl group of one CYMAL-5 molecule protruded into this peripheral pocket, located in

close proximity to F202W and oriented in a similar manner as observed in several other structures. The CYP2B4 (F202W) structure in the presence of CYMAL-5 revealed a closed conformation that was nearly identical to that of ligand-free wild-type CYP2B4 (PDB ID: 3MVR) with a root mean square deviation (RMSD) of 0.29 Å (Supplemental Figure 2). Our crystallization attempts in the absence of CYMAL-5 were unsuccessful.

To find an alternate detergent for the crystallization of F202W that would not bind in the peripheral pocket, the condition that yielded wild type CYP2B4 crystals in a ligand free state in the presence of CYMAL-5 was used as a starting point [8]. The protein was concentrated to 550 µM, and crystallization was performed using the Hampton Research detergent screen with 96 unique detergents to screen for conditions that yielded crystals. One condition was identified that yielded crystals in the presence of C₈E₆ at 18 °C after incubating the protein in a 1.0:0.8:0.2 ratio of protein:precipitant:detergent according to the screening protocol. Protein crystallization on a larger scale was carried out in the presence of C₈E₆ at 1X CMC as a substitute for CYMAL-5 in the crystallization protocol, using the sitting drop vapor diffusion method and various screens. X-ray analysis of the crystals of CYP2B4 F202W with C₈E₆ yielded a structure similar in a Cα overlay to the overall structure solved in the presence of CYMAL-5 with an RMSD of 0.32 Å (Figure 1A). One molecule of C₈E₆ was found in the active site of the complex with F202W (Supplemental Figure 1). This detergent molecule coordinates with the heme iron and extends to the protein surface through the 2*f* substrate access channel [9], occupying similar space as the two molecules of amlodipine in CYP2B4 (PDB ID: 3TMZ) as shown in Figure 1B. Detailed comparison of the CYP2B4 (F202W)-C₈E₆ structure with the F202W-CYMAL-5 complex revealed some considerable differences as shown in Figure 2. The A-A' loop near the access channel region was shifted as much as 5 Å, and the A-helix was displaced by 2.5 Å in the

F202W-C₈E₆ structure. The β_4 -loop region that usually comprises part of the roof of the active site was shifted up to ~ 3 Å in the complex with C₈E₆. In addition, differences of ~ 1 - 1.5 Å were observed in an overlay in several parts of the N-terminal region, the C-D loop, the F-G cassette that includes F' and G' helices, and the H helix.

Residues within 5 Å of C₈E₆ were located mainly on the B/C loop, helices F, I, A, and A', and on the β_{1-1} , β_{1-2} and β_{1-4} sheets. In comparison to the F202W structure in the presence of CYMAL-5, several of these residues are either shifted, the side-chain orientation is altered, or both (Figure 3A). Examination of residues within 5 Å of the CYMAL-5 molecule found in the peripheral pocket of the F202W-CYMAL-5 complex show either changes in side-chain orientation or backbone displacement in the presence or absence of the detergent molecule (Figure 3B). The side-chain of W202 in the F202W-C₈E₆ complex rotates $\sim 90^\circ$ around C β , causing it to protrude into the peripheral pocket in the absence of CYMAL-5. The side-chains of residues V194, F195, F198, and L199 were also observed to shift in or towards the peripheral pocket, which may reduce the overall volume of the peripheral pocket in a cumulative manner.

Differential Solvent Exposure upon Detergent Binding as Measured by Deuterium Incorporation. DXMS was used to yield further insight into conformational changes due to the binding of C₈E₆, and the results were compared with the previous studies performed with CYMAL-5. While X-ray crystal structures of protein-ligand complexes give a static glimpse of possible structural arrangements, DXMS provide a means to probe the solution behavior of the enzyme plastic regions, and in this case to elucidate the role of detergents in conformational dynamics. Similar to previous H/D exchange experiments, the regions of CYP2B4 showing the highest rates of deuterium incorporation were the N-terminal end of the protein (residues 20-45), the B'-C loop and C-helix (residues 131-140), the F-, F'-, G'-, and G-helices (residues 203-238,

270-290), the H-helix and *N*-terminal end of the I-helix, and the terminal loop (residues 468-483) (Figures 4-6) [8, 31]. Furthermore, addition of each detergent produced marked differences in the H/D exchange profile of CYP2B4.

In the presence of CYMAL-5, CYP2B4 displays an increase in the deuterium incorporation rate toward the *C*-terminal end of the C-helix, across the C-D loop, D-helix with fragment coverage and the majority of the F-G cassette, as highlighted by the difference plot in Figure 4. An increase in the deuterium incorporation rate is also seen in the loop containing the heme cysteine (residues 432-437) and in the terminal loop (residues 471-484). In contrast, the *N*-terminal residues (amino acids 22-57) and a portion of the I-helix (residues 299-313) show a slower rate of H/D exchange in the presence of CYMAL-5. In the presence of C₈E₆, increased H/D exchange is seen in the E-F loop (residues 182-195), the G- and H-helices (residues 221-290), and across residues 401-437 (Figure 5). Slowing of the H/D exchange rate as seen across the *N*-terminal residues (amino acids 22-57) and to a lesser extent across the *C*-terminal end of the F-helix (residues 209-219) and across the I-helix (residues 307-312). Comparison of CYP2B4 in the presence of CYMAL-5 or C₈E₆ indicates that the deuterium incorporation rate is faster across the F-, F'-, G'-, G-, and H-helices (residues 182-290) when CYMAL-5 is present (Figure 6). Differences in the rate of H/D exchange are also seen across residues 401-437 with lower H/D rates in much of this region in the presence of CYMAL-5 than in the presence of C₈E₆, but the loop containing the heme cysteine (residues 432-437) shows higher rates of deuterium incorporation in the presence of CYMAL-5.

In our previous DXMS study, the large majority of CYP2B4 was found to adopt a more open conformation in the absence of ligand [8]. However, the crystal structure of ligand free CYP2B4 in the presence of CYMAL-5 illustrated a closed conformation, suggesting that the minor

populations of closed conformer in solution would not be detected by averaging across the entire population via DXMS. Unlike the presence of a small ligand which slowed H/D exchange rates across previously described plastic regions of CYP2B4 [32], the presence of either CYMAL-5 or C₈E₆ generally increased the H/D exchange rate across the plastic regions. Furthermore, CYMAL-5 generally caused a greater increase in the H/D exchange rate for most regions than C₈E₆, indicating less protection from solvent access to backbone amide protons. Overall, it is important to consider the effect of detergent in any solution studies carried out either for optimum enzyme stability, or for crystallization of CYP enzyme complexes due to the possibility of competitive binding in the active site. In this context, it is interesting to note the recent DXMS study of CYP3A4 that demonstrated the reduced accessibility of the F- and G- helices in a lipid bilayer vs. the protein in detergent solubilized state [33].

Comparison of the DXMS results of CYP2B4 in CYMAL-5 vs. C₈E₆ with the relevant structural snapshots obtained via X-ray crystallography revealed some intriguing similarities and differences. As illustrated in Figure 6, the differences in the rate of deuterium incorporation in the presence of C₈E₆ were observed mainly in the F-G cassette region (residues ~180-260) that includes the F, F', G', and G-helices, the H-helix, the C-D loop region and the β_4 loop. Differences in these regions were also detected by X-ray crystallography (Figure 2), demonstrating that the solution behavior of CYP2B4 revealed by DXMS is consistent with the predicted behavior from the crystal structures. The one notable exception is the N-terminus and the A-A' region (residues ~29-60), where the remarkable difference between the two crystal structures (Figure 2) is not evident in the DXMS studies. In conclusion, both CYMAL-5 and C₈E₆ promote a more open conformation of CYP2B4, as seen by the increase in H/D exchange rate across much of the protein in the presence of either detergent. The presence of a single C₈E₆

molecule extending all the way from the CYP2B4 heme iron to the protein surface confirms our prior conclusion based on a dual-ligand amlodipine complex that access channel 2*f* is a major route for substrate entry into the active site. On the whole, the DXMS study of CYP2B4 in the presence of C₈E₆ or CYMAL-5 in solution is in close accord with the differences seen in the crystal structures. Whether the crystals of the CYP2B4-C₈E₆ complex in an open protein conformation can be used to exchange larger ligands with tight binding affinity as recently demonstrated with CYP2D6 [34] remains to be elucidated.

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REFERENCES

- [1] S.C. Lee, B.C. Bennett, W.X. Hong, Y. Fu, K.A. Baker, J. Marcoux, C.V. Robinson, A.B. Ward, J.R. Halpert, R.C. Stevens, C.D. Stout, M.J. Yeager, Q. Zhang, Steroid-based facial amphiphiles for stabilization and crystallization of membrane proteins, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) E1203-1211.
- [2] Y. Sonoda, S. Newstead, N.J. Hu, Y. Alguel, E. Nji, K. Beis, S. Yashiro, C. Lee, J. Leung, A.D. Cameron, B. Byrne, S. Iwata, D. Drew, Benchmarking membrane protein detergent stability for improving throughput of high-resolution X-ray structures, *Structure* 19 (2011) 17-25.
- [3] R.M. Bill, P.J. Henderson, S. Iwata, E.R. Kunji, H. Michel, R. Neutze, S. Newstead, B. Poolman, C.G. Tate, H. Vogel, Overcoming barriers to membrane protein structure determination, *Nat. Biotechnol.* 29 (2011) 335-340.
- [4] C.G. Tate, Practical considerations of membrane protein instability during purification and crystallisation, *Methods Mol. Biol.* 601 (2010) 187-203.
- [5] A.J. Annalora, D.B. Goodin, W.X. Hong, Q. Zhang, E.F. Johnson, C.D. Stout, Crystal structure of CYP24A1, a mitochondrial cytochrome P450 involved in vitamin D metabolism, *J. Mol. Biol.* 396 (2010) 441-451.
- [6] S.C. Gay, M.B. Shah, J.C. Talakad, K. Maekawa, A.G. Roberts, P.R. Wilderman, L. Sun, J.Y. Yang, S.C. Huelga, W.-X. Hong, Q. Zhang, C.D. Stout, J.R. Halpert, Crystal structure of a cytochrome P450 2B6 genetic variant in complex with the inhibitor 4-(4-chlorophenyl)imidazole at 2.0-Å resolution, *Mol. Pharmacol.* 77 (2010) 529-538.

- [7] P.R. Wilderman, M.B. Shah, H.H. Jang, C.D. Stout, J.R. Halpert, Structural and thermodynamic basis of (+)- α -pinene binding to human cytochrome P450 2B6, *J. Am. Chem. Soc.* 135 (2013) 10433-10440.
- [8] P.R. Wilderman, M.B. Shah, T. Liu, S. Li, S. Hsu, A.G. Roberts, D.R. Goodlett, Q. Zhang, V.L. Woods, Jr., C.D. Stout, J.R. Halpert, Plasticity of cytochrome P450 2B4 as investigated by hydrogen-deuterium exchange mass spectrometry and X-ray crystallography, *J. Biol. Chem.* 285 (2010) 38602-38611.
- [9] M.B. Shah, P.R. Wilderman, J. Pascual, Q. Zhang, C.D. Stout, J.R. Halpert, Conformational adaptation of human cytochrome P450 2B6 and rabbit cytochrome P450 2B4 revealed upon binding multiple amlodipine molecules, *Biochemistry* 51 (2012) 7225-7238.
- [10] H.H. Jang, J. Liu, G.Y. Lee, J.R. Halpert, P.R. Wilderman, Functional importance of a peripheral pocket in mammalian cytochrome P450 2B enzymes, *Arch. Biochem. Biophys.* 584 (2015) 61-69.
- [11] J. Liu, M.B. Shah, Q. Zhang, C.D. Stout, J.R. Halpert, P.R. Wilderman, Coumarin derivatives as substrate probes of mammalian cytochromes P450 2B4 and 2B6: assessing the importance of 7-alkoxy chain length, halogen substitution, and non-active site mutations, *Biochemistry* 55 (2016) 1997-2007.
- [12] L. Schrodinger, The PyMOL molecular graphics system, MacPyMOL ed., Schrodinger, LLC, 2010.
- [13] E.E. Scott, M. Spatzenegger, J.R. Halpert, A Truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function, *Arch. Biochem. Biophys.* 395 (2001) 57-68.

- [14] E.E. Scott, Y.A. He, M.R. Wester, M.A. White, C.C. Chin, J.R. Halpert, E.F. Johnson, C.D. Stout, An open conformation of mammalian cytochrome P450 2B4 at 1.6-Å resolution, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 13196-13201.
- [15] M.B. Shah, J. Pascual, Q. Zhang, C.D. Stout, J.R. Halpert, Structures of cytochrome P450 2B6 bound to 4-benzylpyridine and 4-(4-nitrobenzyl)pyridine: insight into inhibitor binding and rearrangement of active site side chains, *Mol. Pharmacol.* 80 1047-1055.
- [16] T. Omura, R. Sato, Carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J. Biol. Chem.* 239 (1964) 2370-2378.
- [17] T. Omura, R. Sato, Carbon monoxide-binding pigment of liver microsomes. 2. Solubilization purification + Properties, *J. Biol. Chem.* 239 (1964) 2379-2385.
- [18] S.M. Soltis, A.E. Cohen, A. Deacon, T. Eriksson, A. Gonzalez, S. McPhillips, H. Chui, P. Dunten, M. Hollenbeck, I. Mathews, M. Miller, P. Moorhead, R.P. Phizackerley, C. Smith, J. Song, H. van dem Bedem, P. Ellis, P. Kuhn, T. McPhillips, N. Sauter, K. Sharp, I. Tsyba, G. Wolf, New paradigm for macromolecular crystallography experiments at SSRL: automated crystal screening and remote data collection, *Acta Crystallogr. D. Biol. Crystallogr.* 64 (2008) 1210-1221.
- [19] A.G.W. Leslie, Integration of macromolecular diffraction data, *Acta Crystallogr. D. Biol. Crystallogr.* 55 (1999) 1696-1702.
- [20] S. Bailey, The CCP4 suite - programs for protein crystallography. *Acta Crystallogr. D. Biol. Crystallogr.* 50 (1994) 760-763.
- [21] A. Gonzalez, Y. Tsai, http://smb.slac.stanford.edu/facilities/software/xds/#autoxds_script (2010).

- [22] A.J. McCoy, R.W. Grosse-Kunstleve, P.D. Adams, M.D. Winn, L.C. Storoni, R.J. Read, Phaser crystallographic software, *J. Appl. Cryst.* 40 (2007) 658-674.
- [23] M.B. Shah, I. Kufareva, J. Pascual, Q. Zhang, C.D. Stout, J.R. Halpert, A structural snapshot of CYP2B4 in complex with paroxetine provides insights into ligand binding and clusters of conformational states, *J. Pharmacol. Exp. Ther.* 346 (2013) 113-120.
- [24] G.N. Murshudov, A.A. Vagin, E.J. Dodson, Refinement of macromolecular structures by the maximum-likelihood method, *Acta Crystallogr. D. Biol. Crystallogr.* 53 (1997) 240-255.
- [25] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtan, Features and development of Coot, *Acta Crystallogr. D. Biol. Crystallogr.* 66 (2010) 486-501.
- [26] A.W. Schuttelkopf, D.M. van Aalten, PRODRG: a tool for high-throughput crystallography of protein-ligand complexes, *Acta Crystallogr. D. Biol. Crystallogr.* 60 (2004) 1355-1363.
- [27] V.B. Chen, W.B. Arendall, 3rd, J.J. Headd, D.A. Keedy, R.M. Immormino, G.J. Kapral, L.W. Murray, J.S. Richardson, D.C. Richardson, MolProbity: all-atom structure validation for macromolecular crystallography, *Acta Crystallogr. D. Biol. Crystallogr.* 66 (2010) 12-21.
- [28] S.C. Gay, A.G. Roberts, K. Maekawa, J.C. Talakad, W.X. Hong, Q. Zhang, C.D. Stout, J.R. Halpert, Structures of cytochrome P450 2B4 complexed with the antiplatelet drugs ticlopidine and clopidogrel, *Biochemistry* 49 (2010) 8709-8720.
- [29] Y. Hamuro, G.S. Anand, J.S. Kim, C. Juliano, D.D. Stranz, S.S. Taylor, V.L. Woods, Mapping intersubunit interactions of the regulatory subunit (RI alpha) in the type I holoenzyme of protein kinase A by amide hydrogen/deuterium exchange mass spectrometry (DXMS), *J. Mol. Biol.* 340 (2004) 1185-1196.

- [30] Z.Q. Zhang, D.L. Smith, Determination of amide hydrogen-exchange by mass-spectrometry - a new tool for protein-structure elucidation, *Protein Sci.* 2 (1993) 522-531.
- [31] S.C. Gay, H. Zhang, P.R. Wilderman, A.G. Roberts, T. Liu, S. Li, H.L. Lin, Q. Zhang, V.L. Woods, C.D. Stout, P.F. Hollenberg, J.R. Halpert, Structural analysis of mammalian cytochrome P450 2B4 covalently bound to the mechanism-based inactivator tert-butylphenylacetylene: insight into partial enzymatic activity, *Biochemistry* 50 (2011) 4903-4911.
- [32] Y. Zhao, J.R. Halpert, Structure-function analysis of cytochromes P450 2B, *Biochim. Biophys. Acta* 1770 (2007) 402-412.
- [33] N.A. Treuheit, M. Redhair, H. Kwon, W.D. McClary, M. Guttman, J.P. Sumida, W.M. Atkins, Membrane interactions, ligand-dependent dynamics, and stability of cytochrome P4503A4 in lipid nanodiscs, *Biochemistry* 55 (2016) 1058-1069.
- [34] A. Wang, C.D. Stout, Q. Zhang, E.F. Johnson, Contributions of ionic interactions and protein dynamics to cytochrome P450 2D6 (CYP2D6) substrate and inhibitor binding, *J. Biol. Chem.* 290 (2015) 5092-5104.

The atomic coordinates and structure factors of these complexes are available in the Research Collaboratory for Structural Bioinformatics Protein Databank (PDB) (<http://www.rcsb.org/>) under PDB entries 5IUZ for CYP2B4 F202W in the presence of CYMAL-5 and 5IUT for CYP2B4 F202W in the presence of C₈E₆.

ABBREVIATIONS

CYP, cytochrome P450; H/D, hydrogen deuterium; CYMAL-5, 5-cyclohexyl-1-pentyl- β -D-maltoside; C₈E₆, hexaethylene glycol mono-octyl ether; FA-4, 3 α ,7 α ,12 α -tris[(β -D-maltopyranosyl)ethyloxy]cholane; IPTG, β -D-1-thiogalactopyranoside; ALA, δ -aminolevulinic acid; β -ME, 2-mercaptoethanol; PMSF, Phenylmethanesulfonyl fluoride; SSRL, Stanford Synchrotron Radiation Lightsource; BL, beamline; PDB, protein data bank; RMSD, root mean square deviation; DXMS, hydrogen/deuterium exchange coupled to mass spectrometry; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; DTT, dithiothreitol; CHAPS, [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate].

FIGURE LEGENDS

Figure 1. Overall structures of CYP2B4 F202W in the presence of CYMAL-5 or C₈E₆. (A) Overlay of CYP2B4 F202W-CYMAL-5 complex (magenta) and CYP2B4 F202W-C₈E₆ complex (yellow) in a ribbon diagram. The C₈E₆ detergent molecule is shown in yellow and CYMAL-5 in magenta lines. (B) Overlay of CYP2B4 F202W-C₈E₆ (yellow) and CYP2B4-amlodipine (green) complexes (RMSD of 0.48 Å). Substrate access channel 2f observed in the amlodipine complexes of CYP2B4 and CYP2B6 is shown in sphere representation [9]. The C₈E₆ detergent and the two amlodipine molecules are shown in blue and red, respectively. CYMAL-5 in the CYP2B4-amlodipine complex is shown in green.

Figure 2. (A) Changes in secondary structural elements upon C₈E₆ binding in an overlay of CYP2B4 F202W-CYMAL-5 complex (magenta), and CYP2B4 F202W-C₈E₆ complex (yellow). The secondary structural elements that demonstrated differences in the structures are shown in thin lines. (B). Orthogonal view of the region that showed differences between the two structures. The CYMAL-5 (magenta) and Heme (red) are shown in thin lines, whereas C₈E₆ (blue) is shown as sticks.

Figure 3. Structural changes due to the presence of C₈E₆ or CYMAL-5 in CYP2B4 F202W. A) An overlay of the active site of CYP2B4 F202W with CYMAL-5 (magenta) or C₈E₆ (yellow). Residue side-chains within 5 Å of the C₈E₆ ligand (blue sticks) are shown in each of the representative structures. The heme is shown as red sticks. B) An overlay of residues found in

the peripheral pocket of CYP2B4 F202W with CYMAL-5 (magenta) or C₈E₆ (yellow). Side-chains of the residues within 5 Å of the CYMAL-5 ligand in the peripheral pocket (cyan sticks) are shown in each of the structures. The heme is shown as red sticks, and C₈E₆ is shown as blue sticks. The inset shows the orientation of W202 in the C₈E₆ complex (yellow) or the CYMAL-5 complex (magenta) with CYMAL-5 in cyan sticks.

Figure 4. Deuterium exchange of CYP2B4 in the presence and absence of CYMAL-5. Under the primary sequence, each condition is divided into rows corresponding to different time points from 10 s to 100,000 s (top to bottom). The color-coding of the absolute deuteration level indicates the percent of H/D exchange during the given time period. The difference is calculated by subtracting the percent deuteration in the second condition from the percent deuteration in the first condition. The color-coding of the difference in deuteration levels indicates the change in percent deuterium incorporation during that time period.

Figure 5. Deuterium exchange of CYP2B4 in the presence and absence of C₈E₆. The time points for each condition, the color coding scales, and method of calculating the difference in deuterium incorporated are the same as Figure 4.

Figure 6. Comparison of deuterium exchange of CYP2B4 in the presence of CYMAL-5 or C₈E₆. The time points for each condition, the color coding scales, and method of calculating the difference in deuterium incorporated are the same as Figure 4.

Table 1. Data Collection and Refinement Statistics: Values for the highest resolution shell are shown in parentheses.

Construct	CYP2B4 F202W	CYP2B4 F202W
Detergent	CYMAL-5	C ₈ E ₆
Crystal Space group	P3 ₁	P3 ₁ 21
Crystal unit cell parameters (Å)		
a = b	91.61	91.16
c	150.93	151.13
$\alpha = \beta$	90	90
γ	120	120
Data Collection statistics		
Beamline	ALS 5.0.1	SSRL 11-1
Wavelength (Å)	0.97	0.97
Resolution range (Å)	54.0-2.73 (2.8-2.73)	39.0-2.33 (2.41-2.34)
Completeness (%)	90.5 (65)	97.4 (72.7)
Redundancy ^a	5.95 (5.3)	6.7 (3.8)
R_{merge} (%) ^{a,b}	10.5 (43.8)	11.8 (46)
I/ σ	16.4 (1.5)	7.3 (1.4)
No. of observations	41760	31717
No. of unique reflections	37793	30766

Refinement Statistics		
R -factor ^c	18 %	21.6 %
R_{free} ^c	24 %	26.5 %
RMS deviations		
Bond lengths (Å)	0.017	0.007
Bond angles (°)	1.747	1.009
Average B factor (Å ²)	39.37	39.83
Ramachandran Plot		
Preferred (%)	94.9	96
Allowed (%)	99.8	100
Number of Atoms		
Protein ^d	7395 (41.37)	3651 (50.1)
Heme ^d	86 (22.17)	43 (32.01)
Solvent ^d	280 (40.04)	179 (48.42)
Detergent ^d	136 (98.8)	27 (90.58)

^aValues for the highest resolution shell are in parentheses.

^b $R_{\text{merge}} = [\sum_h \sum_i |I_h - I_{hi}| / \sum_h \sum_i I_{hi}]$ where I_h is the mean of I_{hi} observations of reflection h .

^c R -factor and $R_{\text{free}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}| \times 100$ for 95% of the recorded data (R -factor) and 5 % of the data (R_{free}).

^dAverage B -factors (Å²) are in parentheses.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Figure 6