Structural and Biophysical Characterization of Human Cytochromes P450 2B6 and 2A6 Bound to Volatile Hydrocarbons: Analysis and Comparison^S

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ABSTRACT

X-ray crystal structures of complexes of cytochromes CYP2B6 and CYP2A6 with the monoterpene sabinene revealed two distinct binding modes in the active sites. In CYP2B6, sabinene positioned itself with the putative oxidation site located closer to the heme iron. In contrast, sabinene was found in an alternate conformation in the more compact CYP2A6, where the larger hydrophobic side chains resulted in a significantly reduced active-site cavity. Furthermore, results from isothermal titration calorimetry indicated a much more substantial contribution of favorable enthalpy to sabinene binding to CYP2B6 as opposed to CYP2A6, consistent with the previous observations with (+)- α -pinene. Structural analysis of CYP2B6 complexes with sabinene and the structurally similar (3)-carene and comparison with previously

solved structures revealed how the movement of the F206 side chain influences the volume of the binding pocket. In addition, retrospective analysis of prior structures revealed that ligands containing –Cl and –NH functional groups adopted a distinct orientation in the CYP2B active site compared with other ligands. This binding mode may reflect the formation of Cl- π or NH- π bonds with aromatic rings in the active site, which serve as important contributors to protein-ligand binding affinity and specificity. Overall, the findings from multiple techniques illustrate how drugs metabolizing CYP2B6 and CYP2A6 handle a common hydrocarbon found in the environment. The study also provides insight into the role of specific functional groups of the ligand that may influence the binding to CYP2B6.

Introduction

Cytochrome P450 (P450)-dependent monooxygenases are heme-containing enzymes that metabolize a vast array of drugs and endogenous chemicals (Johnson and Stout, 2013). The 57 P450 enzymes found in humans are divided into 18 families and 44 subfamilies (Nelson, 2009). Cytochromes that play a dominant role in the metabolism of drugs and xenobiotics, including environmental toxins, are members of the 1, 2, and 3 families, including CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 (Ortiz de Montellano, 2005). Each of these enzymes binds, metabolizes, and is inhibited by a unique set of compounds of different size, shape, and stereochemistry. A single compound may interact with multiple cytochrome enzymes in different

orientations relative to the heme and with different binding affinities consistent with the different active site topologies. Additionally, these enzymes are also known for their high degree of conformational flexibility, which allows them to accommodate a broad range of ligands within the active site (Johnson and Stout, 2013).

Enzymes from the CYP2B subfamily were among the first mammalian microsomal P450 enzymes that were isolated and studied. Human CYP2B6 metabolizes approximately 3 to 12% of all available drugs and is inhibited by many clinically relevant drugs and small-molecule inhibitors (Wang and Tompkins, 2008). This enzyme, found in liver, lung, kidney, and brain, is highly polymorphic in nature with 51 known alleles (Zanger et al., 2014). The most commonly observed single nucleotide polymorphisms yield the Q172H, K262R, and R487C variants (Zanger et al., 2007). Our laboratory has used N-terminally truncated and engineered constructs of CYP2B enzymes with specific internal mutations to obtain increased stability, solubility, and purity of the protein (Scott et al., 2001; Gay et al., 2010a). Structural and biophysical studies on human CYP2B6 remain focused on a construct

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ABBREVIATIONS: BME, 2-mercaptoethanol; 4-BP, 4-benzylpyridine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FA-7, 234-chol, 3 α -hydroxyl-7 α ,12 α -di-((2-(trimethylamino)ethyl)phosphoryl)ethoxy)-cholane; 4-CPI, 4-(4-chlorophenyl)imidazole; CYMAL-5, 5-cyclohexyl-1-pentyl- β -D-maltoside; DTT, dithiothreitol; ITC, isothermal titration calorimetry; 4-NBP, 4-(4-nitrobenzyl)pyridine; P450, cytochrome P450; PDB, protein data bank; PMSF, phenylmethylsulfonyl fluoride; RMSD, root-mean-square deviation; TCEP, tris(2-carboxyethyl)phosphine.

containing two mutations (K262R/Y226H) that has demonstrated superior yield and stability (Gay et al., 2010a).

Recent improvements in protein expression and purification methods (Shah et al., 2011) have led to the determination of more than 15 crystal structures from the CYP2B subfamily in the last 5 years. The structures of rabbit CYP2B4 and human CYP2B6 in complex with two molecules of amlodipine helped elucidate for the first time the substrate access channels in CYP2B enzymes (Shah et al., 2012). More recently, we investigated the binding of the environmentally important monoterpene (+)- α -pinene to human CYP2B6 via X-ray crystallography, and the thermodynamics of ligand binding to the relatively more rigid human CYP2A6 or to the more flexible CYP2B6 were compared using isothermal titration calorimetry (ITC) (Wilderman et al., 2013). CYP2A6 demonstrated entropy-driven binding of (+)- α -pinene, in sharp contrast to the predominant enthalpic contributions to ligand binding in the case of CYP2B6.

Both CYP2B6 and CYP2A6 were shown previously to be involved in metabolism of several monoterpenes found in various plant oils (Miyazawa et al., 2003; Miyazawa and Gyoubu, 2006). To date, a crystal structure of CYP2A6 with environmentally important monoterpenes and a direct structural comparison of CYP2B and 2A enzymes in the presence of such hydrocarbons have been lacking. Sabinene is a major olefinic monoterpene derived from the volatile oils found in plants and is often used as an odorant and perfume additive and as an important constituent of plant oils in alternative medicine (Karp et al., 1987; Mercier et al., 2009; Harrison et al., 2013). In the current study, we sought to determine the first crystal structure of human CYP2A6 in complex with sabinene and compare it with the CYP2B6 complex. In addition, we used ITC to investigate the thermodynamic basis of sabinene binding to each enzyme. To probe further the hydrocarbon binding to P450 enzymes, we determined the structure of CYP2B6 in complex with a second monoterpene, (+)-3-carene. The results presented here provide insight into the differences and similarities of 1) how two different subfamilies of P450 enzymes interact with a monoterpene; and 2) how multiple monoterpenes with similar chemical structures, sabinene, (+)-3-carene, and the previously reported (+)- α -pinene bind to CYP2B6. Understanding the structural and biophysical basis for small hydrocarbon binding across multiple P450 subfamilies and within the same subfamily will help us to understand the role of various P450 enzymes in the metabolism of environmental toxicants and natural products.

Materials and Methods

Sabinene (4-methylene-1-(1-methylethyl)bicyclo[3.1.0]hexane) was purchased from Indofine chemicals (Hillsborough, NJ) and (+)-3-carene (3,7,7-trimethylbicyclo[4.1.0]hept-3-ene) from Sigma-Aldrich (St. Louis, MO). 3-[(3-cholamidopropyl) dimethylammonia]-1-propanesulfonate (CHAPS), 5-cyclohexyl-1-pentyl- β -D-maltoside (CYMAL-5) and isopropyl β -D-1-thiogalactopyranoside were purchased from Calbiochem (EMD Chemicals, San Diego, CA) and Anatrache (Maumee, OH), respectively. Amicon ultrafiltration devices were from Millipore (Billerica, MA). Nickel-nitrilotriacetic acid (Ni-NTA) affinity resin was from Thermo Scientific (Rockford, IL), and Macro-Prep CM cation exchange resin was received from Bio-Rad Laboratories (Hercules, CA). The plasmid pGro7 harboring the GroEL/ES chaperones was from Takara Bio (Shiba, Japan). The cell lines used

to express *Escherichia coli* were JM109 and TOPP3 cells purchased from Stratagene (La Jolla, CA). 3 α -Hydroxyl-7 α ,12 α -di-((2-(trimethylamino)ethyl)phosphoryl)ethoxy)-cholane (FA-7/234-chol) is a facial amphiphile used as described previously (Lee et al., 2013). D-Aminolevulinic acid, phenylmethylsulfonyl fluoride (PMSF), lysozyme, dithiothreitol (DTT), 2-mercaptoethanol (BME), and TCEP (tris(2-carboxyethyl)phosphine) were from Sigma-Aldrich. The CYP2A6 plasmid (pCW2A6dH) was a gift from Dr. Eric Johnson at the Scripps Research Institute (La Jolla, CA). All figures present in this article were created using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4; Schrödinger, LLC, New York).

Protein Expression and Purification. Human CYP2B6 was expressed and purified as described previously (Shah et al., 2012). Protein expression was carried out for 72 hours at 30°C in *E. coli* JM109 cells coexpressing pKK2B6dH (Y226H/K262R) and pGro7 plasmid after induction with isopropyl β -D-1-thiogalactopyranoside and D-aminolevulinic acid ($A_{600} \sim 0.7$ at 37°C). The cells were centrifuged at 4000g for 15 minutes, and 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% glycerol, 10 mM BME, and 0.5 mM PMSF. Lysozyme (0.2 mg/ml) was added to the resuspended culture, which was stirred at 4°C for 30 minutes. The cells were centrifuged again before resuspension in the same buffer containing 500 mM potassium phosphate. The cells were then sonicated on ice, and the lysate was stirred at 4°C in the presence of CHAPS (0.8% w/v) for 90 minutes before ultracentrifugation at 245,000g for 45 minutes in an Optima L-80 XP ultracentrifuge (Beckman Coulter, Fullerton, CA). The concentration was measured using the reduced CO difference spectra (Omura and Sato, 1964). The CYP2A6 plasmid was transformed into the TOPP3 cell line, and the coexpression protocol was carried out in a similar fashion as with CYP2B6, except that the cells were grown for 48 hours at 30°C. For nickel-affinity chromatography in the presence of CHAPS, protein was loaded 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 BME, 0.5 mM PMSF, 0.5% CHAPS, and 5 mM histidine. CYP2B6 or 2A6 was eluted using the same buffer containing 50 mM histidine. The fractions containing protein of the highest purity as measured by the $A_{417/280}$ ratios were pooled. The cytochrome concentration was measured as described already, and the pooled protein was diluted in buffer with 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% (w/v) CHAPS. A Macro-Prep CM cation exchange column was used to further purify the protein using the buffer containing 50 mM potassium phosphate (pH 7.4 at 4°C), 20% (v/v) glycerol, 1 mM EDTA, 0.2 mM DTT, and 20 mM NaCl, followed by elution using buffer containing 500 mM NaCl. It is important to note that the reducing agent 0.2 mM TCEP was used in place of DTT in all the preceding buffers used for the purification of CYP2A6. The fractions containing protein of the highest purity as measured by the $A_{417/280}$ ratios were pooled, and the cytochrome concentration was measured as described earlier.

Crystallization and Data Collection. CYP2B6 and CYP2A6 were crystallized as described previously (Shah et al., 2011). The pooled protein from the CM column was further diluted to 18 μ M in 50 mM potassium phosphate (pH 7.4 at 4°C), 500 mM NaCl, 500 mM sucrose, 1 mM EDTA, and 0.2 mM DTT or 0.2 mM TCEP. Sabinene or (+)-3-carene for CYP2B6 and sabinene for CYP2A6 was added to a final concentration of 180 μ M, and the samples were concentrated to 280 and 150 μ M, respectively, by centrifugation using 50-kDa cutoff Amicon ultrafiltration devices (Millipore). The protein aliquots were again diluted to 18 μ M using the above buffer containing ligand at a final concentration of 180 μ M. The procedure was repeated twice before concentrating the protein to the final concentration of 280 μ M for CYP2B6 and 150 μ M for CYP2A6. These samples were supplemented with 4.8 mM CYMAL-5 and 0.077% (w/v) 234-chol before crystallization, and crystal screening was performed by the sitting drop vapor diffusion method. The crystals of CYP2B6 were obtained from the Hampton Research (Aliso Viejo, CA) Index screen

(HR2-144) in 0.1 M HEPES pH 7.5, 25% w/v polyethylene glycol 3350 (sabinene complex), and 0.2 M lithium sulfate monohydrate, 0.1 M HEPES pH 7.5, 25% w/v polyethylene glycol 3350 (3-carene complex) over a period of 4–6 days at 18°C. CYP2A6 crystals grew from the Molecular Dimensions (Altamonte Springs, FL) MD1-31 screen in 0.3 M sodium acetate, 0.1 M sodium cacodylate (pH 8.5), and 25% w/v poly(ethylene glycol) 2000 mono-methyl ether after 10 days of incubation at 18°C. Crystals were transferred to the mother liquor with 20% sucrose for cryoprotection before flash freezing in liquid nitrogen. Crystallographic data on CYP2B6 crystals in the presence of sabinene and (+)-3-carene were collected remotely at Stanford Synchrotron Radiation Lightsources using beam line BL7-1 with a ADSC Quantum 315r charge-coupled device detector, whereas the data on CYP2A6 with sabinene were collected at the Advanced Light Source in Berkeley (BL 5.0.3, ADSC Q315R detector). In both cases, data sets were collected using 1-degree oscillations over 240 frames and 20-second exposure at 100 K. Data were integrated using iMOSFLM (Battye et al., 2011) at 2.1 and 2.2 Å resolution for CYP2B6-sabinene and CYP2B6-(+)-3-carene, respectively, and 2.6 Å resolution for CYP2A6-sabinene and were further scaled via SCALA in CCP4i (Bailey, 1994).

Structure Determination and Refinement. The structure of CYP2B6 in complex with sabinene and (+)-3-carene was determined via the molecular replacement program Phaser (McCoy et al., 2007) using an ensemble of all five previously solved CYP2B6 structures (PDB ID 3IBD, 3QOA, 3QU8, 3UA5, and 4I91). The Matthews coefficient suggested the presence of two molecules per asymmetric unit in the $P3_2$ space group with 62% solvent content for each of the CYP2B6 complexes. The output model from Phaser was subjected to rigid body and restrained refinement in REFMAC (Murshudov et al., 1997), followed by model building using COOT (Emsley and Cowtan, 2004) and refinement using $F_o - F_c$ and $2F_o - F_c$ maps contoured at 3- σ and 1- σ , respectively. The crystallographic information library description files for sabinene and (+)-3-carene were created using sketcher in CCP4 (Bailey, 1994). Iterative model building and refinement was performed until the R-factor and R-free stopped improving. The R-factor of 0.21 and R-free of 0.25 were achieved for the sabinene complex, and R-factor of 0.17 and R-free of 0.22 were achieved for the (+)-3-carene complex of CYP2B6. MOLPROBITY (Davis et al., 2004) ranked the CYP2B6-sabinene complex at 91st percentile and (+)-3-carene complex at 92nd percentile in terms of overall geometry compared with other structures at similar resolution. In addition, there were no Ramachandran outliers or bad bond lengths and angles. In the sabinene complex, 448 water molecules were seen and 459 in (+)-3-carene structure, and three CYMAL-5 molecules were observed per molecule in the asymmetric unit for each of the structures, with one present in the peripheral pocket near residues F202 and F296. Residues from 28 to 492 were present in the final model of the CYP2B6-sabinene complex, and one C-terminal histidine was located as part of the poly-His-tag in each chain. In the CYP2B6-(+)-3-carene complex, residues 28–491 were present and density for residues 136–138 and C-terminal histidines were disordered and thus were not modeled in the final structure.

The structure of CYP2A6 in complex with sabinene was determined using an ensemble of four previously solved structures (PDB ID 1Z11, 3T3R, 1Z10, and 2FDY) merged in Phaser. The space group was $P1$, and Matthews coefficient analysis indicated 57% solvent content, assuming six molecules in the asymmetric unit. REFMAC was used to refine the model further from COOT until the R-factor and R-free reached 0.19 and 0.26, respectively. In the CYP2A6 structure with six chains, a total of 312 water molecules were added, and the structure was further analyzed using MOLPROBITY, which scored the structure at 89th percentile among structures of comparable resolution with no Ramachandran outliers or bad bond lengths or angles. The final model of CYP2A6 consisted of residues 31–495 in each chain with a terminal histidine being part of the larger poly-His tag. Electron density was not sufficient to build histidine residues beyond 491 in CYP2B6-sabinene and beyond 495 in the CYP2A6-sabinene complex due to conformational disorder.

Spectral Binding Titrations. The absorbance spectra were measured with an MC2000-2 multichannel charge-coupled device rapid scanning spectrometer (Ocean Optics, Dunedin, FL) equipped with one absorbance and one fluorescence channel, a pulsed Xe-lamp PX-2 light source, and a homemade thermostated cell chamber with a magnetic stirrer. A semimicro quartz cell with a stirring compartment (10 × 4-mm light path) from Hellma GmbH (Müllheim, Germany) was used in the titration experiments. All titration experiments were carried out at 25°C with continuous stirring in buffer containing 50 mM potassium phosphate (pH 7.4 at 4°C), 500 mM NaCl, 1 mM EDTA, and 1 mM TCEP. Ligand was dissolved in acetone, and total solvent concentration at the end of each titration was less than 1%. A baseline was recorded between 340 and 700 nm using this buffer. A spectrum was recorded after the addition of protein to the buffer. Spectra were recorded after the addition of aliquots of ligand to the sample cuvette.

Principal component analysis combined with least-squares approximation of the spectra of principal components with a linear combination of appropriate prototype spectral standards was used to interpret changes in absorbance spectra in spectral titration experiments, as previously described (Davydov et al., 1995; Renaud et al., 1996). The spectral standards used in the titrations included the spectra of ferric high-spin, ferric low-spin, and ferric P420 states obtained for full length CYP2B4 (Davydov et al., 1995). The spectral dissociation constants (K_S) were generally obtained by fitting the data to the equation for ligand binding for high affinity ligands $2\Delta A = (\Delta A_{\max}/[E_0])((K_D + [I_0] + [E_0]) - (K_D + [I_0] + [E_0])^2 - 4[E_0][I_0])^{1/2}$, where $\Delta A_{\max}$ is the maximum change in the fraction of high-spin enzyme, E_0 is total enzyme concentration, and I_0 is total inhibitor concentration when data are normalized to total cytochrome enzyme concentration using SPECTRALAB (Davydov et al., 1995).

ITC Experiments. ITC experiments were performed on a VP-ITC calorimeter (Malvern Instruments, Worcestershire, UK). The volume of the calorimetric cell was 1.4 ml, and the titrations were conducted by adding the titrant in steps of 10 μ l. Experiments were performed at 25°C with a 180-second initial delay. All solutions were thoroughly degassed to prevent bubble formation in the cell from stirring. Freshly prepared CYP2B6 or CYP2A6 was dialyzed extensively against degassed buffer containing 50 mM potassium phosphate (pH 7.4 at 4°C), 500 mM NaCl, 1 mM EDTA, and 1 mM TCEP, as previously reported (Wilderman et al., 2013). The protein concentration was 10 μ M, and the ligand concentration was 25 μ M. Cold methanol was added to a final concentration of 2% immediately before temperature equilibration. Ligand stock solutions were prepared in 100% methanol. Care was taken to prevent methanol evaporation from each of the solutions. The stability and CO binding properties of the protein were not altered in the buffer containing 2% methanol for the duration of the ITC experiments. Protein and ligand samples were quickly preincubated to the required temperature using a ThermoVac (Malvern Instruments) and loaded into the calorimetric cell and titration syringe, respectively. The titration cell was stirred continuously at 305 rpm. Reference titrations were carried out by injecting each ligand into buffer alone in the calorimetric cell, and the heat of dilution was subtracted from the ligand-protein titration data. The binding isotherms were best fit to a one-set binding site model by Marquardt nonlinear least-squares analysis to obtain the binding stoichiometry (N), association constant (K_A), and thermodynamic parameters of the interaction using ORIGIN, Version 7 (Malvern Instruments). Fitting of the data to a two-site model or sequential binding model did not improve the fit and yielded unreasonable error values. When total heme protein concentration was used to calculate enthalpic changes, thermodynamic parameters remained unchanged, but the stoichiometric ratio decreased ~20% for CYP2B6 and ~10% for CYP2A6. This observation is consistent with our prior proposal that the 1:1 stoichiometric binding ratio represents the active pool of cytochrome determined by reduced carbon monoxide difference spectra, as shown in the binding of imidazole ligands to rabbit CYP2B4 (Muralidhara et al., 2006).

Results

Overall Structures of CYP2B6 and CYP2A6 in Complex with Monoterpenes. The crystal structures of CYP2B6 in the presence of sabinene or (+)-3-carene were solved at 2.1 and 2.2 Å resolution, respectively. An unbiased electron density for either ligand was present in close proximity to the heme in the active site (Supplemental Fig. 1, a and b). The two CYP2B6 chains found in the asymmetric unit of each complex were identical to each other with a root-mean-square deviation (RMSD) of 0.1 and 0.3 Å, respectively, for the C α atoms between each chains. A 2.6 Å structure of human CYP2A6 determined in complex with sabinene contained six chains that were quite similar, with an average C α RMSD of 0.1 Å. Moreover, an unbiased electron density in CYP2A6 structure clearly showed sabinene present near the heme iron in chain D (Supplemental Fig. 1c); the chains A, B, C, E, and F displayed varying degrees of disorder with partial density for the monoterpene at the present resolution. The data collection and refinement statistics for these structures are summarized in Table 1. The atomic coordinates and structure factors for CYP2B6 in complex with sabinene and (+)-3-carene (PDB ID [4RQL](#) and [4RRT](#), respectively) and CYP2A6 in complex with sabinene (PDB ID [4RUI](#)) were deposited in the protein data bank (PDB). The overall structures displayed the global P450 fold as shown in Supplemental Figs. 1, d–f.

Comparison of CYP2B6 Structures. The closed conformation structures of CYP2B6 in complex with (+)-3-carene and sabinene are nearly identical and superimposed onto each other with an RMSD of 0.25 Å in a C α overlay. A total of 11 residues were found within a 5 Å radius of either ligand (Fig. 1A). These include I101, I114, F115, F206, I209, F297, A298, T302, L363, V367, and V477 in both complexes in addition to residue F108 in the sabinene complex. The overall structures were virtually identical to the previously solved structures of CYP2B6 in complex with (+)- α -pinene, 4-benzylpyridine (4-BP), or 4-(4-nitrobenzyl)pyridine (4-NBP) and superimposed with an RMSD of ~0.15 Å in a C α overlay onto each of the structures. However, marked differences were observed between new structures and the complexes with 4-(4-chlorophenyl)imidazole (4-CPI) (PDB ID [3IBD](#); RMSD ~0.55 Å), or amlodipine (PDB ID [3UA5](#); RMSD ~1 Å). Considerable shifts in the A-, B', C-, H, I-helices, the F-G cassette, and the C-D, H-I and β_4 -loops illustrate protein motion captured via crystallographic snapshots upon binding various ligands (Fig. 1B). Furthermore, as noted recently with the (+)- α -pinene complex of CYP2B6 (Wilderman et al., 2013), the orientation of the side chains of F206 and F297 was similar to that in the 4-BP or 4-NBP complexes but different from that in the complexes with either 4-CPI or amlodipine. The active site volumes calculated using VOIDOO (Kleywegt and Jones, 1994) for the CYP2B6-(+)-3-carene and CYP2B6-sabinene complexes

TABLE 1
Data collection and refinement statistics

Construct	CYP2B6	CYP2B6	CYP2A6
Ligand	Sabinene	(+)-3-Carene	Sabinene
Crystal space group	P3 ₂	P3 ₂	P1
Crystal unit cell parameters			
a (Å)	78.3	78.0	113.7
b (Å)	78.3	78.0	132.9
c (Å)	202.9	203.0	133.0
α (°)	90	90	62.4
β (°)	90	90	99.0
γ (°)	120	120	80.9
Data collection statistics: values for highest resolution shell are shown in parentheses.			
Beam line	SSRL 7-1	SSRL 7-1	ALS 5.0.1
Wavelength (Å)	0.97	0.97	0.97
Resolution range (Å)	67.8–2.1 (2.15–2.1)	67.7–2.0 (2.26–2.2)	65.77–2.6 (2.67–2.6)
Completeness (%)	95.9 (93.6)	95.9 (89.3)	90.2 (65.7)
Redundancy	3.58 (3.5)	3.30 (3.1)	3.53 (2.5)
R_{merge} (%)	16 (52.6)	6.2 (21.6)	11.3 (30.1)
I/σ	7.2 (2.2)	11.6 (4.5)	4.7 (1.1)
No. of observations	287,998	221,984	390,869
No. of unique reflections	80,742	67,137	110,464
Refinement statistics: values for highest resolution shell are shown in parentheses.			
R -factor	21% (26%)	17% (20%)	19% (36%)
R_{free}	25% (32%)	22% (27%)	26% (40%)
RMS deviations			
Bond lengths (Å)	0.02	0.02	0.01
Bond angles (°)	1.9	1.9	1.6
Average B factor (Å ²)	26.8	34.5	65.2
Ramachandran plot			
Preferred (%)	96.4	96.6	90.6
Allowed (%)	3.2	3.0	8.3
No. of atoms			
Protein ^a	7499 (26.9)	7402 (33.2)	21882 (61.8)
Heme ^a	86 (16.3)	86 (23.1)	258 (51.3)
Solvent ^a	448 (33.1)	459 (39.1)	312 (45.6)
CYMAL-5 ^a	141 (78.3)	116 (73.7)	—
Ligands (Sabinene or (+)-3-Carene) ^a	20 (55.4)	20 (54.2)	60 (76.1)

ALS, Advanced Light Source; SSRL, Stanford Synchrotron Radiation Lightsource.

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

Fig. 1. Superimposed structures of CYP2B6-sabinene and CYP2B6-(+)-3-carene and representation of active-site cavity volume. (A) Overlay of CYP2B6-sabinene (green) and CYP2B6-(+)-3-carene (red) structures in a cartoon representation showing active-site residues in sticks within a 5 Å radius of the ligand. Heme is shown in red sticks. Sabinene is shown in blue and (+)-3-carene in red sticks. (B) Overlay of CYP2B6-sabinene (green), CYP2B6-(+)-3-carene (red), and CYP2B6-amlodipine (yellow) structures in surface representation with secondary structural elements that are displaced shown as ribbon. For clarity purposes, ligands are not shown. (C and D) Overall structures of CYP2B6-sabinene and CYP2B6-(+)-3-carene with active-site cavity volume shown as brown and pink mesh, respectively. Heme, sabinene, and (+)-3-carene are shown in red, blue, and yellow sticks, respectively.

were 350 and 376 Å³ (Fig. 1, C and D), respectively. This value is remarkably less than that found in the 4-CPI (582 Å³) and amlodipine (755 Å³) complexes of CYP2B6 (Gay et al., 2010a; Shah et al., 2012). The cavity volume for CYP2B6-(+)- α -pinene, CYP2B6-4-BP, and CYP2B6-4-NBP active sites were calculated and were comparable to the current structures (Supplemental Fig. 2).

Figure 2A represents the active site overlay of all CYP2B6 structures and demonstrates the movement of phenylalanine side chains. The phenylalanine residues in the active site of the CYP2B6-4-CPI and CYP2B6-amlodipine complexes show the greatest movement, whereas minimal or negligible differences were observed in the CYP2B6 structures in complex with 4-BP, 4-NBP, or monoterpenes. Detailed analysis of the active site in all CYP2B6 structures to date reveals two distinct occupancy spaces for the ligand that depend on the orientation of F206 and F297 side chains (Fig. 2B). Most of the ligands observed in the crystal structures of CYP2B6 occupy space “A” where F206 moves in and F297 moves out. Ligands that occupy space “B” (4-CPI and amlodipine) were found in complexes showing the greatest movement of F206 (out) and F297 (in) within the active site. Comparison with several CYP2B4 structures further demonstrates similar space “B” architecture with 4-CPI (Scott et al., 2004), ticlopidine (Gay et al., 2010b), and paroxetine (Shah et al., 2013).

Comparison of CYP2B6 and CYP2A6 Structures in Complex with Sabinene. The structural overlay of CYP2A6 and CYP2B6 in complex with sabinene resulted in an RMSD of ~0.78 Å (Fig. 3A), which is slightly larger than the RMSD observed between CYP2B4 and CYP2B6 in complex with either 4-CPI (0.65 Å) or amlodipine (0.66 Å) (Halpert, 2011; Shah et al., 2012), and CYP2A6 and CYP2A13 with pilocarpine (0.63 Å) (DeVore et al., 2012). However, the sequence identity between CYP2A6 and CYP2B6 is only 51%, which is significantly less than that between CYP2B4 and CYP2B6 (78%) or CYP2A6 and CYP2A13 (94%), suggesting that the ability to conform to these ligands outweighs sequence differences in the amino acid sequences of CYP2B6 and CYP2A6. The differences between the CYP2B6 and CYP2A6 sabinene complexes were located mainly at the A-, A'-, B'-, F-, F'-, G'-, and G-helices; the β_1 -sheets; and the H-I and β_4 loop regions. In addition, comparison of the active site revealed distinct differences in the orientation of sabinene in each enzyme, with a total of 13 residues falling within 5 Å of the ligand (Fig. 3B). Sabinene rotated 180 degrees in CYP2A6 active site relative to its orientation in CYP2B6, which avoids any clashes with the larger hydrophobic side chains in CYP2A6. V104 and V477 in CYP2B6 correspond to F107 and F480 in CYP2A6, and the side chains of these residues protrude into the active site of CYP2A6, making it more compact. Such substitution of the amino acids in the

Fig. 2. Overlay of active-site phenylalanine residues from all the available CYP2B6 complex structures in the PDB. Phenylalanine residues found within 5 Å of the ligand in CYP2B6-amlodipine (yellow), CYP2B6-4-CPI (gray), CYP2B6-4-BP (orange), CYP2B6-4-NBP (dark green), CYP2B6- α -pinene (pink), CYP2B6-sabinene (green), and CYP2B6-(+)-3-carene (red) structures are shown. Ligands amlodipine (blue), 4-CPI (gray), 4-BP (orange), 4-NBP (dark green), α -pinene (pink), sabinene (blue), and (+)-3-carene (red) are shown in thin lines. Heme is shown in red sticks. (A) Phenylalanine residue side chains (sticks) from CYP2B6-4-CPI and CYP2B6-amlodipine complexes demonstrated the greatest movement in the active site on binding respective ligands. (B) Dual-occupancy spaces, "space A" and "space B" shown in sphere representation, were found that constitute various ligands orienting in either of the two directions in the active site and may depend on the orientation of F206 and F297 side chains. The 4-CPI and amlodipine complexes of CYP2B6 and also ticlopidine complex of CYP2B4 containing a "-Cl" and "-NH" moiety are observed to occupy space B. All phenylalanine residues are shown as thin lines and ligands as sticks.

more rigid CYP2A6 enzyme may have further reduced the room available for sabinene to orient in a similar fashion as in CYP2B6. Moreover, the observed alternate conformation now locates sabinene in the region near I300 in CYP2A6, where there is more space than in CYP2B6, which contains F297. Furthermore, a total of nine amino acid differences

were found from 13 residues in the active site. These include V104, I114, I209, S294, F297, A298, L363, V367, and V477 in CYP2B6, which align to F107, V117, T212, N297, I300, G301, I366, L370, and F477 in CYP2A6. The distinct active site topology of CYP2A6 with larger hydrophobic side-chain substitutions led to a reduced active-site cavity

Fig. 3. Overlay of CYP2B6-sabinene (red) and CYP2A6-sabinene (cyan) structures. Sabinene in CYP2B6 is shown in red stick representation; sabinene from CYP2A6 is shown in cyan sticks. Sabinene in CYP2B6 is shown in purple sticks and that in CYP2A6 is shown in blue sticks. Heme is shown in red sticks. (A) Superimposed structures of CYP2A6 and CYP2B6 in complex with sabinene showing differences in overall protein conformation between two P450 enzymes on binding the same ligand sabinene. (B) Stereoview of CYP2B6 and CYP2A6 active site located within 5 Å radius of sabinene showing differences in the active-site residues and the alternate orientation of sabinene in each enzyme. Residues V104, I114, F115, I209, S294, F297, A298, L363, V367, and V477 labeled in CYP2B6 are substituted with F107, V117, F118, T212, N297, I300, G301, I366, L370, and F480 in CYP2A6. (C) Overlay of the active-site cavity volume of CYP2B6 (red mesh) and CYP2A6 (cyan mesh).

volume of $289 \text{ \AA}^3$ compared with CYP2B6 ($376 \text{ \AA}^3$) (Fig. 3C) as calculated using VOIDOO.

Binding Interactions of Sabinene with CYP2B6 and CYP2A6. Binding of sabinene to CYP2B6 produced a type I spectral shift characterized by a decrease at 417 nm accompanied by an increase at 390 nm. The spectral dissociation constant (K_S) of sabinene binding to CYP2B6 is $0.75 \pm 0.32 \text{ } \mu\text{M}$, with a maximal increase in the fraction of high-spin enzyme of $62.0 \pm 2.0\%$ (Table 2). The binding affinity of this small hydrocarbon for CYP2B6 is similar to that for (+)- α -pinene ($0.38 \text{ } \mu\text{M}$) or (+)-3-carene ($1.60 \text{ } \mu\text{M}$) with a comparable large increase in high-spin enzyme (~ 62 versus ~ 49 or $\sim 42\%$) (Wilderman et al., 2013). The high-affinity binding of sabinene is remarkable for a compound without functional groups that interact specifically with CYP2B6. Interestingly, the predicted dissociation constant from the mean value of experimentally determined octanol/water-partitioning coefficients ($\log P$) of monoterpenes (4.34 ± 0.06) and based solely on enthalpy of desolvation ($-1.364 \times \log P = \sim -5.9 \text{ kcal/mol}$) (Griffin et al., 1999) is $\sim 45 \text{ } \mu\text{M}$. As in the cases of (+)- α -pinene and (+)-3-carene, this calculated value is more than 50 times higher than the experimentally derived spectral binding constant (Wilderman et al., 2013).

To assess whether the different structures of (+)- α -pinene, (+)-3-carene, and sabinene affect the thermodynamics of binding, ITC was performed. The results of a typical titration of CYP2B6 with sabinene are shown in Fig. 4A, with the upper panel illustrating the monotonic decrease in exothermic heat of binding and the lower panel showing the integrated enthalpic changes for each injection fit to a one-site binding model. The thermodynamic constants of ligand binding are given in Table 2. The stoichiometric binding ratio was 1:1; the K_D value was $\sim 0.08 \text{ } \mu\text{M}$. Previous dynamic light scattering experiments revealed no significant effect of monoterpene ligands on CYP2B6 in the conditions used for the ITC experiments (Wilderman et al., 2013). The results of monoterpene binding to CYP2B6 suggest that enthalpy drives formation of these protein-ligand complexes in addition to favorable entropy (Wilderman et al., 2013).

Spectral titrations of CYP2A6 with sabinene produced a type I spectral shift and yielded a K_S of $5.22 \pm 0.40 \text{ } \mu\text{M}$ and a $87.0 \pm 14.9\%$ maximal increase in high-spin ferric enzyme content (Table 2). A representative ITC binding isotherm is shown in Fig. 4B, and the thermodynamic constants are contained in Table 2. The stoichiometric binding ratio was 1:1, and the K_D was $\sim 4.20 \text{ } \mu\text{M}$. Dynamic light scattering also confirmed that the aggregation state of CYP2A6 was unchanged

by the addition of monoterpenes in the conditions used for ITC. As expected, binding of sabinene to CYP2A6 is entropy driven, similar to the results of hydrocarbon binding the case of (+)- α -pinene (Wilderman et al., 2013).

Discussion

Of more than 100 X-ray crystal structures of mammalian cytochrome enzymes available in the PDB, 24 are of CYP2B subfamily enzymes and 19 of CYP2A subfamily enzymes. The structures represent complexes with a wide variety of small molecules, including clinically relevant drugs, substrates, and several organic and natural compounds. The previous structural work has mainly focused on comparison of CYP2B6 with CYP2B4 or on comparison of CYP2A6 with CYP2A13 in the absence or presence of various ligands. Interestingly, the ligands that interact with CYP2A6 and CYP2B6 show significant similarity, with overlapping ranges of molecular masses (CYP2A6 $M_r = 136\text{--}580$; CYP2B6 $M_r = 86\text{--}450$) but with marked differences in potency resulting from hydrogen bond formation with active-site residues (CYP2A6) or molecular size and relative molecular mass (CYP2B6) (Lewis, 2003). This trend is also true for the interaction of monoterpenes with CYP2A6 and CYP2B6 Miyazawa et al., 2003; Miyazawa and Gyoubu, 2006). The current study provides a new dimension to our understanding by using X-ray crystallography and ITC to explore sabinene binding to CYP2A6 and CYP2B6.

The X-ray crystal structures of the sabinene complexes of CYP2A6 and CYP2B6 revealed significant differences in the active site architecture between the enzymes. The orientation of sabinene in CYP2A6 is not consistent with the demonstrated oxidation at the allylic carbon of the six-member ring by plant biosynthetic P450 enzymes (Karp et al., 1987). In contrast, sabinene illustrated the likely productive mode of binding in the active site of CYP2B6, where the putative oxidation site was located closer to the heme iron. In addition to the binding orientation in the enzyme-substrate complex, the high reactivity of allylic carbons will influence the products formed from sabinene (Korzekwa et al., 1990; Afzelius et al., 2007; Ortiz de Montellano, 2010). Key residue differences between CYP2A6 (active site volume = $289 \text{ \AA}^3$) and CYP2B6 ($376 \text{ \AA}^3$) that make the former more compact include F107 versus V104 and F480 versus V477. Moreover, the different orientations of sabinene may reflect the presence of I300 in CYP2A6 as opposed to F297 in CYP2B6.

Detailed analysis of the active site architecture of the seven CYP2B6 structures solved to date enabled us to extract two

TABLE 2

Isothermal titration calorimetry and spectral titration derived thermodynamic parameters of interaction

Enzyme	ΔH	ΔS	ΔG	N	K_D^a	K_S	ΔF_h
	<i>kcal/mol</i>	<i>cal/mol per K</i>	<i>kcal/mol</i>	<i>unit-less</i>	<i>10⁻⁶ M</i>		<i>%</i>
2A6 ^b	-5.8 (0.9) ^d	5.3 (3.2)	-7.3 (0.1)	1.0 (0.1)	4.20 (0.86)	5.22 (0.40)	87.0 (14.9)
2B6 ^b	-8.2 (1.0)	4.6 (1.5)	-9.5 (0.6)	1.1 (0.1)	0.08 (0.02)	0.75 (0.32)	62.0 (2.0)
2B6 ^c	-6.3 (2.2)	3.2 (2.3)	-7.5 (1.4)	1.2 (0.3)	1.82 (0.61)	1.60 (0.14) ^e	42.5 (3.4)

^a K_A^{-1} from isothermal titration calorimetry.

^bSabinene.

^c(+)-3-Carene, previously reported values (Wilderman et al., 2013).

^dValues represent mean of three independent experiments. The confidence interval for $P = 0.05$ is displayed in parentheses.

^eMeasured using the Hill equation giving the S_{50} with $n = 2$.

Fig. 4. Calorimetric titrations (upper panels) and binding isotherms (lower panels) of sabinene binding to (A) CYP2B6 or to (B) CYP2A6 at 25°C.

interesting findings: 1) how the rotation of the F206 side chain has a major effect on the active site cavity volume and 2) how ligands with specific functional groups interact and influence the active site. The F206 side chain demonstrated remarkable movement that led to the increased volume in the 4-CPI and amlodipine complexes. In the other five complexes, the F206 side chain seems to “shut off” the active-site region between the E- and F-helices, previously predicted to be the region for metabolite egress or to be a solvent channel (Fig. 5) (Wade et al., 2004). Another interesting observation from the seven structures is how the presence of a “-Cl” or “-NH” functional

group influences the orientation of F206 and F297 and, consequently, the overall volume of the active site. Recent analysis of Cl- π and NH- π interactions in protein ligand complexes found in the PDB revealed the presence of two different geometries when these groups interact with aromatic rings: edge-on, where the -Cl or -NH group approaches the aromatic atoms or bonds on the periphery of the ring, or face-on, where the group interacts with the electron density at the center of the aromatic ring (Scrutton and Raine, 1996; Imai et al., 2008; Dalkas et al., 2014). The aromatic ring of the phenylalanine side chain preferred edge-on geometry for -Cl

Fig. 5. Analysis of F206 side-chain orientation in CYP2B6 structures. (A) The movement of the F206 side chain (stick representation) in the 4-CPI (orange) and amlodipine (pink) complexes of CYP2B6 is compared with the (+)- α -pinene (green) complex. The CYP2B6 structures with the F206 side chain protruding into the previously proposed solvent channel (brown mesh) demonstrated reduced active site cavity volume compared with the 4-CPI and amlodipine complexes of CYP2B6. The ligands 4-CPI and (+)- α -pinene are shown in red and blue, respectively, in a stick representation. For clarity, amlodipine is not shown, and heme is shown in red sticks. (B) Orthogonal view of the same figure.

atoms, which were found predominantly in crystallographic structures at a distance range of 3.7–4.1 Å (Imai et al., 2008). This is in agreement with CYP2B6 structures, where the -Cl atom faces the edge of the aromatic ring of F297 at a distance of 3.7 to 3.8 Å from the closest bond as shown in Fig. 6. The NH- π interaction found in the CYP2B6-amlodipine complex (Fig. 6) may also influence binding and stabilization of the overall complex. Moreover, a -Cl functional group bound to an aromatic ring, such as the chlorophenyl moiety, has a greater propensity for a stable Cl- π interaction than a Cl bound to nonaromatic moieties in a ligand (Matter et al., 2009). Therefore, the Cl- π or NH- π interactions are important contributors to protein-ligand binding affinity and, combined with the observed movement of F206 and F297 side chains, may enhance ligand-binding properties, leading to more potent inhibitors or efficient substrates. In that context, it is noteworthy that some of the best known substrates of CYP2B6 or those that are metabolized more efficiently by CYP2B6 than by other P450 enzymes, such as efavirenz, bupropion, sertraline, and ticlopidine, contain -Cl and -NH functional groups.

Extensive studies of CYP2A6 and CYP2A13, which show 94% amino-acid sequence identity (94%) and high structural similarity ($C\alpha$ RMSD 0.25–0.65 Å), have revealed significant differences in the metabolism of multiple substrates. For example, with nicotine, CYP2A6 demonstrated 5- to 23-fold lower catalytic efficiency and 8.7-fold lower binding affinity than CYP2A13 (Bao et al., 2005; Murphy et al., 2005). Crystal structures revealed a key hydrogen bond between the side chain of N297 and nicotine in CYP2A13 but not CYP2A6 (DeVore and Scott, 2012). The polar side chain of N297 was also involved in hydrogen bonding with pilocarpine in the structures of CYP2A6 and CYP2A13 (DeVore et al., 2012). The identity of the nonconserved residue at position 300 is also crucial, as mutating I300 of CYP2A6 to phenylalanine as in CYP2A13, significantly increased binding affinity with nicotine (DeVore and Scott, 2012). In the current CYP2A6-sabinene complex, the pure hydrocarbon lacks a hydrogen bonding interaction with N297, and the ethyl group of

sabinene is positioned near N297 at a distance of ~ 3.6 Å and close to I300.

Recent thermodynamic analysis of (+)- α -pinene binding to CYP2B6 and CYP2A6 indicated that binding was driven by enthalpy in the more plastic CYP2B6 and entropy in the less plastic CYP2A6 (Wilderman et al., 2013). Similar to (+)- α -pinene, sabinene displayed a much higher affinity for CYP2B6 than for CYP2A6, both from ITC and spectral titrations. Both ΔH and $T\Delta S$ for (+)- α -pinene binding to CYP2B6 are roughly -9 kcal/mol relative to the same constants for CYP2A6. These values are much closer for sabinene binding to CYP2B6 and CYP2A6, roughly -2.5 kcal/mol and 0.2 kcal/mol, indicating that the 50-fold greater binding affinity of sabinene for CYP2B6 is almost entirely due to enthalpy. As proposed to account for differences in (+)- α -pinene interactions with CYP2A6 and CYP2B6 (Wilderman et al., 2013), differences in the thermodynamics of sabinene binding may be due to effects of conformational changes, not to the binding interaction per se. These conformational changes could involve the overall protein or local residues within the active site and/or multiple conformations of sabinene in the less compressed CYP2B6 active site.

Comparison of sabinene and (+)-3-carene binding to CYP2B6 and CYP2A6 provided insight into the contribution of ligand structure to binding affinity. Previous analysis of CYP2B4 by ITC, enzyme inhibition, and spectral binding titrations found that the physical structure of imidazole ligands produced marked changes in the binding parameters (Muralidhara et al., 2006). Each of the imidazole ligands in that study was roughly planar, but differences in where the functional group was bound to the imidazole ring and the identity of the functional group produced marked changes in the binding affinity and the enthalpy/entropy profile for interaction with CYP2B4. Monoterpene interactions with CYP2B6 and CYP2A6 showed distinct enthalpy/entropy profiles as well. For CYP2B6, the ΔH values for (+)- α -pinene, sabinene, and (+)-3-carene binding were -13.1 , -8.2 , and -6.3 kcal/mol, and $T\Delta S$ values were -3.6 , 1.4 , and 1.0 kcal/mol, respectively. These constants yielded similar ΔG values for (+)- α -pinene and sabinene but a significantly different value for (+)-3-carene, $-9.4 \approx -9.5$ versus -7.5 kcal/mol. For CYP2A6, the ΔH values for (+)- α -pinene and sabinene binding were -3.8 and -5.8 kcal/mol, and $T\Delta S$ values were 5.4 and 1.6 kcal/mol respectively. This led to significantly different ΔG values for (+)- α -pinene and sabinene binding to CYP2A6, -9.3 versus -7.3 kcal/mol. The compact (+)- α -pinene showed little preference for CYP2B6 or CYP2A6. With the more planar sabinene and (+)-3-carene, either a preference for one enzyme or significant change in affinity with a change in the chemical structure of the ligand occurred. Interestingly, for sabinene binding to CYP2B6 (Table 2) and (+)- α -pinene binding to CYP2A6 (Wilderman et al., 2013), the K_D resulting from ITC was 10-fold lower than the K_S from spectral titrations. In contrast, no such discrepancy was observed for 3-carene binding to either enzyme or (+)- α -pinene binding to CYP2B6. The K_D and K_S are both dissociation constants. The K_D from ITC is the reciprocal value of the K_A produced from data analysis of the thermodynamic changes as a result of protein-ligand interactions. The K_S is a dissociation constant that is

Fig. 6. The -Cl- π interactions in the CYP2B6-4-CPI (orange) and -NH- π interactions in the CYP2B6-amlodipine (yellow) structures are shown in a structural overlay. The -Cl of the chlorophenyl moiety in 4-CPI that interacts with the aromatic ring of F297 (3.7 Å), and the -NH of pyridine moiety in amlodipine that interacts with the aromatic ring of F206 (4.1 Å) are shown. The F206, F297, 4-CPI, amlodipine, and heme molecules are shown in stick representation.

extracted from changes in the UV-visible absorbance spectrum of the protein with increasing ligand concentration. Ligand-induced displacement of water coordinated to a hexacoordinate low-spin heme iron in the enzyme active site results in a pentacoordinate high-spin heme, but ligand binding does not necessarily produce spin-state changes in mammalian cytochrome enzymes. The concentration of ligand needed to create a spin-state transition in the absorbance spectra of P450 enzymes is not necessarily linked to the concentration of ligand needed to occupy protein-binding sites fully. This intriguing difference between the enzymes and compounds may reflect structural rearrangements required for the low-to-high spin transition in the absorbance spectra of cytochrome enzymes (Haines et al., 2001).

In conclusion, our structural analysis revealed different binding orientations of sabinene in CYP2A6 versus CYP2B6 and similar orientations of sabinene and (+)-3-carene in CYP2B6. ITC revealed a greater contribution of favorable enthalpy to monoterpene binding to CYP2B6 than CYP2A6. Furthermore, -Cl- and -NH- π interactions between CYP2B6 ligands and the enzyme are proposed to influence binding orientation and/or affinity. Such interactions may help resolve the puzzling question of how CYP2B6 can bind certain substrates with high specificity despite the lack of any functional groups in the enzyme capable of forming hydrogen or ionic bonds.

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Authorship Contributions

Participated in research design: Shah, Wilderman, Halpert.

Conducted experiments: Shah, Wilderman, Liu, Jang.

Contributed new reagents or analytic tools: Zhang.

Performed data analysis: Shah, Wilderman.

Wrote or contribute to the writing of the manuscript: Shah, Wilderman, Stout, Halpert.

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