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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-benzyloxyresorufin *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.



## 1. Introduction

Cytochrome P450 (CYP) 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

*Abbreviations:* 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; $b_5$, cytochrome $b_5$; IPTG, β-D-1-thiogalactopyranoside; ALA, δ-aminolevulinic acid; β-ME, 2-mercaptoethanol; PDB, protein data bank; 4-CPI, 4-(4-chlorophenyl) imidazole; 1-PBI, 1-biphenyl-4-methyl-1H-imidazole; 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.

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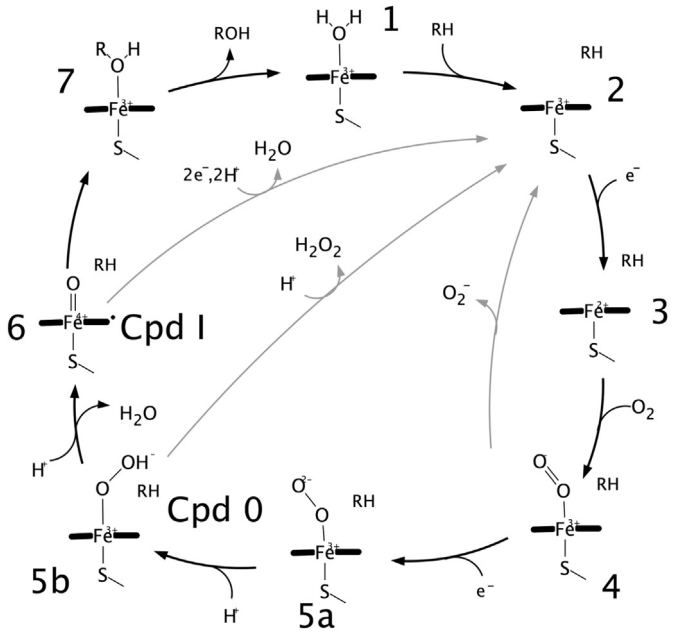


**Scheme 1.** Cytochrome P450 reaction cycle. The productive pathway is shown as solid arrows. The three unproductive shunts are shown as dashed arrows. Cpd 0: Compound 0, Cpd I: Compound I, RH: substrate, ROH: hydroxylated product.

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 physio-chemical 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 3–12% 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 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, CYP2B4dH[3] (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 (Mr ~75–900) 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 (Fig. 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-benzyloxyresorufin (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.

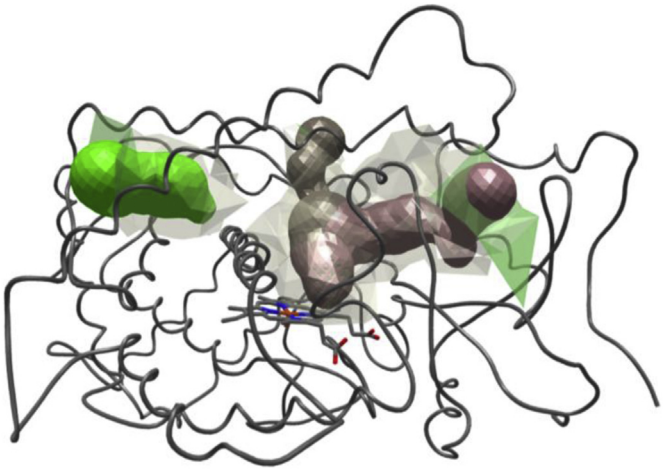

**Fig. 1.** Observed peripheral pocket in CYP2B4. Cavities found in the CYP2B4-paroxetine complex (4JLT) using Mole 2.0 are depicted as surfaces, and the protein backbone is shown as a gray ribbon. The active site (dark gray/maroon) is physically separate from the peripheral pocket occupied by CYMAL-5 (light green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[3] In 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].