CHARACTERIZATION OF SUBSTRATE BINDING TO CYTOCHROME P450 1A1 USING MOLECULAR MODELING AND KINETIC ANALYSES: CASE OF RESIDUE 382

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ABSTRACT:

Key residue Val-382 in P450 1A1 has been predicted to interact with the alkoxy chain of resorufin derivatives. Therefore, we undertook a detailed analysis of substrate mobility in the active site of the P450 1A1 homology model and assessed the effect of mutations at position 382. Dynamic trajectories of 7-methoxy-, 7-ethoxy-, and 7-pentoxyresorufin indicated that 7-ethoxyresorufin would be oxidized most efficiently by the wild-type enzyme. The Val-382→Ala mutation would increase the O-dealkylation of 7-pentoxyresorufin but decrease the oxidation of other substrates. In the case of the V382L mutant, the large bulk of Leu would block alkoxyresorufins from productive binding orientations leading to lowered activities. Binding free energy calculations for three substrates with 1A1 WT and two mutants indicated that binding constants would be similar for all enzyme-substrate combinations.

Cytochrome P450 (P450) enzymes are ubiquitous in living organisms, and more than 400 isoforms have been identified and sequenced from plants, animals, bacteria, and yeast (Nelson et al., 1996). These enzymes are very versatile catalysts that play a pivotal role in the metabolism of a wide variety of xenobiotic and endogenous compounds. Of major importance is the involvement of these enzymes in toxicant biotransformation and drug metabolism. Human P450 1A1 is mainly present in lungs and plays an important role in the metabolic activation of chemical carcinogens. The enzyme is able to oxidize benzo[a]pyrene and other polycyclic aromatic hydrocarbons to their toxic derivatives (Guengerich, 1995; Kawajiri and Hayashi, 1996; Shou et al., 1996a). For example, dibenzo[a,l]pyrene, considered the most potent carcinogen among all polycyclic aromatic hydrocarbons, is oxidized almost exclusively by P450 1A1 to highly mutagenic diol-epoxides (Shou et al., 1996b). Like many other P450s, 1A1 appears to be subject to genetic polymorphism and thus may play a role in determining cancer susceptibility (Houlston, 2000). Moreover, the enzyme is strongly induced by cigarette smoking and thereby associated with lung cancer (Shou et al., 1996b; Rendic and Di Carlo, 1997). Therefore, the elucidation of the structural basis of P450 1A1 substrate specificity is of great importance in understanding enzyme function and mechanism and may provide a foundation for the rational design of drugs and inhibitors.

One of the approaches to study structure-function relationships of cytochromes P450 involves a combination of molecular modeling and experimental techniques (Szklarz and Halpert, 1997a; Szklarz et al., 2000). Using homology models, residues involved in substrate recognition/specifcity can be identified in a P450-substrate complex, and predictions concerning various aspects of enzyme function advanced. The utility of the homology model as a basis for predicting enzyme-substrate interactions can be verified experimentally using site-directed mutagenesis, heterologous expression, and biochemical analyses.

We have recently constructed a homology model of P450 1A1 based on the structure of P450 2C5 (Szklarz and Paulsen, 2002). Docking of enzyme substrates in the active site allowed us to identify amino acid residues that might be important for activity. One of those residues, Val-382, has been postulated to affect P450 1A1 activity.
with 7-methoxy- and 7-ethoxyresorufin through its interaction with the alkoxy chain of the substrate (Szklarz and Paulsen, 2002). In the present study, we have modeled enzyme-substrate interactions in P450 1A1 WT with three compounds, 7-methoxy-, 7-ethoxy-, and 7-pentoxyresorufin and postulated changes in enzymatic activity upon the substitution of Val-382 with Ala or Leu. We have also calculated binding free energies for all enzyme-substrate combinations. Upon experimental verification using E. coli-expressed, purified enzymes, these modeling predictions were found to be reasonably accurate.

Materials and Methods

Materials. The DNA sequencing and mutagenic primers were synthesized at the Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA. Restriction endonucleases, the GeneEditor in vitro site-directed mutagenesis system and E. coli DH5α competent cells were obtained from Promega (Madison, WI). 7-Methoxyresorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, resorufin, NADPH, ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (ALA), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), dilauroyl-3-phosphatidyl choline (DLPC) and phenylmethylsulfonyl fluoride (PMSF) were all from Sigma-Aldrich (St. Louis, MO). Ni-NTA Agarose, plasmid mini-kit and gel extraction kit were purchased from Qiagen (Valencia, CA). All other chemicals were of analytical grade and were obtained from standard commercial sources.

Construction of the P450 1A1 His-tag Clone (P450 1A1 WT). A plasmid for the expression of human cytochrome P450 1A1 was obtained courtesy of Dr. R.W. Estabrook of University of Texas Southwestern Medical Center. The pCWori expression plasmid contained the cDNA for human P450 1A1 in which the 5′ coding sequence was modified for expression in E. coli as described by Fisher et al. (1992). In that construct amino acids 1 to 18 were replaced with the amino acids sequence MALLAVL (coding sequence 5′-ATGGCTCTTAGTATGCAGATTTCTG-3′). For the present studies, this construct was modified to contain a histidine domain on the carboxy-terminus with a serine-threonine dipeptide linker encoding a 5′ restriction site. To generate this construct, the original human P450 1A1 pcW expression construct was amplified with a primer encoding a modified 3′ end of the coding sequence (5′-AGGTATGTCGACGGCGAGCGTGCATTGTTCG-3′). The resulting DNA product was digested with SalI and EcoRI to produce a 107 bp fragment. The substrate was subcloned into a SalI and EcoRI I and the 1435 bp insert isolated. The insert was then ligated into a SalI and EcoRI I and the insert isolated. The insert was then ligated into a NdeI-SalI digested pcWori+ expression construct containing the modified bovine 17α-hydroxylase with a six-histidine carboxy-terminus. The scheme of the construction of the P450 1A1 His-tag clone is shown in Fig. 1.

Construction of P450 1A1 V382A and V382L Mutants. The P450 1A1 V382A and V382L mutants were constructed using the GeneEditor in vitro site-directed mutagenesis system (Promega), with P450 1A1 WT plasmid containing the His-tag as a template. The selection oligonucleotide, bottom primer V382A and V382L mutants were constructed using the GeneEditor in vitro mutagenesis system and used as a template to produce a new additional antibiotic resistance to the GeneEditor Antibiotic Selection Mix. The antisense mutagenic primers used to create Val-382A and Val-382L were 5′-GGTAGGTCGACGGCGAGCGTGCATTGC-3′ and 5′-GGGATGTTGTAAGGCGAGCGAAGGATGTCG-3′, respectively, with mutations underlined. All the procedures were performed according to the manufacturer’s instructions. The mutations were verified by DNA sequencing performed at the Molecular Genetics Instrumentation Facility, University of Georgia (Athens, GA).

P450 Expression and Purification. The preliminary experiments were performed with the mono- and bisctronic clones for human P450 1A1 expression that were generously provided by Dr. F.P. Guengerich (Vanderbilt University, Nashville, TN). The enzymes were expressed in E. coli and partially purified to obtain membrane preparations as described (Guo et al., 1994; Sandhu et al., 1994). Further studies were conducted with the I1A clones containing the His-tag, since it provides easy means of enzyme purification. Like mono- and bicistronic 1A1 clones, the His-tag-containing 1A1 wild-type and mutant enzymes were expressed in E. coli DH5α cells. The cultures were grown in 1 L of Terrific Broth medium containing 10 mg ampicillin liter−1, 1 mM δ-aminolevulinic acid (ALA), and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 48 h at 30 °C with shaking at 120 to 150 rpm (Fisher et al., 1992; Guo et al., 1994). Membrane preparations were obtained essentially as described (Guo et al., 1994), by solubilization for 3 h at 4 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 1% Emulgen 911 and 1 mM PMSF. The solution was centrifuged at 100,000g for 45 min to remove insoluble material, and the supernatant containing His-tagged P450 protein was purified by affinity chromatography with Ni-NTA Agarose (Qiagen). The enzyme was eluted with 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 1% Emulgen 911, 0.05% CHAPS, 200 mM imidazole, and 50 mM NaCl. P450-containing fractions were pooled and concentrated by ultrafiltration (Millipore Corporation, Bedford, MA, YM-30 membrane). The removal of imidazole was achieved by multiple ultrafiltration steps using 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 20% glycerol, 0.1% CHAPS, and 0.05% Emulgen 911. The final purity was assessed by SDS-PAGE (Laemmli, 1970). Western blots were performed (Kedzie et al., 1993) using anti-human P450 1A1/1A2 (Oxford Biomedical Research, Oxford, MI) and visualizing P450 proteins as described (Kedzie et al., 1991). P450 content was determined by reduced CO/reduced difference spectra (Omura and Sato, 1964), and protein was measured by the method of Lowry et al. (1951).

NADPH-P450 Reductase Expression and Purification. The cdNA encoding rat cytochrome P450 reductase was obtained courtesy of Dr. J.R. Halpert, University of Texas Medical Branch at Galveston (Galveston, TX). Rat cytochrome P450 reductase was expressed in E. coli strain HMS174, solubilized from the membranes and purified on a 25% PEG affinity column, as described previously (Shen et al., 1989; Harlow and Halpert, 1997). Protein was measured by the method of Lowry et al. (1951), and the reductase activity was assayed with cytochrome c as a substrate at 28 °C (Shen et al., 1989). Total flavoprotein obtained from 1 liter of the culture was 137 to 148 nmol. In the last step of purification, the preparation was concentrated by ultra filtration to give 1 ml of purified reductase with total activity of about 280 units and specific activity about 44 units/mg of protein. The purity of the preparation was verified with SDS-PAGE (Laemmli, 1970).

P450 Activity Assays. 7-Methoxy-, 7-ethoxy-, and 7-pentoxyresorufin dealkylase activities of P450 1A1-WT, and its two mutants were assayed at 37 °C by fluorometric detection of resorufin (Burke and Mayer, 1983) using excitation and emission wavelengths of 550 nm and 585 nm, respectively. The reaction mixture contained 50 mM P450 1A1 protein, 100 nM P450 reductase, 100 mM NADPH, 1% 200 mM imidazole, 50 mM NaCl, 1 mM isopropyl-β-D-thiogalactopyranoside, 200 mM imidazole, and 50 mM NaCl. P450-containing fractions were pooled and concentrated by ultrafiltration (Millipore Corporation, Bedford, MA, YM-30 membrane). The final purity was assessed by SDS-PAGE (Laemmli, 1970). Western blots were performed (Kedzie et al., 1993) using anti-human P450 1A1/1A2 (Oxford Biomedical Research, Oxford, MI) and visualizing P450 proteins as described (Kedzie et al., 1991). P450 content was determined by reduced CO/reduced difference spectra (Omura and Sato, 1964), and protein was measured by the method of Lowry et al. (1951).

Molecular Modeling. Computer modeling was performed using a Silicon Graphics Octane workstation with InsightII/Discover software (Accelrys, San Diego, CA) using consistent valence force field. The parameters for heme and ferryl oxygen were as described earlier (Paulsen and Ornstein, 1991, 1992). The homology model of P450 1A1 based on the crystal structure of P450 2C5 was constructed previously (Szklarz and Paulsen, 2002), and was used to dock three substrates, 7-methoxy-, 7-ethoxy-, and 7-pentoxyresorufin. The compounds were docked in the active site in productive binding orientations leading to their O-dealkylation using the Docking module of InsightII, and nonbond enzyme-substrate interaction energies were estimated, as described previously (Szklarz et al., 1995; Szklarz and Halpert, 1997b; Szklarz and Paulsen, 2002). To evaluate the stability of resorufin substrates in productive binding orientations in the WT and mutants, molecular dynamics simulations were performed for 5 ps at 300 K in vacuo, essentially as described before (Kent et al., 1997; Strobel et al., 1999), except that both the substrate and active
site residues within 8 Å from the substrate were allowed to move. These simulations were performed for the iron-oxo form of the P450.

Binding free energy calculations were performed for each combination of enzyme and substrate (i.e., P450 1A1 wild-type and V382A and V382L mutants with 7-methoxy-, 7-ethoxy-, and 7-pentoxyresorufin substrates). For each substrate and enzyme, a pair of simulations was carried out, one with the substrate in an aqueous environment and one with the substrate bound in the active site of the 1A1 model, using the consistent valence force field, as described earlier (Paulsen and Ornstein, 1996; Szklarz and Paulsen, 2002). Briefly, for the aqueous simulation, each substrate was solvated in a periodic box of water 28 Å on a side. The system was minimized with 1000 steps of conjugate gradient, and then subjected to molecular dynamics at 300 K, with the equilibration phase of 25 ps, followed by 50 ps of simulations during which data were collected. For protein-bound simulations, the starting point was the

The details are in the text.

Fig. 1. Construction of P450 1A1 clones containing the His-tag.
toxyresorufin is green, heme is red, and Val-382 purple. Three resorufin compounds, 7-methoxy- and 7-ethoxyresorufin substrates in the active site of the wild-type P450 1A1 (Fig. 3) predicts that all three compounds may bind without major steric hindrance. However, only in the case of 7-ethoxyresorufin, the hydrogens at the oxidation site remain at about 3 Å from the ferryl oxygen throughout the 5 ps simulation, thus promoting substrate deethylation. 7-Methoxyresorufin is likely to be oxidized with lower efficiency since the distance between its hydrogens at the oxidation site and the oxygen fluctuates between 4 and 8 Å, with hydrogen atoms only infrequently approaching the ferryl oxygen. In the case of 7-pentoxyresorufin, the oxidation site is far from the oxygen (hydrogens at the oxidation site are at the average distance of ~6 Å) and is sterically screened by other atoms of the pentoxy tail throughout the 5 ps simulation. Thus, the oxidation of this compound would be very difficult.

In the case of P450 1A1 V382A mutant, the active site cavity is somewhat enlarged compared with the wild type, which results in the increased mobility of 7-ethoxy and 7-methoxyresorufin (Fig. 4). The oxidation site of 7-ethoxyresorufin now remains farther from the ferryl oxygen, which may indicate the decrease in activity. In contrast, for 7-pentoxyresorufin, the oxidation site is occasionally close to heme, with hydrogen atoms approaching within 3 to 4 Å of the ferryl oxygen thus allowing for substrate dealkylation.

The substitution of Val-382 with Leu drastically affects the position of smaller alkoxyresorufins within the active site (Fig. 5). 7-Methoxyresorufin may move out from the productive binding orientation and the distance between oxidation site hydrogens and the ferryl oxygen increases to 8 to 10 Å. The corresponding distances for larger compounds also increase, albeit to a somewhat lesser extent. In general, the large bulk of Leu seems to push alkoxyresorufins out of productive binding orientations leading to a decrease in activities.

Binding characteristics of the three resorufin substrates were further investigated by calculating binding free energies for various enzyme-substrate combinations. As shown in Table 1, all the values were similar, within 4 kcal/mol. This result in the dissociation constants on the order 1 μM using the equation $AG = -RT \ln K_d$. Interestingly, the electrostatic component energies were comparable in all cases, whereas the electrostatic energy components showed some differences. In the case of methoxyresorufin, the substitution of Val-382 with Ala leads to some decrease in van der Waals energy contribution, but the electrostatic component increases leading to nearly identical binding free energy. Upon mutation of this Val to Leu, the electrostatic contribution is more favorable than for the wild type resulting in slight decrease in binding free energy. Some variation of this kind can also be observed for other substrates studied, but the net result is little or no change in binding free energy. Therefore, since the dissociation constant may be approximated by $K_m$, little or no change in $K_m$ can be expected for the three resorufin substrates upon Val-382 mutation. The predictions from these modeling analyses were then tested experimentally in the second part of our studies.

Expression and Purification of P450 1A1 WT and V382A and V382L Mutants. Prior to the construction of the mutants, the P450 1A1 cDNA was modified at the 5′ end to facilitate expression in E. coli, whereas the 3′ terminus was altered to contain an extra six-histidine domain. The resulting C-terminal (His)$_6$-tag expedites P450 purification by reducing the number of chromatographic separations to one, namely affinity chromatography with Ni-NTA agarose. Similar strategy has been successfully used with P450 1A2 and its mutants (Yun et al., 2000). The P450 1A1 V382A and V382L mutants used in this study were constructed by site-directed mutagenesis using the His-tag containing plasmid. The expression levels of the enzymes and some characteristics of purified preparations are shown in Table 2. The expression levels of all enzymes were fairly high, with that of the wild type close to 0.5 μM, whereas the mutants were expressed at lower levels of 0.15 to 0.11 μM. It should be noted that these values are derived from P450 measurements in sonicated spheroplasts. These levels are higher than those reported by Guo et al. (1994) who first expressed recombinant human P450 1A1 in E. coli. As seen in Table 2, the total amount of purified P450 obtained from 1 liter culture was again the highest for the wild-type enzyme, ~90 nmols, with close to a half of that amount in the case of the mutants. The overall yield of the entire separation procedure was ~20–35%, with the yield of the affinity chromatography step itself averaging 70%. The total yield was calculated using spheroplasts as the first step in the purification protocol and included multiple ultrafiltration steps. The purified wild-type protein displayed a high specific content of P450 with close to 90% of the holoenzyme present. In contrast, in the case of both V382A and V382L mutants, the corresponding values were much lower, suggesting a significant loss of heme. In fact, the V382A mutant was quite unstable and was easily reduced to the P420 form during the purification procedure. For each of the final P450 prepa-
rations, SDS-PAGE and Western blots revealed the presence of a single band confirming protein purity (data not shown).

Kinetic Parameters of Purified P450 1A1 WT and Val-382 Mutants. Purified P450 1A1 WT and the V382A and V382L mutants were used to measure the O-dealkylase activity with 7-methoxy-, 7-ethoxy-, and 7-pentoxyresorufin using a range of substrate concentrations. In these reactions, the substrates undergo demethylation, deethylation, and depentylation, respectively, to form resorufin product. Kinetic parameters for these reactions are shown in Table 3. The activity of the P450 1A1 WT was the highest with 7-ethoxyresorufin, with $V_{\text{max}}$ close to 11 nmol of product formed min$^{-1}$ (nmol P450)$^{-1}$, and the lowest with 7-pentoxyresorufin, with a corresponding value of about 0.4. Since $K_m$ values varied little, the catalytic efficiency ($V_{\text{max}}/K_m$) of the wild-type enzyme was likewise the highest with 7-ethoxyresorufin, whereas the analogous values for the other substrates were 10 times lower. It is worth mentioning that the apparent $K_m$ and $V_{\text{max}}$ values for 7-ethoxyresorufin $O$-deethylation observed in this study [i.e., 0.61 $\mu$M and 11 nmol product formed min$^{-1}$ (nmol P450)$^{-1}$, respectively, corresponded to those reported by Guo et al. (1994) for their E. coli-expressed human P450 1A1, which were 0.58 $\mu$M and 8.3 nmol product formed min$^{-1}$ (nmol P450)$^{-1}$, respectively].

Upon the substitution of Val-382 with Ala, the activities toward 7-methoxy- and 7-ethoxyresorufin fell 10-fold, whereas the $V_{\text{max}}$ for 7-pentoxyresorufin increased almost twice. Little change in $K_m$ values was observed in the mutant compared with the wild-type enzyme. This is illustrated in Fig. 6, which shows the difference between the rates of 7-pentoxyresorufin dealkylation by the wild-type and the V382A mutant. Although the catalytic efficiencies of the mutants decreased about 10 times with smaller substrates, the value of $V_{\text{max}}/K_m$ for 7-pentoxyresorufin increased over 3-fold. The V382A mutant displayed the highest catalytic efficiency with the latter substrate, although the $V_{\text{max}}$ value was lower than that for 7-ethoxyresorufin. The differences in the metabolism between 7-ethoxy- and 7-pentoxyresorufin are illustrated in Fig. 7, which shows the relevant Lineweaver-Burk kinetic plots. In general, these results suggest that the replacement of Val with Ala at position 382 permits better oxidation of the larger substrate, 7-pentoxyresorufin.

In the case of the P450 V382L mutant, a significant decrease in activity and catalytic efficiency was observed for all substrates com-
pared with the wild-type enzyme, while $K_m$ changed little. The effect of this mutation on oxidation of 7-ethoxyresorufin is shown in Fig. 8, which demonstrates considerable decrease in activity when compared with the wild type. We had some difficulties with obtaining the relevant data for this mutant, especially with 7-pentoxyresorufin as a substrate. In that case, we observed a prolonged lag phase during the reaction (3–6 min), which might have had an effect on the final values. Since this mutant displayed very low heme content (see Table 2), the presence of large amounts of apoprotein might have resulted in unproductive binding to the reductase and/or protein aggregation. Overall, the V382L mutation significantly decreased oxidation of all alkoxyresorufin substrates.

**Discussion**

The overall objective of our study was to evaluate molecular modeling as a tool to predict certain aspects of P450 catalysis, such as

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**TABLE 1**

Calculated binding free energies (kcal/mol) for resorufin substrates docked into the active site of P450 1A1 WT and V382A and V382L mutants

<table>
<thead>
<tr>
<th>Methoxyresorufin</th>
<th>Ethoxyresorufin</th>
<th>Pentoxyresorufin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td><strong>V382A</strong></td>
<td><strong>V382L</strong></td>
</tr>
<tr>
<td>$\Delta V_{vdW}$</td>
<td>$3.75$</td>
<td>$6.54$</td>
</tr>
</tbody>
</table>

Binding free energies were calculated according to the equation: $\Delta G_{bind} = 0.5 \Delta V_{el} + \alpha \Delta V_{vdW}$, where $\Delta V_{el}$ is the difference in the electrostatic interaction energy between the ligand and its surroundings in the protein and in aqueous solution, and $\Delta V_{vdW}$ represents the difference in van der Waals interaction energies. The value of parameter $\alpha$ was 1.043 (Paulsen and Ornstein, 1996). The data for binding free energies for methoxy- and ethoxyresorufin with the wild-type P450 1A1 enzyme are from Szklarz and Paulsen (2002).

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**TABLE 2**

Purification results of P450 1A1 WT, V382A, and V382L mutants

<table>
<thead>
<tr>
<th>P450 1A1</th>
<th>P450 expression (nmol/l)</th>
<th>Total purified P450 (nmol)</th>
<th>Yield (%)</th>
<th>Specific content (nmol of P450/mg of protein)</th>
<th>Holoenzyme P450 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>471.2</td>
<td>89.6</td>
<td>19.4</td>
<td>15.2</td>
<td>88</td>
</tr>
<tr>
<td>V382A</td>
<td>151.4</td>
<td>43.3</td>
<td>28.6</td>
<td>5.7</td>
<td>33</td>
</tr>
<tr>
<td>V382L</td>
<td>113.6</td>
<td>39.2</td>
<td>34.5</td>
<td>3.3</td>
<td>19</td>
</tr>
</tbody>
</table>

* For all P450s, the P420 content was less than 5%.

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**TABLE 3**

Comparison of kinetic parameters of P450 1A1 WT and V382A and V382L mutants with resorufin substrates

<table>
<thead>
<tr>
<th>P450 1A1</th>
<th>Methoxyresorufin</th>
<th>Ethoxyresorufin</th>
<th>Pentoxyresorufin</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$V_{max}$</td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td></td>
<td>$2.42 \pm 1.07$</td>
<td>$1.27 \pm 0.76$</td>
<td>$1.91$</td>
</tr>
<tr>
<td>V382A</td>
<td>$0.18 \pm 0.08$</td>
<td>$0.78 \pm 0.38$</td>
<td>$0.23$</td>
</tr>
<tr>
<td>V382L</td>
<td>$0.14 \pm 0.02$</td>
<td>$2.06 \pm 1.41$</td>
<td>$0.07$</td>
</tr>
</tbody>
</table>

* Values are derived from duplicate assays performed as described under Materials and Methods. $V_{max}$ is expressed in picomoles of product formed per minute (pmol P450)$^{-1}$, and $K_m$ is shown in micromolar concentrations.

* The data were obtained from the rates following the 3- to 6-min lag phase, as mentioned under Results.
substrate binding and mobility in the active site, which, in turn, affect kinetic parameters of the reaction. In our previous work (Szklarz and Paulsen, 2002), we have shown that binding free energy calculations in homology models can be successfully used to predict binding constants. We have also identified Val-382 as a key residue of P450 1A1 and proposed its role in binding of alkoxyresorufin substrates. In the present study, we have explored the possible function of this residue in more detail. In the first part of our investigation, we performed molecular modeling analyses of substrate motion in the active site of the enzyme model and binding free energy calculations, and predicted the effect of mutations on catalysis. The substrates chosen were three resorufin derivatives, 7-methoxy-, 7-ethoxy-, and 7-pentoxyresorufin, which differ in the length of the alkoxy chain. Moreover, 7-ethoxyresorufin is efficiently metabolized by P450 1A1, while 7-methoxy compound oxidized to a lesser extent, whereas the metabolism of 7-pentoxyresorufin is very poor. We explored the effect of substituting Val-382 with smaller Ala or larger Leu on O-dealkylation of alkoxyresorufins. In the second part of our investigation, these theoretical studies were followed by experimental research to verify modeling predictions.

The results of this study show a good agreement between the predictions and experimentally determined P450 activities in vitro, and complement each other quite well. Thus, high activity of P450 1A1 WT toward 7-ethoxyresorufin can be explained by the substrate remaining close to heme and ferryl oxygen, which promotes its oxidation. Unfavorable distances between the oxidation sites of other alkoxyresorufins and ferryl oxygen are in agreement with lowered activities of the enzyme toward these compounds. In the case of the V382A mutant, the corresponding distances increase for the smaller resorufins, but the enlarged active site allows for better oxidation of 7-pentoxyresorufin, consistent with the observed changes in Vmax. When Val-382 is replaced by Leu, the bulk of this residue pushes all three alkoxyresorufin substrates out of the productive binding orientations, in agreement with lower activity observed for this mutant. In most cases, the mutation led to a decrease in Vmax, except for the V382A mutant with 7-pentoxyresorufin as a substrate. Calculated binding free energies for all enzyme-substrate combinations were quite similar, with dissociation constants of the order of 10-9 M. The Km values observed varied somewhat, but were usually of similar order of magnitude, more so in view of large standard deviations observed in several cases.

P450 1A1 is closely related to another enzyme of 1A family, P450 1A2, with which it shares 72% sequence identity (Kawajiri and Hayashi, 1996). However, P450 1A2 displays different substrate specificity and inhibitor susceptibility, and preferentially oxidizes heterocyclic and aromatic amines (Hammons et al., 1997; Turesky et al., 1998). Some substrates can be metabolized by both enzymes, but with different efficiencies. P450 1A1 effectively oxidizes 7-ethoxyresorufin, while P450 1A2, although also able to metabolize this substrate, exhibits preference toward the 7-methoxy analog (Nerurkar et al., 1993; Kawajiri and Hayashi, 1996). Interestingly, Val-382 of P450 1A1 corresponds to Leu-382 of P450 1A2. Although our 1A1 V382L mutant displayed decreased activity with 7-methoxyresorufin compared with the wild-type enzyme, the active site of

**Fig. 6.** Rate of formation of resorufin by 1A1-WT (■) and 1A1-V382A (▲) as a function of the 7-pentoxyresorufin concentration.

Data were obtained from duplicate assays.

**Fig. 7.** Lineweaver-Burk plots of the kinetic analysis of 7-ethoxyresorufin (■) and 7-pentoxyresorufin (▲) oxidation to resorufin by P450 1A1 V382A mutant.

Data are derived from duplicate assays.

**Fig. 8.** Rate of formation of resorufin by P450 1A1 WT (A) and the V382L mutant (B) as a function of the 7-ethoxyresorufin concentration.

Data were obtained from duplicate assays.
P450 1A2 is likely to be different, with residues other than Leu-382 playing a role in substrate binding. It would be of interest to examine a reciprocal mutation, Leu-382 → Val, in P450 1A2, and its effect on dealkylation of alkoxyresorufins. In fact, our recent data suggest that this substitution increases the activity toward 7-ethoxyresorufin, while O-dealkylation of the 7-methoxy compound drastically decreases so that it is lower than for the oxo derivative (unpublished results). Thus, the P450 1A2 L382V mutant exhibits activities similar to those of P450 1A1 WT with the two resorufin substrates. The structural basis for differences in specificity among related P450 enzymes has been studied in 2B subfamily. Modeling and experimental studies with highly related P450 2B4 and 2B5 showed that single reciprocal mutants displayed altered substrate specificities (Szklarz et al., 1996), although multiple active-site mutations were necessary to completely interconvert activities (He et al., 1996).

Alkoxyresorufin dealkylation has been widely used to selectively investigate P450 activities. The O-dealkylations of ethoxy- and propoxyresorufin are generally accepted as selective probes for measuring O-dealkylation of the 7-methoxy compound drastically decreases so that it is lower than for the oxo derivative (unpublished results). Thus, the P450 1A2 L382V mutant exhibits activities similar to those of P450 1A1 WT with the two resorufin substrates. The structural basis for differences in specificity among related P450 enzymes has been studied in 2B subfamily. Modeling and experimental studies with highly related P450 2B4 and 2B5 showed that single reciprocal mutants displayed altered substrate specificities (Szklarz et al., 1996), although multiple active-site mutations were necessary to completely interconvert activities (He et al., 1996).

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Shou M, Krausz KW, Gonzalez FJ, and Gelboin HV (1996b) Molecular activation of the potent binding constants for several substrates bound in the active site of the P450 1A1 model (Szklarz and Paulsen, 2002). Such methods provide more detailed information about enzyme-substrate interactions in the active site and allow for a mechanistic interpretation of various aspects of P450 catalysis. The trend toward a more rigorously computational approach to study P450 function can be expected to grow and has a potential to powerfully enhance our understanding of P450 structure-function relationships.


