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Functional Importance of a Peripheral Pocket in Mammalian Cytochrome P450 2B Enzymes*

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Abstract

The functional importance of a peripheral pocket found in previously published X-ray crystal structures of CYP2B4 and CYP2B6 was probed using a biophysical approach. Introduction of tryptophan within the pocket of CYP2B4 at F202 or I241 leads to marked impairment of 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) or 7-benzoyloxyresorufin *O*-dealkylation efficiency; a similar substitution at F195, near the surface access to the pocket, does not affect these activities. The analogous CYP2B6 F202W mutant is inactive in the 7-EFC *O*-dealkylation assay. The stoichiometry of 7-EFC deethylation suggested that the decreased activity of F202W and I241W in CYP2B4 and lack of activity of F202W in CYP2B6 coincided with a sharp increase in the flux of reducing equivalents through the oxidase shunt to produce excess water. The results indicate that the chemical identity of residues within this peripheral pocket, but not at the mouth of the pocket, is important in substrate turnover and redox coupling, likely through effects on active site topology.

Keywords

cytochrome P450; structure-function relationship; site-directed mutagenesis; monooxygenase coupling

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Introduction

Cytochrome P450 (CYP)¹ enzymes are a ubiquitous superfamily of mixed function oxidases responsible for the oxidation of a wide range of important endogenous compounds such as steroids, fatty acids, and prostaglandins, and of exogenous chemicals including drugs, carcinogens, and environmental pollutants [1]. Many members of this superfamily, generally those involved in biosynthetic processes, metabolize a single substrate into a single or small number of products. Other examples, including the mammalian xenobiotic metabolizing enzymes, are able to metabolize multiple chemically distinct substrates [2, 3]. CYP enzymes generally metabolize hydrophobic substrates leading to increased water solubility and higher clearance of the modified compound, and those enzymes involved in mammalian detoxification often exhibit overlapping substrate specificities [4].

Despite the breadth of substrates and possible reactions, oxidations catalyzed by CYP enzymes generally involve consumption of reducing equivalents from one molecule of NADPH and utilization of one molecule of oxygen, where one oxygen atom is inserted into a product and one forms a water molecule (Scheme 1). Release of hydrogen peroxide is a result of either hydrogen peroxide release (peroxide shunt) or by release of superoxide anion (autoxidation shunt) that dismutates to hydrogen peroxide. Mutation of active site residues often alters the coupling of electron transfer to product production and product profile for CYP enzymes [5–7].

Across the kingdoms of life, the single-domain fold of CYP enzymes is remarkably well conserved despite their broad range of substrates and biological roles [8]. The mammalian drug metabolizing CYP enzymes display a high degree of conformational flexibility, and the active site, generally buried at the center of protein, possesses varying sizes and physiochemical properties [9, 10].

The CYP2B subfamily enzymes are versatile catalysts with a broad range of substrates, preferring angular, medium-sized neutral or basic compounds [11]. Compared with several other CYP subfamilies, the CYP2B subfamily exhibits a relatively low degree of catalytic preservation across mammalian species, providing an excellent model system for structure-function analysis [12, 13].

In humans, CYP2B6 contributes to the metabolism of 3 to 12% of all drugs and metabolizes a number of important pharmaceuticals including bupropion, efavirenz, propofol, selegiline, and artemisinin [14]. Moreover, this enzyme is highly polymorphic, and most of the single nucleotide polymorphisms (SNPs) are located outside the active site [15]. Q172H, K262R, and R487C are the most common SNPs in CYP2B6, occurring alone or in combination with one another or other SNPs. Some of these alleles show differential binding or metabolism of

¹Abbreviations used are: CYP, cytochrome P450; ITC, isothermal titration calorimetry; H/D, hydrogen deuterium; DXMS, H/D exchange coupled to mass spectrometry; SNP, single nucleotide polymorphism; CYMAL-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; 7-BR, 7-benzyloxyresorufin; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; 7-HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; POR, NADPH-cytochrome P450 reductase; b5, cytochrome b5; IPTG, β-D-1-thiogalactopyranoside; ALA, d-aminolevulinic acid; β-ME, 2-mercaptoethanol; PDB, protein data bank; 4-CPI, 4-(4-chlorophenyl)imidazole; 1-PBI, 1-biphenyl-4-methyl-1Himidazole; DXMS, hydrogen/deuterium exchange coupled to mass spectrometry; H/D, hydrogen/deuterium; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; GuHCl, guanidine hydrochloride; MD, molecular dynamics; POR, cytochrome P450 reductase.

clinically relevant drugs [14, 16]. Furthermore, previous studies of non-active site residues in CYP2B enzymes also demonstrated that mutations located distal from the active site can significantly affect ligand binding or enzyme function [17–20].

X-ray crystal structures and solution studies of the CYP2B subfamily have provided insight into the high degree of conformational plasticity of the enzymes [12, 13, 21]. X-ray crystal structures of an engineered form of rabbit CYP2B4, CYP2B4dH² (*N*-terminally modified and containing a C-terminal tetra-His tag), highlight the ability of this enzyme to accommodate ligands of a broad size range ($M_r \sim 75\text{--}900$) via rearrangements in protein secondary structure, especially the B'-C Loop and the F-G cassette, which includes the F-, F'-, G'-, and G-helices.

Solution structural studies of CYP2B enzyme using isothermal titration calorimetry (ITC) demonstrate the link between enzyme plasticity and the thermodynamic parameters of ligand binding [21–23]. Mutations in the active site of CYP2B4 altered the relative contributions of entropy and enthalpy to ligand binding [21]. While ITC demonstrated changes in the thermodynamics driving ligand binding, Hydrogen/Deuterium (H/D) Exchange coupled to Mass Spectrometry (DXMS) provided evidence of conformational rearrangement to accommodate ligand binding in solution [24, 25]. The regions of the protein showing the greatest changes in H/D exchange rates were also those that showed rearrangements in X-ray crystal structures to accommodate binding of ligands of different sizes, namely the B'-C loop and the F- and G-helices.

Interestingly, multiple X-ray crystal structures of CYP2B enzymes show the cyclohexyl group of the detergent 5-cyclohexyl-1-pentyl- β -D-maltoside (CYMAL-5) occupying a peripheral binding site between the F- and G-helices [23, 24, 26, 27]. Residues lining this peripheral site in CYP2B4 are S176, C180, F188, F195, L198, L199, F202, I241, F244, I245, F296, and T300 (Figure 1) [26]. Interestingly, effects of ligand binding at a peripheral site in CYP3A4 were tied to allosteric modulation of enzyme activity [28].

In order to investigate the functional role of the peripheral binding site in CYP2B enzymes, we replaced residues F195, F202, and I241 in CYP2B4 and F202 in CYP2B6 with tryptophan by site-directed mutagenesis. Following purification of the mutants from *Escherichia coli*, steady-state kinetics parameters were determined with the typical CYP2B substrates 7-benzoyloxyresorufin (7-BR), and 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC). The effect on coupling of reducing equivalents to product formation was measured for the *O*-deethylation of 7-EFC. Comparison of steady-state rates of water formation and product production for the rabbit CYP2B4 and human CYP2B6 provide new insight into the functional effects of altering CYP2B non-active site amino acid residues.

²In this manuscript, CYP2B4 wild type will refer to CYP2B4 H226Y and CYP2B6 wild type will refer to CYP2B6 Y226H/K262R unless otherwise indicated. These are an N-terminally truncated and modified and C-terminally His-tagged forms of CYP2B4 and CYP2B6, respectively. These are the backgrounds in which all mutations were made. Previous studies have demonstrated these mutations in CYP2B4 and CYP2B6 do not alter enzyme catalysis and facilitate monomeric protein crystallization [26, 59].

Experimental Procedures

Materials

7-Hydroxy-4-(trifluoromethyl)coumarin (7-HFC), and 7-EFC were purchased from Life Technologies (Carlsbad, CA). β -NADPH, RNase A, DNase I, resorufin, and 7-BR were purchased from Sigma-Aldrich (St. Louis, MO). Ni^{2+} -NTA 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 QuikChange XL site-directed mutagenesis kit and TOPP3 cells were obtained from Agilent Technologies (Santa Clara, CA). Recombinant NADPH:cytochrome P450 reductase (POR) [29] and cytochrome b_5 (b_5) from rat liver [30, 31] were prepared as described previously. All other chemicals and supplies used were from standard sources. All protein model figures were created using MacPyMOL [32]. Channel/cavity analysis was performed using the Graphical User Interface for the Mole 2.0 program on Windows [33].

Site-directed Mutagenesis

CYP2B mutants were generated by PCR using the pKK2B4dH (H226Y) or the pKK2B6dH (Y226H/K262R) plasmid [31] as a template, appropriate forward and reverse primers (Table 1), and Agilent's QuikChange XL site-directed mutagenesis kit. All mutants generated in this study were verified by sequencing at Retrogen Inc. (San Diego, CA) to confirm the presence of the intended mutations and the absence of extraneous mutations.

Protein Expression and Purification

Enzymes were expressed in *Escherichia coli* TOPP3 cells (2B4) or JM109 cells (2B6) as previously described [31] and purified by the protocol used by Shah et al. [34]. Protein expression took place in Terrific Broth medium ($A_{600} \sim 0.7$ at 37 °C) in the presence of ampicillin by induction using isopropyl β -D-1-thiogalactopyranoside (IPTG, 0.5 mM) and δ -aminolevulinic acid (ALA, 1 mM). The cells were grown for 68–72 h at 30 °C and were harvested by centrifugation ($4,000 \times g$). Protein purification was carried out at 4 °C according to a protocol described previously [34]. The pellet was resuspended in 10% of the original culture volume in buffer containing 20 mM potassium phosphate (pH 7.4 at 4 °C), 20% (v/v) glycerol, 10 mM 2-mercaptoethanol (β -ME), and 0.5 mM PMSF. The resuspended cells were further treated with lysozyme (0.3 mg/ml) and stirred for 30 min, followed by centrifugation for 30 min at $7500 \times g$. After decanting the supernatant, spheroplasts were resuspended in 5 % of the original culture volume 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 and were sonicated for 3×45 s on ice. CHAPS was added to the sample at a final concentration of 0.8%, and this solution was allowed to stir for 30 min at 4 °C. After ultracentrifugation for 1 h at $245,000 \times g$, the supernatant was collected; the CYP enzyme concentration was determined by measuring a difference spectrum of the ferrous carbonyl complex of the heme protein [35, 36].

The supernatant was applied to Ni^{2+} -NTA resin, and the column was washed with 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. The protein was

eluted using 40 mM histidine in the same buffer described above. Protein fractions containing protein of the highest quality as measured by the A_{417}/A_{280} ratios were pooled, and the CYP enzyme concentration in the eluted fractions was measured using the reduced CO difference spectra. Pooled fractions containing CYP enzyme were 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 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 were pooled, and the P450 concentration was determined using the reduced CO-difference spectra.

Steady-State Kinetics

The rate of *O*-dealkylation of 7-EFC was measured as described previously [37]. The reconstituted enzyme system contained 10 pmol cytochrome P450, 40 pmol POR, and 20 pmol b_5 in 50 mM HEPES, 15 mM $MgCl_2$, 0.1 mM EDTA (pH 7.6) in a final reaction volume of 100 μ l. The concentrations of 7-EFC were in the range of 2 ~ 200 μ M for CYP2B4 and 0.5 ~ 50 μ M for CYP2B6. The samples were preincubated for 3 min at 37 °C before initiation of the reaction by addition of 1 mM NADPH. After a 5 min assay time at 37 °C, the reaction was stopped by the addition of cold acetonitrile (50 μ l). For determination of 7-HFC production rates, 50 μ l of the reaction mixture was diluted into 950 μ l of Tris-HCl buffer (pH 9.0). The content of 7-HFC was determined from the intensity of fluorescence at $\lambda_{ex} = 410$ nm and $\lambda_{em} = 510$ nm. The rate of 7-BR *O*-dealkylation was examined using a modified protocol in a reconstituted system [38]. The total reaction volume was 250 μ l, and the concentrations of 7-BR were in the range of 0.5 ~ 10 μ M. The composition of the reaction mixture was similar to that described above for 7-EFC *O*-deethylation. The reaction was quenched using 1 ml of methanol. Formation of resorufin was measured fluorometrically using $\lambda_{ex} = 550$ nm and $\lambda_{em} = 585$ nm. The K_M and k_{cat} values were calculated using Michaelis-Menten nonlinear regression analysis with GraphPad Prism (GraphPad Software, San Diego, CA).

NADPH Oxidation

The reaction was performed in a spectrophotometric cuvette maintained at 30 °C. A series of absorbance spectra covering the 340–700-nm range was used to monitor the NADPH concentration during the assay. Because 7-EFC and 7-HFC absorb strongly at 340 nm, the rate of NADPH oxidation was calculated using principal component analysis (PCA), also known as singular value decomposition (SVD)), as described previously [39, 40]. Changes in NADPH concentration were interpreted using a least-squares approximation of the spectra of the principal components by a basis set of spectral standards including spectra of NADPH ($\epsilon = 6.22 \times 10^3$ M⁻¹cm⁻¹), 7-EFC ($\epsilon = 14.0 \times 10^3$ M⁻¹cm⁻¹), and 7-HFC ($\epsilon = 16.0 \times 10^3$ M⁻¹cm⁻¹).

Oxygen Consumption

Consumption of molecular oxygen was measured at 30 °C using a fluorescence-based oxygen sensing system consisting of an MC2000-2 multichannel CCD rapid scanning spectrometer (Ocean Optics, Dunedin, FL, USA) equipped with one absorbance and one fluorescence channel, a Foxy-18G probe (Ocean Optics), a LEDD1B T-cube LED driver connected to a M505F1 fiber-coupled LED (Thor Labs, Newton, NJ, USA), a home-made thermostated cell chamber with a magnetic stirrer, and a semi-micro quartz cell (5 × 5 mm light path) from Hellma GmbH (Mülheim, Germany). The oxygen sensing system measures oxygen partial pressure by the fluorescence intensity of a ruthenium complex suspended in a sol-gel. Fluorescence intensity is dependent upon the ability of dissolved oxygen to quench the fluorescence of the ruthenium complex and is measurable by the spectrometer. The cuvette was sealed using a rubber septum during the reaction. The oxygen measurements were standardized using two standard solutions that were prepared fresh daily: air-saturated water and 20 mM dithionite solution. To initiate the reaction, NADPH was injected into the respiration cell through the septum with the aid of a micro-syringe.

Hydrogen Peroxide Production

The H₂O₂ produced in NADPH oxidation and O₂ consumption reactions was determined using the xylenol orange iron (III) colorimetric assay [41] with some modifications. Solution A was 25 mM ammonium ferrous (II) sulfate and 2.5 M H₂SO₄. Solution B consisted of 100 mM sorbitol and 125 μM xylenol orange in water. Working reagent was prepared by combining 1 volume of Solution A with 100 volumes of Solution B. An assay sample was prepared by mixing 100 μl of the quenched reaction mixture from either the NADPH oxidation sample or oxygen consumption sample with 900 μl of working reagent and incubating at room temperature for 1 h. The calibration curve was prepared from a series of solutions each of which contained 100 μl of the quenched reaction mixture, 900 μl of working reagent, and 10 μl of H₂O₂ standard solution of different concentrations. The H₂O₂ standard solutions were prepared by dilution of a 30% H₂O₂ stock solution with deionized water. The exact concentration of H₂O₂ in the stock solution was calculated using the molar extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm [41]. Control experiments confirmed that added H₂O₂ was recovered quantitatively from reaction mixtures.

7-HFC Production for Stoichiometry Measurements

An aliquot of each NADPH oxidation or O₂ consumption reaction was transferred to a glass tube containing 0.1 M Tris, pH 9.0, and fluorescence was determined with $\lambda_{\text{ex}} = 410$ nm and $\lambda_{\text{em}} = 510$ nm for 7-HFC quantities using a Cary Eclipse Fluorimeter. A blank was run for each set of samples, and the final activity was calculated by comparison with a standard curve that was prepared daily for the respective product.

Stoichiometry measurements

Direct measurement of either NADPH consumption or oxygen consumption was performed. Quantitation of 7-HFC and H₂O₂ production for each sample was used as an internal control for consistency between measurement methods. Reactions were carried out using the previously described reconstituted enzyme system in the same 1:4:2 ratio of enzymes and 50

pmol of cytochrome P450 in a 300 μ l final volume using either 0 or 150 μ M 7-EFC for CYP2B4 and 60 μ M 7-EFC for CYP2B6. Assays were performed in buffer containing 50 mM HEPES, pH 7.4, and 15 mM MgCl_2 , initiated by addition of NADPH to a final concentration of 0.2 mM and allowed to proceed for 10 min with continuous monitoring of NADPH or oxygen as described above. After 10 min a 100 μ l aliquot of the assay was immediately transferred to 900 μ l of peroxide color developing solution, and a 50 μ l aliquot was transferred to 950 μ l of 0.1 M Tris, pH 9.0.

Results

Steady-State Kinetic Analysis with 7-EFC and 7-BR

Steady-state kinetic analysis of 7-EFC *O*-deethylation by F195W in CYP2B4 showed increased catalytic efficiency (k_{cat}/K_M) compared to wild-type CYP2B4 (0.39 vs. 0.28), mainly due to a higher k_{cat} with little change in K_M (Table 2). F202W and I241W displayed significantly lower catalytic efficiency. F202W displayed a k_{cat} of about 1/20th that of CYP2B4, and the K_M was about one half that of the wild-type protein. For I241W, the k_{cat} was about 1/3 that of wild type, but the K_M for 7-EFC was more than 6-times higher than that of wild-type enzyme. In CYP2B6, the F202W mutant displayed no *O*-deethylation activity with 7-EFC.

F195W showed activity in the *O*-debenzylation of 7-BR, but no product was detected in this assay with F202W or I241W. The catalytic efficiency of the metabolism of 7-BR by F195W is similar to that of wild-type CYP2B4 despite small decreases in both k_{cat} and K_M . Since CYP2B6 F202W was inactive in the 7-EFC *O*-deethylation assay and CYP2B4 F202W was inactive in the 7-BR *O*-debenzylation assay, CYP2B6 F202W activity with 7-BR was not tested. An X-ray crystal structure of ligand-free CYP2B4 F202W with CYMAL-5 bound in the peripheral pocket has been solved that is virtually superimposable with the previously solved ligand-free CYP2B4 structure (PDB ID: 3MVR), so the lack of catalytic activity in either of the F202W mutants is likely not due to an improperly or partially folded enzyme (unpublished data).

Since CYMAL-5 was seen in the peripheral pocket of these enzymes, the effect of CYMAL-5 on catalysis was explored. The IC_{50} for CYP2B6 turnover of 7-EFC was 738.5 ± 42.5 μ M (Figure 2A). In the presence of 750 μ M CYMAL-5, CYP2B6 displayed a hyperbolic response in turnover to increased substrate concentration (Figure 2B). The k_{cat} of 7-EFC oxidation by CYP2B6 was almost equal to that in the absence of the detergent (Table 2). However, the K_M was increased by more than three times. Thus, CYMAL-5 interferes with 7-EFC binding, but not catalytic turnover.

Stoichiometry of 7-EFC Deethylation

Table 3 shows the rates of NADPH oxidation, oxygen consumption, product formation, and hydrogen peroxide production without substrate and during 7-EFC *O*-deethylation by wild type and mutant CYP2B4 enzymes. Due to the reactivity and unstable nature of superoxide, formation of this anion as an uncoupling product was not directly measured [42]. Addition of superoxide dismutase to the reaction mixture did not increase the amount of hydrogen

peroxide detected, indicating that any superoxide produced in the reaction dismutated to hydrogen peroxide at a faster rate than could be measured in these experimental conditions; the oxy-ferric heme complex could also autoxidize at a much slower rate than system cycling at a steady-state [5]. Thus, measurements of hydrogen peroxide production serve as an indicator of contributions from both the autoxidation shunt and the peroxide shunt. The rates of formation of excess water found in Table 3 were calculated from A) the difference between the rate of NADPH consumption and the rates of hydrogen peroxide plus product production, B) the difference between the rate of oxygen consumption and the rates of hydrogen peroxide plus product formation, and C) NADPH oxidation and oxygen consumption alone [6]. Results from each method of calculation were in agreement within experimental error, confirming that no major products other than 7-HFC were formed during the reaction.

With the CYP2B4 enzymes in the absence of substrate, substitution of F195 leads to an increased consumption of NADPH and O₂ and increased water production. Mutation of F202 or I241 leads to decreased rates of NADPH and O₂ consumption and H₂O₂ and H₂O production. In each system tested without substrate, water is produced indicating the formation of Cpd I even in the absence of substrate, as previously reported for CYP3A4 [43]. Compared to CYP2B4, the fraction of O₂ converted into H₂O does not change appreciably in the F202W or I241W mutants (~35–40% of total oxygen consumed), but the F195W mutant displays an elevated fraction of O₂ converted to H₂O (~75%).

Adding substrate to the reaction increases all measured rates for each enzyme. When examining coupling rates in CYP3A4 testosterone hydroxylation, the fraction of Cpd I used in productive hydroxylation events was maximized near 100 μ M or ~3-times the K_M for testosterone hydroxylation, so a concentration of ~5-times the K_M for CYP2B4 and ~10-times the K_M for CYP2B6 was used in these experiments [43]. The product production rates from the stoichiometry experiments agree with the results from steady-state kinetics experiments and previously reported results (Tables 2 and 3) [20]. For each enzyme, the NADPH consumption and O₂ consumption rates were ~3-times that seen in the absence of substrate, and the H₂O production rates were ~3-times higher than the substrate-free condition for F195W and I241W and ~7-times higher for CYP2B4 and F202W. The rate of H₂O₂ production increased by 50–100% in the presence of substrate. The fraction of O₂ diverted into excess water (H₂O/O₂) by F195W or I241W increases, but not to the same extent as seen in CYP2B4 or F202W. Mutation of F202 in CYP2B6 also leads to decreased rates of NADPH and O₂ consumption and water production were lower than those of CYP2B6 with little change in the rate of H₂O₂ production (Table 4). CYP2B6 and CYP2B6 F202W had similar fractions of O₂ diverted into the oxidase shunt, which was higher than that seen in CYP2B4 and CYP2B4 F202W.

Uncoupling to Hydrogen Peroxide and Water by Peripheral Pocket Mutants

The efficiency of coupling of reducing equivalents to product formation for CYP2B4 and the peripheral pocket mutants is shown in Table 5 and for CYP2B6 and the F202W mutant in Table 6 as the ratio of product formation:NADPH oxidation. Furthermore, the ratios of H₂O₂ production:NADPH oxidation, H₂O formation:NADPH oxidation, H₂O₂

production:O₂ consumption, and H₂O formation:product formation are also found in Tables 5 and 6.

Compound 0 (5b in Scheme 1) is a good Lewis base and upon the second protonation event of the CYP catalytic cycle, it may decay with release of hydrogen peroxide (peroxide shunt) or form Compound 1 (Cpd I) with release of water (6 in Scheme 1) providing the second branch point in the cycle. Dividing the hydrogen peroxide produced by the oxygen consumed, not the NADPH consumed, provides a measure of flux through the peroxide shunt. Compared with CYP2B4, F195W had ~18% less flux through the peroxide shunt (~0.07 lower H₂O₂:O₂), and F202W and I241W had increases in flux of ~63% (~0.24 higher H₂O₂:O₂) and ~82% (~0.31 higher H₂O₂:O₂), respectively. In 2B6, F202 displayed ~51% more flux through the peroxide shunt (~0.17 higher H₂O₂:O₂). Similar changes in flux through the peroxide shunt were seen in active site mutants of CYP2B1 [6]. For these mutants, these changes in the percentage of O₂ equivalents failing to undergo dioxygen bond scission at the second branch in Scheme 1 indicate changes in the oxygen activation chemistry, with F195W enhancing and F202W and I241W impairing this chemistry, as previously postulated for CYP101 V247A [5].

Flux diversion in the CYP catalytic cycle may also occur at Cpd I with substrate oxidation versus water release via oxidase activity. As seen in CYP3A4, water formation is the main result of Cpd I decay, even in the presence of substrate [43]. Changes in the partitioning between the oxidase shunt and productive hydroxylation reflect changes access to the heme [5]. A measure of CYP enzyme efficiency that addresses this partitioning is excess water produced divided by product produced. Wild type CYP2B4 and F195W both produce roughly four molecules of excess water for each productive substrate oxidation. In both enzymes, overall coupling to substrate hydroxylation for the mutants varies ~16%, but CYP2B4 F202W and I241W and CYP2B6 F202W produce excess water to a much greater extent than the respective wild-type enzymes. In CYP3A4 testosterone hydroxylation, a greater amount of water is produced per productive hydroxylation event at substrate concentrations below the K_M than at higher substrate concentrations [43]. Thus the coupling of reducing equivalents to product production in CYP2B4 I241W is underestimated. Previous analysis of the effects of amino acid substitutions on the stoichiometry of CYP enzyme catalysis were focused on mutations in the active site of the enzyme [5, 6]. In mutants where reducing equivalents partitioned to oxidase activity at the expense of productive substrate oxidation (T101M and V295I in CYP101 and V363A in CYP2B1), the ratio of H₂O:product was in excess of 50:1. Some mutants had substantial, but less severe, effects on partitioning at the third branch point (V247M and T101I in CYP101 and F206L and G478S in CYP2B1). While these mutants were located in the respective enzyme active site, residues 202 and 241 in CYP2B enzymes are not. Thus, the theoretical water to product production ratios for CYP2B4 F202W (~105:1) and CYP2B6 F202W (infinite due to the lack of detected product) are striking for residues not directly interacting with the substrate or redox partners.

Discussion

Previous structure-function studies of CYP2B enzymes utilizing site-directed mutagenesis focused on determinants of substrate specificity, metabolite profiles, or stability or on the contribution of non-active site residues to CYP enzyme plasticity [12, 13, 20, 44]. As expected, most of the amino acid residues that affect enzyme interaction with substrates and product production are located within the previously defined substrate recognition sites (SRS) [45]. Furthermore, substitutions that alter enzyme stability and plasticity are often found outside the enzyme active site [20, 46, 47]. However, some non-active site mutants exhibited significant changes in enzyme-ligand interactions [20]. Results from site-directed mutagenesis were strengthened by X-ray crystal structures of CYP2B enzymes that provided structural snapshots identifying amino acids within the active site that interact with bound ligands [12, 13].

Analysis of X-ray crystal structures of CYP2B4 revealed a peripheral pocket where the detergent CYMAL-5 bound. Residues in the pocket show no differences in orientation among structures where CYMAL-5 was observed in the peripheral pocket. These include the ligand-free closed conformation of CYP2B4 (PDB ID: 3MVR) and CYP2B4 in complex with paroxetine (PDB ID: 4JLT), (Figure 3A). However, in the absence of CYMAL-5 in this pocket, represented by structures of CYP2B4 in complex with clopidogrel (PDB ID: 3ME6) or 1-(4-chlorophenyl)imidazole (1-CPI, PDB ID: 2Q6N), eleven of the roughly twenty amino acids that were found lining the peripheral cavity, including C180, F188, V194, L198, L199, F202, F203, F206, F244, F296, F297, and T300, demonstrated considerable spatial shift or rotation (Figure 3B). In structures without CYMAL-5 in the peripheral pocket, residue 202 protruded into the pocket.

Overlays containing CYP2B4 structures with CYMAL-5 bound in the peripheral pocket and structures from other members of the CYP2 family of enzymes, including CYP2A6, CYP2A13, CYP2C9, CYP2C19, CYP2D6, and CYP2E1, revealed two structural features that are unique to CYP2B4. In each of these other enzymes, the residue corresponding to L198 in CYP2B4 protruded into the space occupied by CYMAL-5 in the CYP2B4 structures. In addition, in CYP family 2 enzymes besides CYP2B4 and 2B6, polar residues at the protein surface near where CYMAL-5 would have to bind prevent access to any unoccupied space from the protein surface. This observation and the absence of detergent bound at this peripheral site in the available structures helped us to conclude that the hydrophobic peripheral pocket is unique to the CYP2B subfamily of enzymes and might be of functional significance.

A plausible link between the functional alterations in F202W in CYP2B4 and CYP2B6 and structural changes due to the tryptophan substitution revolves around the close proximity of F297 in the active site and F202 in the peripheral cavity. As shown in Figure 3C, the orientations of these two residues are intertwined. F297 forms part of one wall of the active site, and when this residue is replaced with Ala, F202 swings toward the active site and compensates for the loss of aromatic bulk in the mutant [37]. In the structure of CYP2B4 complexed with clopidogrel, which does not have CYMAL-5 present in the peripheral pocket, F297 swings away from the active site and the ligand. In contrast, in the ticlopidine

complex, which has CYMAL-5 in the peripheral pocket, F202 orients more toward the active site, and F297 extends more into the active site (Figure 3D). Presumably, binding of ligands in the peripheral pocket might lead to a change in active site topology that could be mimicked by the F202W mutation and results in unproductive orientation of 7-EFC or 7-BR. I241W also shows severely impaired activity, but the connection to active site topology may not be as direct as that of F202W. In CYP2B4 residue 241 is located on the G-helix. This structural element, along with the F-, F'-, and G'-helices, form the previously described F-G cassette. When the global conformation of CYP2B enzymes changes upon ligand binding, this set of helices is thought to form a "roof" over the enzyme active site [48]. Greatly increasing the bulk and rigidity of residue 241 with tryptophan instead of isoleucine likely prevents efficient closure of the enzyme active site. For 7-EFC, this is reflected in the increase in K_M of more than 6 times and a catalytic efficiency $1/26^{\text{th}}$ that of wild type enzyme. This mutant also did not catalyze 7-BR dealkylation.

Interestingly, unlike F195, both F202 and I241 are involved in helix-helix interactions and show severely impaired function when substituted with tryptophan. The increased bulk of tryptophan at residue 202 may impair efficient movement of the F- and G-helices around the I-helix to allow for closure of the active site, and increasing bulk at residue 241 may prevent efficient movement at the interface of the E-, F-, and G-helices. Introduction of leucine at residue 202 reduced the catalytic efficiency of 7-EFC *O*-deethylation by CYP2B1 (0.29 vs. 0.40) [17, 49]. The ability of the enzyme to utilize H_2O_2 to support catalytic activity was also compromised. The catalytic efficiency of 2B1 F202L 7-BR turnover was higher than CYP2B1 (2.3 vs. 1.4), but for testosterone, the catalytic efficiency was lower (0.03 vs. 0.14). Thus, the effect on catalysis of altering F202 appears to be dependent on both the identity of the amino acid substituent and the substrate of interest.

Similar to previous analyses of CYP active site mutants, flux through the peroxide shunt, branch two in Scheme 1, is roughly 20% lower for F195W and 60% and 80% higher for F202W and I241W than for wild type [5, 6]. The major change in constituent flux is seen at the third branch point. Wild type CYP2B4 and F195W theoretically produce roughly four molecules of excess water for each molecule of substrate oxidized. However, this value increases three times for I241W and more than twenty times for F202W, indicating a shift towards oxidase activity. A similar uncoupling of reducing equivalent flux to product production is seen in CYP2B6 F202W. These measurements should be seen as estimates as discussed in Grinkova et al. [43]. In microbial CYP enzymes, these values may be found from literature data, as for CYP101 [5], or based on the reaction of Cpd 1 + Substrate $\rightarrow$ Product, as for CYP119 [50]. Since the reactivity rates of Cpd 1 formed in CYP2B enzymes have not been measured, any water production rate is theoretical in nature.

Broader implications of these results apply to single nucleotide polymorphisms (SNPs) of CYP2B6, since the resulting mutations are located outside the enzyme active site. The clinical relevance of SNPs in drug metabolizing enzymes has been of substantial recent interest [51, 52]. In CYP enzymes and other proteins, non-active site residues likely maintain three dimensional protein structure, influence active site topology, and contribute to intrinsic protein dynamics. Data from multiple complementary methods describe the importance of protein dynamics in ligand binding and catalysis for many enzymes [53–56].

Furthermore, the presence of peripheral binding sites in CYP enzymes has been previously seen in X-ray crystal structures of CYP3A4 in complex with progesterone [57]. Possible functional effects of ligand binding to a peripheral pocket include differences in enzyme activity levels or non-Michaelis-Menten kinetics [58].

In conclusion, mutation of residues within, but not near, a peripheral pocket in CYP2B4 produces significant functional changes in the enzyme that can be explained on the basis of indirect effects on active site topology. The presumed change in active site topology produced striking changes in both oxidative substrate turnover and coupling of reducing equivalents to productive substrate oxidation. As of yet, the biological role of the peripheral pocket documented here is not known, but it could play a role in the regulation of CYP2B activity or contribute to non-Michaelis-Menten kinetic effect seen in some substrate oxidations. In both cases, the role of the peripheral pocket may have implications for drug turnover or drug-drug interactions.

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Highlights

- A peripheral pocket was observed in crystal structures of CYP2B enzymes.
- Mutation of residues located within the pocket markedly altered enzyme function.
- In mutants F202W and I241W substrate turnover is uncoupled from reducing equivalent use.
- Changes in this peripheral pocket may lead to altered active-site topology.

Figure 1. Observed peripheral pocket in CYP2B4. Cavities found in the CYP2B4-paroxetine complex (4JLT) using Mole 2.0 are depicted as surfaces, and the protein backbone is shown as a gray ribbon. The active site (dark gray/maroon) is physically separate from the peripheral pocket occupied by CYMAL-5 (light green).

Figure 2.

Inhibition of CYP2B6 by CYMAL-5. A) The dose-response curve for CYMAL-5 inhibition of CYP2B6. B) Steady-state kinetics of 7-EFC *O*-deethylation by CYP2B6 in the absence (red circles) or presence (blue diamonds) of 750 μ M CYMAL-5.

Figure 3.

General structural changes in the peripheral pocket of CYP2B4 due to the presence or absence of CYMAL-5. A) Peripheral pocket overlay of the structures of wild-type CYP2B4 ligand free (3MVR, green) and CYP2B4 in complex with paroxetine (4JLT, magenta) showing the residue side-chains located within 5 Å of the CYMAL-5 in the cavity. The heme is shown as red sticks and CYMAL-5 as thin lines. B) Peripheral pocket overlay of CYP2B4 in complex with clopidogrel (3ME6, orange) and CYP2B4 in complex with 1-CPI (2Q6N, cyan). CYMAL-5 was not observed in the peripheral pocket of any of these structures. The respective ligands in the active site of each structure are not shown for clarity in the figure. C) Residues 202, 296, and 297 from the X-ray crystal structure of CYP2B4 in complex with clopidogrel shown in sticks and transparent spheres to highlight their spatial proximity and the ligand orientation. D) Overlay of CYP2B4 in complex with clopidogrel (orange) or ticlopidine (3KW4, blue) highlighting the relationship between spatial rearrangements of F202, F296, and F297 and ligand orientation.

Scheme 1.

Cytochrome P450 reaction cycle. The productive pathway is shown as solid arrows. The three unproductive shunts are shown as dashed arrows. Cpd 0: Compound 0, Cpd I: Compound I, RH: substrate, ROH: hydroxylated product.

Table 1

Oligonucleotides used for construction of CYP2B4 mutants using PCR. Nucleotides changed from the wild-type CYP2B4 sequence are indicated in bold italics.

Mutants	Oligonucleotide
CYP2B4	
F195W	5'-AAG GAC CCC GTG TGG CTG CGG CTG CTG G-3' 5'-CAG CAG CCG CAG CCA CAC GGG GTC CTT-3'
F202W	5'-CGG CTG CTG GAC TTG TGG TTC CAG TCC TTC TCC C-3' 5'-G GGA GAA GGA CTG GAA CCA CAA GTC CAG CAG CCG -3'
I241W	5'-AAC CTG CAG GAG TGG AAC ACT TTC AT-3' 5'-AT GAA AGT GTT CCA CTC CTG CAG GTT-3'
CYP2B6	
F202W	5'-TC CTG AAG ATG CTG AAC TTG TGG TAC CAG ACT TTT TCA CTC ATC-3' 5'-GAT GAG TGA AAA AGT CTG GTA CCA CAA GTT CAG CAT CTT CAG GA-3'

Table 2

Steady state kinetics of substrate oxidation by CYP2B4 enzymes

Enzyme	7-EFC			7-BR		
	k_{cat} (min ⁻¹) ^a	K_M (μM)	k_{cat}/K_M	k_{cat} (min ⁻¹) ^a	K_M (μM)	k_{cat}/K_M
CYP2B4	7.4 ± 1.3	28.4 ± 7.6	0.26	1.96 ± 0.01	1.03 ± 0.22	1.90
F195W	12.9 ± 0.7	33.5 ± 14.1	0.39	1.83 ± 0.02	0.78 ± 0.07	2.35
F202W	0.3 ± 0.1	15.4 ± 8.8	0.02 ^b	N.D.		
I241W	2.4 ± 0.7	189.1 ± 73.8	0.01	N.D.		
CYP2B6	8.1 ± 0.2	6.2 ± 0.6	1.31			
F202W	N.D.					
CYMAL-5	8.5 ± 0.2	18.3 ± 2.4	0.46			

Results are the average ± confidence interval calculated for p=0.05 of 3–4 independent experiments done in duplicate. N.D., not detectable above background; maximum fluorescence was less than twice the zero product value, corresponding to 7-HFC production rates <0.05 min⁻¹ or resorufin production rates <0.1 min⁻¹.

^a k_{cat} is a representation of nmoles of product produced per minute per nmole of CYP enzyme in the reaction.

^b Global fitting of data from three experiments was used due to the very low turnover of 7-EFC. Therefore the mean and standard deviation refer to the fit to the Michaelis-Menten equation not differences among experiments.

Table 3

Rates determined in the absence and presence of 7-ethoxy-4-(trifluoromethyl) coumarin for CYP2B4 and three mutants.

Enzyme	[7-EFC] (μM)	Rate (nmol/min/nmol P450) ^a				Excess H ₂ O ^a		
		NADPH	Product	H ₂ O ₂	O ₂	Eq. 1 ^{b,c}	Eq. 2 ^{b,c}	Eq. 3 ^{b,c}
CYP2B4	0	17.9 ± 1.4	NA ^d	12.0 ± 0.9	15.3 ± 0.6	5.9 ± 1.3	6.7 ± 1.7	5.1 ± 3.7
F195W	0	26.3 ± 0.6	NA ^d	11.7 ± 0.4	19.2 ± 0.9	14.6 ± 1.0	14.9 ± 1.3	14.3 ± 2.8
F202W	0	11.7 ± 0.3	NA ^d	8.1 ± 0.3	10.2 ± 0.3	3.5 ± 0.7	4.1 ± 0.7	3.0 ± 1.1
I241W	0	14.8 ± 0.6	NA ^d	10.0 ± 0.5	12.7 ± 0.9	4.8 ± 1.2	5.3 ± 1.8	4.3 ± 2.4
CYP2B4	150	64.4 ± 0.7	9.5 ± 0.5	17.6 ± 0.6	46.3 ± 1.2	37.2 ± 1.0	38.5 ± 1.8	36.0 ± 1.0
F195W	150	72.5 ± 3.9	11.6 ± 0.6	15.2 ± 0.2	49.7 ± 0.7	45.7 ± 3.2	45.7 ± 2.5	45.7 ± 8.4
F202W	150	38.4 ± 5.4	0.2 ± 0.1	17.3 ± 0.7	27.7 ± 0.9	20.9 ± 5.4	20.3 ± 0.7	21.5 ± 10.2
I241W	150	36.4 ± 2.4	1.1 ± 0.1	20.0 ± 0.3	29.1 ± 0.7	15.3 ± 2.7	15.8 ± 1.6	14.8 ± 3.8

^aResults are the average ± confidence interval calculated for p= 0.05 of 3–6 independent experiments done in duplicate.

^bEq. 1: H₂O = NADPH – H₂O₂ – Product. Eq. 2: H₂O = 2(O₂ – H₂O₂ – Product). Eq. 3: H₂O = 2(NADPH – O₂).

^cUnits = nmol/min/nmol P450

^dNA = Not Applicable. This condition did not have substrate, so no product is formed.

Table 4

Rates determined in the absence and presence of 7-ethoxy-4-(trifluoromethyl)coumarin for CYP2B6 and F202W.

Enzyme	[7-EFC] (μM)	Rate (nmol/min/nmol P450) ^a			Excess H ₂ O ^a		
		NADPH	Product	H ₂ O ₂	O ₂	Eq. 1 ^{b,c}	Eq. 2 ^{b,c}
CYP2B6	60	54.4 ± 0.7	8.1 ± 0.2	12.1 ± 0.5	37.3 ± 2.0	34.1 ± 1.0	34.2 ± 3.2
F202W	60	34.7 ± 3.7	ND ^d	12.0 ± 1.2	24.5 ± 1.5	22.7 ± 2.5	24.9 ± 5.0

^a Results are the average ± confidence interval calculated for p= 0.05 of 3–6 independent experiments done in duplicate.

^b Eq. 1: H₂O = NADPH – H₂O₂ – Product. Eq. 2: H₂O = 2(O₂ – H₂O₂ – Product). Eq. 3: H₂O = 2(NADPH – O₂).

^c Units = nmol/min/nmol P450.

^d ND = Not Detected. The amount of product produced did not have a fluorescence intensity greater than twice the background intensity; the detection limit is <0.05 nmoles/min/nmole P450.

Table 5

Effect of mutations on coupling to product formation and uncoupling to hydrogen peroxide and water formation in CYP2B4.

Enzyme	[7-EFC] (μM)	Product/NADPH	H ₂ O ₂ /NADPH	H ₂ O ₂ /O ₂	H ₂ O/NADPH	H ₂ O/Product ^a	H ₂ O/Product ^b
CYP2B4	150	0.15	0.27	0.38	0.58	3.9	3.8
F195W	150	0.16	0.21	0.31	0.63	3.9	3.9
F202W	150	0.0052	0.45	0.62	0.54	104	107
I241W	150	0.03	0.55	0.69	0.42	14	13

^aExpressed as ratio of H₂O:Product using excess H₂O formation from Eq. 1 in Table 3.

^bExpressed as ratio of H₂O:Product using excess H₂O formation from Eq. 3 in Table 3.

Table 6

Effect of F202W on coupling to product formation and uncoupling to hydrogen peroxide and water formation in CYP2B6.

Enzyme	[7-EFC] (μM)	Product/NADPH	H ₂ O ₂ /NADPH	H ₂ O ₂ /O ₂	H ₂ O/NADPH	H ₂ O/Product ^a	H ₂ O/Product ^b
CYP2B6	60	0.15	0.22	0.32	0.63	4.2	4.2
F202W	60	0	0.35	0.49	0.65	∞	∞

^a Expressed as ratio of H₂O:Product using excess H₂O formation from Eq. 1 in Table 4.

^b Expressed as ratio of H₂O:Product using excess H₂O formation from Eq. 3 in Table 4.