Preferred Binding Orientations of Phenacetin in CYP1A1 and CYP1A2 Are Associated with Isoform-Selective Metabolism

Qingbiao Huang, Rahul S. Deshmukh, Spencer S. Ericksen, Youbin Tu, and Grazyna D. Szklarz

Department of Basic Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, West Virginia

Received June 13, 2012; accepted September 4, 2012

ABSTRACT:
Human cytochromes P450 1A1 and 1A2 play important roles in drug metabolism and chemical carcinogenesis. Although these two enzymes share high sequence identity, they display different substrate specificities and inhibitor susceptibilities. In the present studies, we investigated the structural basis for these differences with phenacetin as a probe using a number of complementary approaches, such as enzyme kinetics, stoichiometric assays, NMR, and molecular modeling. Kinetic and stoichiometric analyses revealed that substrate specificity ($k_{cat}/K_m$) of CYP1A2 was approximately 18-fold greater than that of CYP1A1, as expected. Moreover, despite higher $H_2O_2$ production, the coupling efficiency of reducing equivalents to acetaminophen formation in CYP1A2 was tighter than that in CYP1A1. CYP1A1, in contrast to CYP1A2, displayed much higher uncoupling, producing more water. The subsequent NMR longitudinal ($T_2$) relaxation studies with the substrate phenacetin and its product acetaminophen showed that both compounds displayed similar binding orientations within the active site of CYP1A1 and CYP1A2. However, the distance between the $OCH_2$ protons of the ethoxy group (site of phenacetin O-deethylation) and the heme iron was 1.5 Å shorter in CYP1A2 than in CYP1A1. The NMR findings are thus consistent with our kinetic and stoichiometric results, providing a likely molecular basis for more efficient metabolism of phenacetin by CYP1A2.

Introduction
Cytochromes P450 (P450s) comprise a superfamily of heme-containing enzymes that have a ubiquitous presence across the biological kingdom. These enzymes are associated with the oxidative metabolism of a large number of structurally diverse chemicals, including drugs, carcinogens, environmental chemicals, and endogenous compounds such as steroids and fatty acids. Each P450 isoform usually has the ability to metabolize a variety of substrates, and different P450s often display overlapping substrate specificities and inhibitor susceptibilities.

The human P450 1A subfamily consists of two members, CYP1A1 and CYP1A2, with CYP1A2 being one of the major P450s in the human liver (~13–15%) (Shimada et al., 1994; Guengerich, 2005). Apart from their role in the metabolism of drugs such as phenacetin, caffeine, and warfarin, these two enzymes are also involved in the metabolic activation of procarcinogenic chemicals and toxins (Androustopoulos et al., 2009; Zhou et al., 2009). CYP1A1 and CYP1A2 share 72% amino acid sequence identity but exhibit different substrate specificities and inhibitor susceptibilities (Kawajiri and Fujii-Kuriyama, 1991; Guengerich, 2005). For example, CYP1A1 metabolizes benzo[a]pyrene and polycyclic aromatic hydrocarbons to their toxic derivatives (Kawajiri and Fujii-Kuriyama, 1991; Shou et al., 1996), whereas CYP1A2 preferentially oxidizes heterocyclic aromatic amines (Hammons et al., 1997; Turesky et al., 1998). The two enzymes are also different in their tissue distribution and expression. Constitutively expressed CYP1A2 is particularly abundant in hepatic tissue, whereas CYP1A1 is distributed throughout many tissues, including the lungs, and is highly inducible. However, both enzymes are regulated, in part, by the aryl hydrocarbon receptor system, which is known to be induced by a variety of chemicals (Kawajiri and Fujii-Kuriyama, 1991; Shimada et al., 2002).

Despite many differences, CYP1A enzymes share functional similarities as well. Therefore, many of the substrate and inhibitor probes do not clearly differentiate between the 1A1 and 1A2 isoforms (Tassaneeyakul et al., 1993). For example, caffeine has been shown to be a nonspecific in vitro probe for CYP1A2, because it is also metabolized by CYP1A1 (Tassaneeyakul et al., 1992). Likewise, the resorufin substrates, 7-ethoxyresorufin and 7-methoxyresorufin, undergo O-dealkylation by CYP1A1 and CYP1A2, although the efficiencies of the reactions are different (Nerurkar et al., 1993). In the case of phenacetin and acetaminophen, both compounds have been well documented as substrates for CYP1A1 and CYP1A2 and are known to be metabolized by these enzymes with different efficiencies. Phenacetin is primarily metabolized to acetaminophen via O-deethylation, although the formation of a minor metabolite acetol (~5%) via acetyl hydroxylation has also been reported (Yun et al., 2000). Acetaminophen can be further metabolized by CYP1A1 and CYP1A2 to a potent...
cytotoxin, N-acetyl-p-benzoquinone imine (NAPQI), along with 3-hydroxy acetaminophen.

Of interest, in each of the cases above, the substrates are nonspecific, yet demonstrably selective with respect to the two isoforms in terms of reaction rates, efficiency, and $K_{m}$. It is likely that the subtle differences in oxidative catalysis arise from isoform-specific substrate binding position and mobility in the active site.

Despite the fact that the crystal structure of CYP1A2 and the homology model of CYP1A1 have been available, there has been no direct physicochemical evidence to assess the binding position(s) of phenacetin within the active sites of CYP1A1 and CYP1A2. One experimental method used to indirectly determine substrate position with respect to the heme iron is NMR $T_1$ relaxation measurement, which was first used to study the orientation of a substrate (xylidine) in a P450 active site by Novak et al. (1977). More recently, this method has been successfully applied toward several ligand-P450 complexes (Myers et al., 1994; Regal and Nelson, 2000; Himmel et al., 2004, 2005; Cameron et al., 2005; Mosher et al., 2008; Gay et al., 2010). On the basis of these findings, $T_1$ relaxation experiments are also suitable to study phenacetin/acetaminophen-CYP1A complexes because these compounds have reasonable numbers of protons with few overlapping NMR signals, characteristics necessary to resolve proton distances from the heme iron.

Therefore, in the current study, we chose phenacetin and acetaminophen to evaluate and compare the substrate binding characteristics in CYP1A1 and CYP1A2. The distance constraints based on NMR $T_1$ relaxation experiments were used to dock the substrates in the active sites of the enzymes. The relationship between substrate binding orientation and CYP1A1 and CYP1A2 substrate selectivity has been further investigated using molecular modeling, enzyme kinetics, and stoichiometry studies. The results help to better rationalize the molecular basis for differences in substrate specificity between these two closely related P450 isoforms.

Materials and Methods

Materials. Phenacetin, acetaminophen, 2-hydroxy acetanilide, aniline, sodium dithionite, dilauroyl-1,3-phosphatidylcholine (DLPC), and PVP were purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate and EDTA were obtained from Thermo Fisher Scientific (Waltham, MA). Emulgen and Chaps were purchased from the Chemical Division of the KAO Corporation (Tokyo, Japan) and EMD Biosciences (La Jolla, CA), respectively. Deuterium oxide ($D_2O$, 99.9%) was from Cambridge Isotope Laboratories (Andover, MA). Centricor 10-kDa molecular mass cutoff filters were obtained from Millipore Corporation (Billerica, MA). Carbon monoxide was purchased from Mountain State Airgas (Morgantown, WV).

Expression and Purification of CYP1A1 and CYP1A2. The enzymes were expressed and purified as described previously (Liu et al., 2003, 2004; Tu et al., 2008; Huang and Szklarz, 2010). In brief, His-tag-containing CYP1A1 and CYP1A2 were expressed in Escherichia coli DH5α cells, and the enzymes were purified by affinity chromatography with nickel-nitrilotriacetic acid agarose. During purification of CYP1A2, 5 mM caffeine was added to all of the buffers to stabilize the enzyme. Caffeine was removed completely from the enzyme preparation during the subsequent ultracentrifugation stage (Huang and Szklarz, 2010). Rat P450 reductase was expressed and purified as described earlier (Liu et al., 2003, 2004). The purified enzyme fractions were stored at −80°C until further use.

Enzyme Kinetics Assay. Phenacetin O-dealkylase activities of CYP1A1 and CYP1A2 in vitro were determined as described previously (Huang and Szklarz, 2010) by quantification of product acetaminophen using high-performance liquid chromatography. The incubation mixture contained 0.5 $\mu$M CYP1A1 enzyme, 1 $\mu$M P450 reductase, and 45 $\mu$M DLPC in 100 mM potassium phosphate buffer (pH 7.5). After a 5-min preincubation, reactions were initiated by the addition of NADPH (1 mM final concentration). Reactions were conducted for 30 min, quenched by addition of 5 $\mu$M of 60% HClO₄, and cooled on ice for 10 min. Then 10 $\mu$l of 100 $\mu$M 2-hydroxy acetanilide was added as internal standard, and the mixture was spun in a centrifuge for 5 min. Acetaminophen in the supernatant was quantified by high-performance liquid chromatography using a C₁₈ column (Alltech Associates, Deerfield, IL). The mobile phase consisted of methanol-0.1% acetic acid (20:80, v/v; flow rate, 1 ml/min), and the eluent was monitored at 254 nm. The product was quantified by comparison with acetaminophen standards. Kinetic parameters ($K_{m}$ and $V_{max}$) were calculated using nonlinear regression with GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Stoichiometry Studies on NADPH oxidation. NADPH oxidation, $H_2O_2$ production, and oxygen consumption were the same as those described previously (Huang and Szklarz, 2010). The reaction mixtures were the same as those used for the enzyme kinetics assay described above. The reaction was initiated by addition of NADPH and conducted at 37°C. NADPH oxidation was determined spectrophotometrically at 340 nm, $H_2O_2$ production was assayed using the xylenol orange iron(III) assay, and oxygen consumption was measured using a Mitocell (Strathkelvin Instruments, Ltd., Glasgow, UK). The ratios of product/NADPH, $H_2O_2/O_2$, and $H_2O/PRODUCT$s were calculated to evaluate the coupling efficiency and uncoupling rates at three branch points of CYP1A1 and CYP1A2 catalytic cycles (Huang and Szklarz, 2010).

Spin State Determination. The percentages of high and low spin in CYP1A1 and CYP1A2 enzymes with phenacetin/acetaminophen were determined as described previously (Locuson et al., 2006). Spectral titrations with phenacetin or acetaminophen in CYP1A1 and CYP1A2 enzymes were performed by spectral scanning between 320 and 500 nm, with the concentrations of phenacetin or acetaminophen identical to those in NMR studies. Absorbance spectra were deconvoluted into three components: a low-spin component, a high-spin component, and the broad $\delta$-band using multiple Gaussian curve fitting with the OriginPro v8 package (OriginLab Corporation, Northampton, MA). To estimate percentages of low-spin components, the three components ($\delta$-bands, low spin, and high spin) were used to fit data for phenacetin or acetaminophen with CYP1A1 and CYP1A2 enzymes.

Enzyme Preparation and Incubation for NMR. Deuterated potassium phosphate buffer (1 M, pH 7.5) was prepared by multiple rounds of resuspension in $D_2O$ and lyophilization. A solution of 1% (w/v) polyvinyl pyrrolidone in 100 mM deuterated phosphate buffer was prepared and applied to the surface of the 10-kDa molecular mass cutoff Centricon filter. The enzyme preparation, initially containing 100 mM phosphate buffer and 20% glycerol, was exchanged and concentrated against 100 mM deuterated phosphate buffer over the Centricon filter by centrifugation (1000g, 4°C) three to four times. The final enzyme preparation contained less than 1% glycerol and approximately 55 $\mu$M CYP1A1 or 20 $\mu$M CYP1A2.

For studies involving cytochrome P450 reductase, the enzyme-reductase complex in the ratio of 1:2 was incubated at room temperature in the presence of 45 $\mu$M DLPC. Before the NMR experiments, all solutions were treated with Chelex 100 to remove any metal ion contaminants. Because the contributions of dioxygen were supposed to remain constant during the experiments, the samples were not degassed.

NMR Spectroscopy. NMR $T_1$ relaxation studies were performed on a Varian Inova NMR spectrometer operating at 600 MHz, internally locked on the deuteron signal of the solvent, $D_2O$, as described previously (Himmel et al., 2004, 2005). Signals were internally referenced to the HDO peak at 4.8 ppm. A standard inversion recovery sequence ($d_1$-180°-$d_2$-90°) was used, along with presaturation of the residual HDO signal. The 90° pulse width was calibrated on each sample. The preacquisition delay ($d_2$) was set to 10 × $T_1$ (40 s) of the longest relaxation time. The spectra associated with at least 10 $d_1$ values were acquired. Line broadening and gaussian fitting were applied for precise calculations of $T_1$ values for the protons. The value of the longitudinal relaxation time was obtained by a nonlinear least-squares fitting of the peak height as a function of the delay $d_2$, using Varian software. $T_1$ values were measured on the substrate solutions without addition of enzyme and after the addition of enzyme to these solutions. The relaxation times $T_1$ ($\text{Fe}^{2+}$-CO) of the ligand protons were measured after in situ conversion of the enzyme to its diamagnetic ferrous carbonyl complex ($\text{Fe}^{2+}$-CO) by bubbling carbon monoxide for 15 min, followed by an addition of a small amount of sodium dithionite (−1 mg). The sample was then allowed to equilibrate 30 min before $T_1$ ($\text{Fe}^{2+}$-CO) measurement. The final $T_1$ ($\text{Fe}^{2+}$-CO) measurement of the ligands in presence of the $\text{Fe}^{2+}$-CO complex and the $T_1$ of the enzyme-free
ligand and were almost identical, which suggests very little paramagnetic contribution due to impurities. The integrity of the enzyme was assayed by measuring the UV-visible spectra of samples maintained under similar conditions. No significant cytochrome P420 formation was observed over the course of the experiment.

To validate the fast-exchange conditions, the temperature dependence of $T_1_p$ was studied. Experiments involving enzyme titration studies with CYP1A1 and CYP1A2 were performed with phenacetin or acetaminophen as a substrate. Concentrated enzyme was added to the substrate in small increments, and the $T_1$ measurements were performed after each addition.

Proton-Heme Iron Distance Calculations. A more precise method for the distance ($r$) calculation using spin-state data was adopted as described in detail earlier (Hummel et al., 2008). In brief, eq. 1 to calculate the distance can be written as

$$r = [9.78 \times 10^{-16} T_{1p} (\alpha_m S = I \tau_m)]^{1/6} \tag{1}$$

The distance estimated by NMR is represented by $r$. The tumbling coefficient $\tau_m$ represents the correlation time of the dipolar interactions of the protein in solution and can be calculated by measuring $T_{1p}$ at several magnetic field strengths (Mildvan and Gupta, 1978). An estimate for $\tau_m$ of P450 1A2 is 3.38 x 10^{-10} s^{-1}, as reported previously (Regal and Nelson, 2000). $T_{1p}$ is the portion of $T_1$ due to paramagnetic effects alone and is given by eq. 2:

$$1/T_{1p} = 1/T_{1p}^{Fe^2+} - 1/T_{1p}^{Fe^3+} \tag{2}$$

assuming that all of the diamagnetic contribution is represented by $1/T_{1p}^{Fe^2+}$ (Regal and Nelson, 2000). The parameter $\alpha_m$, the fractional binding coefficient, is obtained from the equation $\alpha_m = [P450]/(K_s + [Substrate])$ under conditions of fast exchange when only one substrate species is present in solution (Regal and Nelson, 2000). $K_s$ values determined from visible spectroscopy were used for the distance calculations rather than $K_D$ determined by NMR for phenacetin/acetaminophen because, as reported by Regal and Nelson (2000), $K_s$ and $K_D$ values were either equal or differed only slightly on the basis of Michaelis-Menten kinetics. The $K_s$ values for phenacetin binding to CYP1A1 and CYP1A2 were 57 and 17 $\mu$M, respectively (Huang and Szklarz, 2010). In the case of acetaminophen, $K_s$ values reported previously in the literature for CYP1A1 and CYP1A2 were 870 and 108 $\mu$M, respectively (Myers et al., 1994) and were used for the purpose of distance calculation for acetaminophen. The $S(S+1)$ term was simplified by eq. 3:

$$S(S+1) = 8.75f_{hs} + 0.75f_{ls} \tag{3}$$

where $f_{hs}$ and $f_{ls}$ refer to the fractions of the high-spin and low-spin iron, respectively (Hummel et al., 2008).

Molecular Modeling: General. All molecular modeling was performed on a Silicon Graphics Octane workstation using Insight II software (Accelrys, San Diego, CA) for molecular dynamics simulations, ligand docking, ligand construction, and minimizations, and Modeler 8v2 was used for homology modeling. Phenacetin and acetaminophen were constructed and minimized using the Insight II/Builder module. Energy minimization and MD simulations were performed using the Insight II/Discover module with the consistent valence force field. The parameters for heme containing Fe^3+ were as described previously (Paulsen and Ornstein, 1991, 1992).

Construction of the Homology Model of CYP1A1. A homology model of CYP1A1 was made with Modeler 8v2 (Sali and Blundell, 1993) using the CYP1A2 crystal structure as the template. CYP1A2 (Protein Data Bank 2H4) coordinates were obtained courtesy of Dr. Eric F. Johnson (The Scripps Research Institute, La Jolla, CA) (Sansen et al., 2007). The CYP1A1 model consisted of core residues 32 to 512, and the N-terminal transmembrane region was disregarded. Modeler’s conjugate gradients optimization method was applied to build five models based on the template-based spatial restraints. The resulting model with the best objective function value was selected for further study. Heme cofactor coordinates for this model were obtained directly from CYP1A2 after structural alignment to the apo-CYP1A1 homology model.

MD Docking with Distance Restraints. Phenacetin and acetaminophen were initially placed into the active sites of both CYP1A1 and CYP1A2 manually to avoid steric overlaps. Automated docking of ligands was then performed with the Insight II/Affinity module using default parameters, as described previously (Liu et al., 2004; Ericksen and Szklarz, 2005; Huang and Szklarz, 2010), except that the ferric form of the heme was used. After 20 positions (or poses) were obtained, the most energetically favorable complex was subjected to MD simulations and minimization with NMR-based distance restraints imposed. To reduce possible protein deformation resulting from restraint forces, the protein backbone was tethered to its initial coordinates by a harmonic restraint force. Substrate protons were guided to NMR-derived distances from the heme iron by a gradually strengthening harmonic restraint ($k = 2-32$ kcal mol^{-1} Å^{-2}) over 5 ps of MD. The nonbond cutoff was set at 15 Å, and a screened Coulomb potential with a distance-dependent dielectric was used to simulate an aqueous solvent environment. After MD simulations, the final frame of the trajectory was minimized to convergence while restraints were maintained.

Results

Purification of CYP1A1 and CYP1A2. The overall yield of purified enzyme was approximately 20 to 40%, similar to that reported previously (Liu et al., 2004; Tu et al., 2008; Huang and Szklarz, 2010). The purity of CYP1A1 and CYP1A2 verified by SDS-polyacrylamide gel electrophoresis and Western blots indicated that the proteins were at least 95% pure. The UV-visible spectrum of the purified enzymes was that of a typical P450, mainly purified in its low-spin form, whereas the spectrum of the Fe^{2+}-CO complex exhibited the characteristic peak at 450 nm, with little or no P420 formation. The holoenzyme content of the enzymes was consistently in the range of 40 to 60%, as observed previously in our laboratory.

Kinetic Parameters of Purified CYP1A1 and CYP1A2 Enzymes. Both CYP1A1 and CYP1A2 catalyze the O-deethylation of phenacetin to the product acetaminophen (Fig. 1), but with different efficiencies. The Michaelis-Menten plots for CYP1A2 enzymes are shown in Fig. 2. Kinetic parameters, $k_{cat}$ and $K_m$, and catalytic efficiencies, $k_{cat}/K_m$, for phenacetin O-deethylation catalyzed by CYP1A1 and CYP1A2, are shown in Table 1. The $k_{cat}$ for phenacetin oxidation by CYP1A2 was ~2.5-fold higher than that for CYP1A1, whereas the $K_m$ was ~8-fold lower in CYP1A2 than in CYP1A1, probably reflecting better substrate affinity of CYP1A2. Overall, the catalytic efficiency for phenacetin metabolism by CYP1A2 was approximately 18-fold higher than that measured in CYP1A1.

Coupling of CYP1A Reactions. The stoichiometry of CYP1 reactions was studied in the presence of phenacetin to compare the coupling efficiencies of the enzymes. The rates of NADPH oxidation, $O_2$ consumption, product (acetaminophen) formation, and $H_2O_2$ and excess water production during phenacetin oxidation by CYP1A1 and CYP1A2 are shown in Table 2. Oxygen consumption was similar for both enzymes, but the consumption rate of NADPH was higher in CYP1A2 than in CYP1A1. Likewise, the formation of product acetaminophen, as well as by-product $H_2O_2$, was greater in CYP1A2. Of note, CYP1A2 produced ~50% less water than CYP1A1. Table 3 presents coupling efficiencies of CYP1A1 and CYP1A2, both overall and at specific P450 uncoupling branching points. The coupling efficiency of the catalytic cycle to product formation was ~2-fold higher in CYP1A2 than in CYP1A1 on the basis of the ratio of

![Phenacetin and Acetaminophen](Image)
The percentages of low and high spin calculated from the Soret bands of CYP1A1 and CYP1A2 in the absence and presence of substrates are shown in Table 4 and Fig. 3. In the absence of substrate, CYP1A1 and CYP1A2 existed primarily in the low-spin state (95 and 88% low spin, respectively). The addition of phenacetin or acetaminophen caused only a slight increase (2–3%) in the proportion of the high-spin state of CYP1A1. In contrast, the occupancy of the high-spin state in CYP1A2 increased by ~9 and 17% upon binding of acetaminophen and phenacetin, respectively. Thus, phenacetin induced a stronger shift toward the high spin than acetaminophen in the case of CYP1A2, whereas both phenacetin and acetaminophen had similar small effects with respect to the induction of the high-spin state in CYP1A1.

NMR T1 Experiments. The spectral binding constant (Kd) of phenacetin for CYP1A1 and CYP1A2 was calculated to be 57.4 and 17.9 μM, respectively, as reported previously (Huang and Szklarz, 2010). The Kd values of 870 and 108 μM for acetaminophen reported previously in the literature for CYP1A1 and CYP1A2, respectively, were used for the purpose of distance calculation for acetaminophen (Myers et al., 1994).

The validity of distances derived from T1 relaxation times of substrate protons is based on the assumption of substrate binding under fast-exchange conditions, meaning that substrate molecules in the active site must be rapidly exchanging with those in the bulk solution on the NMR time scale. To verify fast-exchange conditions, the temperature dependence of the T1 relaxation times of the substrate protons was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).

The mean distances between the substrate protons and the heme iron for phenacetin and acetaminophen with CYP1A1 and CYP1A2 obtained from T1 relaxation experiments are shown in Tables 5 and 6. In general, there was a substantial difference in T1 relaxation times between the samples with and without carbon monoxide. T1 values for the CO-reduced samples were larger for all protons of both phenacetin and acetaminophen. The distance of the OCH2 proton(s) of phenacetin was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).

The mean distances between the substrate protons and the heme iron for phenacetin and acetaminophen with CYP1A1 and CYP1A2 obtained from T1 relaxation experiments are shown in Tables 5 and 6. In general, there was a substantial difference in T1 relaxation times between the samples with and without carbon monoxide. T1 values for the CO-reduced samples were larger for all protons of both phenacetin and acetaminophen. The distance of the OCH2 proton(s) of phenacetin was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).

The mean distances between the substrate protons and the heme iron for phenacetin and acetaminophen with CYP1A1 and CYP1A2 obtained from T1 relaxation experiments are shown in Tables 5 and 6. In general, there was a substantial difference in T1 relaxation times between the samples with and without carbon monoxide. T1 values for the CO-reduced samples were larger for all protons of both phenacetin and acetaminophen. The distance of the OCH2 proton(s) of phenacetin was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).

The mean distances between the substrate protons and the heme iron for phenacetin and acetaminophen with CYP1A1 and CYP1A2 obtained from T1 relaxation experiments are shown in Tables 5 and 6. In general, there was a substantial difference in T1 relaxation times between the samples with and without carbon monoxide. T1 values for the CO-reduced samples were larger for all protons of both phenacetin and acetaminophen. The distance of the OCH2 proton(s) of phenacetin was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).

The mean distances between the substrate protons and the heme iron for phenacetin and acetaminophen with CYP1A1 and CYP1A2 obtained from T1 relaxation experiments are shown in Tables 5 and 6. In general, there was a substantial difference in T1 relaxation times between the samples with and without carbon monoxide. T1 values for the CO-reduced samples were larger for all protons of both phenacetin and acetaminophen. The distance of the OCH2 proton(s) of phenacetin was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).

The mean distances between the substrate protons and the heme iron for phenacetin and acetaminophen with CYP1A1 and CYP1A2 obtained from T1 relaxation experiments are shown in Tables 5 and 6. In general, there was a substantial difference in T1 relaxation times between the samples with and without carbon monoxide. T1 values for the CO-reduced samples were larger for all protons of both phenacetin and acetaminophen. The distance of the OCH2 proton(s) of phenacetin was measured. A positive slope in the double reciprocal plot of T1P versus temperature indicates that the fast-exchange condition is being met. The double reciprocal plot of T1P versus temperature for CYP1A1 with phenacetin shown in Fig. 4 demonstrates that the fast-exchange requirement has also been satisfied. Similar results were obtained for CYP1A1 in the presence of acetaminophen and CYP1A2 in the presence of either phenacetin or acetaminophen (data not shown).
substrate proton distances obtained from modeling simulations are included in Tables 5 and 6 and are, in general, somewhat longer than those from NMR. Phenacetin and acetaminophen display similar binding orientations in both CYP1A1 and CYP1A2. However, the protons of the OCH₂ group in phenacetin, the site of metabolism, are closer to the heme iron in CYP1A2 than in CYP1A1.

Discussion

The objective of the present study was to explore the structural basis for the differences in substrate specificity between CYP1A1 and CYP1A2 using phenacetin as a probe. These two enzymes are closely related to each other, with 72% amino acid sequence identity. The differences in activities probably arise from structural differences between CYP1A1 and CYP1A2. Our previous studies indicated that five key residues in the active site that differ between the enzymes play a role in the determination of substrate specificity with alkoxy-resorufins (Liu et al., 2003, 2004; Tu et al., 2008) and phenacetin (Huang and Szklarz, 2010). In CYP1A1, the unique active site residues are Ser122, Asn221, Gly225, Leu312, and Val382, which correspond to Thr124, Thr223, Val227, Asn312, and Leu382 of CYP1A2, respectively. These residues line the active-site cavity of the enzymes and may directly interact with substrates and thus influence binding orientations.

The relationship between enzyme specificity and binding orientation of the substrate phenacetin within the active site of CYP1A1 and CYP1A2 was investigated using a number of complementary approaches, such as enzyme kinetics, stoichiometry, NMR, and molecular modeling. Kinetic studies have shown that CYP1A2 displayed a higher turnover rate and substrate specificity ($k_{cat}/K_m$) with phenacetin than CYP1A1 (Table 1), as expected. Of interest, the $K_m$ value that we obtained here is 10-fold higher than the value reported previously, whereas $k_{cat}$ and $K_s$ values are similar (Yun et al., 2000), in contrast to our recent studies (Huang and Szklarz, 2010), which corroborate the current data. Moreover, as indicated by $K_s$, the affinity for phenacetin is approximately three times higher with CYP1A2 than with CYP1A1, consistent with their respective $K_m$ values. Stoichiometry studies revealed that CYP1A2 displayed higher coupling efficiency than CYP1A1 because of the formation of less water, despite higher production of H₂O₂ (Tables 2 and 3).

---

**TABLE 2**

Rates determined for phenacetin metabolism by CYP1A1 and CYP1A2

Data are means of triplicate determinations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>NADPH Oxidized (nmol)</th>
<th>O₂ Consumed (nmol)</th>
<th>Product Formed (nmol)</th>
<th>H₂O₂ Produced (nmol)</th>
<th>Excess H₂O₂ (nmol)</th>
<th>Excess H₂O (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>33 ± 4</td>
<td>22.4 ± 3.0</td>
<td>0.84 ± 0.08</td>
<td>8 ± 0</td>
<td>24 ± 0</td>
<td>26 ± 0</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>43 ± 4</td>
<td>22.7 ± 0.9</td>
<td>2.20 ± 0.10</td>
<td>14 ± 2</td>
<td>15 ± 2</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

* Excess water (H₂O) was calculated from the equation: H₂O = NADPH – H₂O₂ – product.

**TABLE 3**

Coupling efficiency of CYP1A1 and CYP1A2 at different branching points of the P₄₅₀ cycle

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Product/NADPH a</th>
<th>H₂O₂/O₂ b</th>
<th>H₂O/Product c</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>0.025</td>
<td>0.37</td>
<td>28.71</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.051</td>
<td>0.62</td>
<td>6.82</td>
</tr>
</tbody>
</table>

a Efficiency of the coupling of reducing equivalents to product formation.
b Reflects uncoupling at the first or second branch point.
c Reflects uncoupling at the third branch point.

---

**TABLE 4**

Percentages of low spin and high spin in CYP1A1 and CYP1A2 in the absence and the presence of phenacetin or acetaminophen at 27°C

<table>
<thead>
<tr>
<th>Enzyme (+Substrate)</th>
<th>Low Spin %</th>
<th>High Spin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1 (no substrate)</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>+ Phenacetin</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>+ Acetaminophen</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>CYP1A2 (no substrate)</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>+ Phenacetin</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>+ Acetaminophen</td>
<td>79</td>
<td>21</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** The percentages of high-spin (385 nm) and low-spin (418 nm) components of CYP1A2 with the absence and presence of phenacetin at 27°C. Abs, absorbance.

**Fig. 4.** Temperature dependence of $T_{1P}$ of phenacetin protons in the presence of CYP1A1. Positive slopes indicate that the substrate bound in the active site is in fast exchange with the surroundings. Similar results were obtained for CYP1A2 in the presence of phenacetin and acetaminophen. Ball, phenacetin protons H2/6; ◦, H3/5; ▼, CH₂; ▽, COCH₃; △, CH₃.
CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.

CYP1A2 is consuming reducing equivalents at a faster rate than CYP1A1 for this particular substrate.

The differences in coupling efficiencies and catalytic rates of P450 enzymes may be related to the position of the substrate in the active site with respect to the heme cofactor. On the basis of NMR data, the distance between protons of phenacetin at the oxidation site (OCH₂) site with respect to the heme cofactor. On the basis of NMR data, the CYP1A1 for this particular substrate.
CYP1A1 model (Ericksen and Szklarz, 2005). Another explanation is that there are multiple substrate binding modes, whereas the distances calculated from NMR T1 experiments represent only the average binding orientation. Furthermore, the distances in NMR T1 relaxation studies have been measured on the nonoxygenated P450 enzymes. Thus, NMR studies may not provide any information about binding of the substrate to the more catalytically relevant oxo-ferryl form of the enzyme. Further- more, given the dynamic nature of the P450 enzymes and the relatively slow catalytic cycle, it is very likely that substrate binding positions in the active site may rearrange during or between catalytic cycles of the enzyme. Thus, the distances obtained from NMR and the corresponding enzyme-substrate models (Fig. 5) reflect nonproductive binding geometry representative of the initial enzyme-substrate com-

plexes, in which the distances between the heme iron and the oxidation sites are too long to support the initial hydrogen abstraction. In the case of phenacetin, the distance between the heme iron and the oxidation site (OCH₂) is shorter for CYP1A2, which may increase the probability of achieving the productive pose and/or reduce the possibility of uncoupling, which is consistent with the higher specificity of this isof orm toward phenacetin.

Potentially, the binding of P450 reductase could allosterically influence active site geometry, which may, in turn, influence substrate binding position and catalysis. To test such a phenomenon, substrate-iron distances in the presence and absence of P450 reductase were measured and compared. However, on the basis of NMR results, the orientation of phenacetin did not change significantly in either CYP1A1 or CYP1A2 (data not shown).

As discussed earlier, phenacetin and acetaminophen are observed to bind in similar nonproductive orientations in the active sites of both CYP1A enzymes, with phenacetin somewhat farther from the heme prosthetic group (Fig. 5). In CYP1A2, the oxidation site of phenacetin is closer to the heme iron atom and in a position that, after a slight repositioning toward heme, would facilitate the rate-limiting hydrogen abstraction in the first step of the catalytic cycle. The reaction product, acetaminophen is found in a similar orientation slightly closer to the heme, which suggests that there is little movement of the compound in the active site except for brief approaches to the heme from the nonproductive binding orientation. Moreover, the acetamido group remains relatively close to the heme, allowing for the subsequent oxidation of acetaminophen at the amide nitrogen, producing a toxic metabolite, NAPQI. This result is in agreement with previous findings by Myers et al. (1994), who proposed a similar reaction mechanism on the basis of NMR-derived iron-nitrogen distances for acetaminophen. Furthermore, an analogous mechanism may operate during the oxidation of acetaminophen to NAPQI by CYP3A4, with the position of the amide group with respect to the heme likely to influence the reaction, as indicated by NMR T₁ relaxation studies (Cameron et al., 2007).

In conclusion, CYP1A2 displayed higher catalytic efficiency of phenacetin O-deethylation than CYP1A1, consistent with stoichiometry results. The time-averaged binding orientations of phenacetin and acetaminophen within the active site of the enzymes based on NMR data showed that the distance between the OCH₂ protons of the ethoxy group (site of phenacetin O-deethylation) and the heme iron was 1.5 Å shorter in CYP1A2 than in CYP1A1. The NMR results are thus in agreement with kinetic and stoichiometry studies and provide direct evidence that may help to explain more efficient metabolism of phenacetin by CYP1A2 than CYP1A1.

Acknowledgments
We thank Dr. Peter Gannett for his advice concerning NMR methods and for critical review of this manuscript. Molecular modeling studies were performed at the Computational Chemistry and Molecular Modeling Laboratory, Department of Basic Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, WV.

Authorship Contributions
Participated in research design: Huang, Deshmukh, Ericksen, and Szklarz.
Conducted experiments: Huang, Deshmukh, Ericksen, and Tu.
Performed data analysis: Huang, Deshmukh, and Szklarz.
Wrote or contributed to the writing of the manuscript: Huang, Deshmukh, Ericksen, Tu, and Szklarz.

References