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

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(Received September 28, 2002; accepted December 20, 2002)

This article is available online at http://dmd.aspetjournals.org

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.

Modeling predictions were tested experimentally. The plasmid containing the cDNA for human P450 1A1 modified for bacterial expression was altered to include a C-terminal PCR-generated six histidine domain to facilitate enzyme purification. The V382A and V382L mutants were constructed by site-directed mutagenesis and Escherichia coli–expressed enzymes purified using Ni-NTA affinity chromatography. The activity of the WT 1A1 was highest toward 7-ethoxyresorufin and lowest toward 7-pentoxyresorufin. Both mutants displayed a decrease in Vmax with 7-methoxy- and 7-ethoxyresorufin, whereas for the V382A mutant, Vmax with 7-pentoxyresorufin was increased. No significant changes in Km were observed relative to the wild-type enzyme. The experimental results are thus in good agreement with modeling predictions.

Cytochrome P450 (P4502) 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/specificity 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), dilauryl-3-phosphatidyl choline (DPLC) and phenylmethanesulfonyl fluoride (PMSF) were 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 used 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 acid sequence MALLAVLF (coding sequence 5′-ATGGCTCTGATTAGAGCTTGC-3′). For the present study, this construct was modified to contain a histidine domain on the carboxy-terminus with a serine-threonine dipeptide linker encoding a Sall restriction site. To generate this construct, the original human P450 1A1 pCW expression construct was amplified with a primer encoding a modified 3′ site. The reaction mixture contained 50 nM P450 1A1 protein, 100 nM P450 reductase, NADPH, 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 2′-5′-ADP 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 ultrafiltration 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 mM P450 reductase, 100 mM potassium phosphate buffer (pH 7.5), 50 mM potassium phosphate buffer, pH 7.5, in a total volume of 1 ml. The reaction was initiated by addition of 10 μl of 100 mM NADPH, and the formation of resorufin was detected as the increase of fluorescence intensity against time. The reaction rate was quantified with resorufin standards. All measurements were performed in duplicate. For kinetic assays, the same reaction conditions were used, except that substrate concentration was varied from 0.01 to 20 μM. Kinetic parameters were determined by classical linearization methods using the Enzyme Kinetics Pro software (ChemSW, Fairfield, CA).

**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 ferriy oxygen were as described earlier (Paulsen and Ornstein, 1991, 1992), except that both the substrate and active sites 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 vacuum, 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

FIG. 1. Construction of P450 1A1 clones containing the His-tag.

The details are in the text.
7-Methoxyresorufin is shown in yellow, 7-ethoxyresorufin is orange, 7-pentoxyresorufin is green, heme is red, and Val-382 purple.

The active site of the P450 1A1 homo-haping model with resorufin substrates docked in an orientation leading to their O-dealkylation.

1A1-homology structure with the substrate bound in a productive binding orientation, which was solvated by soaking with water. For molecular dynamics simulations, only amino acid residues and solvent within a 16 Å radius from the substrate were permitted to move, whereas the remainder of the protein was fixed. All substrate-P450 complexes were modeled with pentacoordinate heme iron (Paulsen and Ornstein, 1996; Szklarz and Paulsen, 2002).

Results

Molecular Modeling Analyses. Our previous modeling studies suggested that Val-382 is a key residue in P450 1A1 that may interact with 7-methoxy- and 7-ethoxyresorufin substrates (Szklarz and Paulsen, 2002). Therefore, in the present work, we explored this issue further. Three resorufin compounds, 7-methoxy- and 7-ethoxy- and 7-pentoxyresorufin, which differ in the length of the alkoxy chain, were docked in the active site of the P450 1A1 model in productive binding orientations that would be expected to lead to their O-dealkylation. As shown in Fig. 2, the orientations of these substrates are very similar, with the planar portion of the molecules superimposed and differing lengths of the alkoxy chain accommodated within the space close to heme and Val-382.

The expression levels of all enzymes were fairly high, with that of the 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 and V382L mutants, the corresponding values were much lower, suggesting a significant loss of heme. In fact, the V382A and V382L mutants, the corresponding values were much lower, suggesting a significant loss of heme. In fact, the V382A and V382L mutants, the corresponding values were much lower, suggesting a significant loss of heme. In fact, the V382A and V382L mutants, the corresponding values were much lower, suggesting a significant loss of heme. In fact, the V382A and V382L mutants, the corresponding values were much lower, suggesting a significant loss of heme. 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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 μM 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 μM 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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**Fig. 5.** The distance between the ferryl oxygen and hydrogens at the oxidation site of alkoxyresorufins bound in the active site of the P450 1A1 V382L mutant plotted as a function of time.

A. 7-Methoxyresorufin; B. 7-ethoxyresorufin; C. 7-pentoxyresorufin. The simulations were initiated for the substrate bound in the productive binding orientation.

**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>Substrate</th>
<th>WT</th>
<th>V382A</th>
<th>V382L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyresorufin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta V_{el} )</td>
<td>-10.88</td>
<td>-13.19</td>
<td>-12.4</td>
</tr>
<tr>
<td>( \Delta V_{vdW} )</td>
<td>3.75</td>
<td>8.63</td>
<td>2.3</td>
</tr>
<tr>
<td>( \Delta G_{bind} )</td>
<td>-9.47</td>
<td>-9.44</td>
<td>-11.78</td>
</tr>
<tr>
<td>Ethoxyresorufin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta V_{el} )</td>
<td>-11.96</td>
<td>-10.13</td>
<td>-10.3</td>
</tr>
<tr>
<td>( \Delta V_{vdW} )</td>
<td>-1.9</td>
<td>4.68</td>
<td>5.8</td>
</tr>
<tr>
<td>( \Delta G_{bind} )</td>
<td>-13.42</td>
<td>2.86</td>
<td>3.3</td>
</tr>
<tr>
<td>Pentoxyresorufin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta V_{el} )</td>
<td>-11.96</td>
<td>-10.13</td>
<td>-10.3</td>
</tr>
<tr>
<td>( \Delta V_{vdW} )</td>
<td>-1.9</td>
<td>4.68</td>
<td>5.8</td>
</tr>
<tr>
<td>( \Delta G_{bind} )</td>
<td>-13.42</td>
<td>2.86</td>
<td>3.3</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).

**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%.

*Percentage of P450 containing heme. Based on 17.2 nmol/mg of protein (M, for His-tagged P450 1A1 is 58,196).

**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></td>
<td>( V_{max} )</td>
<td>( K_m )</td>
<td>( V_{max}/K_m )</td>
</tr>
<tr>
<td>WT</td>
<td>2.42 ± 1.07</td>
<td>1.27 ± 0.76</td>
<td>1.91</td>
</tr>
<tr>
<td>V382A</td>
<td>0.18 ± 0.08</td>
<td>0.78 ± 0.38</td>
<td>0.23</td>
</tr>
<tr>
<td>V382L</td>
<td>0.14 ± 0.02</td>
<td>2.06 ± 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 the 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, with the 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 $V_{\text{max}}$. 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 low activity observed for this mutant. In most cases, the mutation led to a decrease in $V_{\text{max}}$, 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 1 M. The $K_m$ values observed varied somewhat, but were usually of a 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
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 ethoxy derivative (unpublished results).

Thus, the P450 1A2 L382V mutant exhibits activity 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 benzoxyresorufin are generally accepted as selective probes for measuring P450 isoforms, P450 1A1 and 2B1, respectively (Burke and Mayer, 1983; Burke et al., 1994). 7-Methoxyresorufin is preferentially metabolized by P450 1A1, whereas another resorufin derivative, benzyloxyresorufin, appears to be relatively specific for the P450 2B subfamily (Nerurkar et al., 1993; Burke et al., 1994). However, since other P450s can also metabolize various alkoxyresorufins, the best discriminator between a given isoform and other P450 enzymes is the ratio between various activities, such as the ratio between methoxyresorufin O-dealkylation and n-propoxyresorufin O-dealkylation for P450 1A2 (Burke et al., 1994).

Our present studies indicate that varying alkoxyresorufin substrate specificities are governed by the spatial fit and substrate mobility in the active site.

Alkoxycoumarins are also frequently employed as P450 probes. Molecular and experimental techniques were used to investigate the effect of the chain length on the metabolism of a series of 7-alkoxy-coumarins by P450 2B1 and its mutants (Kobayashi et al., 1998). The ability of the wild-type enzyme to oxidize a specific compound was found to depend upon the length of the alkyl chain. Changes in activity of 2B1 mutants were satisfactorily explained by the analysis of enzyme-substrate interactions in the active site of the enzyme model in conjunction with stoichiometry studies. This approach is similar to that used in our present investigation. However, our current modeling analyses relied on more rigorous molecular dynamics simulations and binding free energy calculations. Future research in this area may include stoichiometry studies, which have potential to allow us to link increased substrate mobility to the uncoupling of the P450 reaction.

Molecular modeling in conjunction with experimental approaches, such as site-directed mutagenesis, have been extensively used to study structure-function relationships of cytochromes P450 (for reviews, see Szklarz and Halpert, 1997a; 1998; Szklarz et al., 2000; Domanski and Halpert, 2001). More recently, modeling methods used in these investigations have become more sophisticated, increasingly employing molecular dynamics simulations, such as those used by Strobel et al. (1999), as well as in the present work. Although the length of the simulations we used was only 5 ps, they should nevertheless provide some information about the motion of the substrate in the active site and its possible effect on catalytic activity. Longer simulations, in the range of nanoseconds, would allow for more quantitative predictions concerning enzymatic activity. Longer simulations have been performed using the crystallized structure of P450cam-camphor complex (Paulsen and Ornstein, 1992) but not with homology models. Another more advanced computational technique involves binding free energy calculations, which have been successfully employed to predict 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 rigorous 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.

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