INFLUENCE OF PHENYLALANINES 77 AND 138 ON THE STEREOSPECIFICITY OF ARYL SULFOTRANSFERASE IV

Jonathan J. Sheng, Atmaja Saxena, and Michael W. Duffel
Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa

Received October 10, 2003; accepted January 16, 2004

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

ABSTRACT:

Aryl sulfotransferase (AST) IV (also named tyrosine-ester sulfotransferase and ST1A1) is a major phenol sulfotransferase in the rat, and it catalyzes the sulfation of many drugs, carcinogens, and other xenobiotics that contain phenol, benzylic alcohol, \(N\)-hydroxy arylamine, and oxime functional groups. Previous work discovered a stereospecificity of AST IV toward the enantiomers of 1,2,3,4-tetrahydro-1-napthol and varying degrees of stereoselectivity with other chiral benzylic alcohols. The studies described here were directed toward understanding the roles of specific amino acid residues at the substrate binding site in determining the stereoselectivity of this sulfotransferase isoform. Docking experiments with a homology model of AST IV revealed three amino acid residues, Phe77, Phe138, and Tyr236, that may potentially be important for interactions with substrates on the chiral carbon of a benzylic alcohol serving as a sulfuryl acceptor, thereby imparting stereoselectivity. To test this hypothesis, mutants were constructed wherein each of the above residues was substituted with alanine. Kinetic studies on the sulfation of the enantiomers of 1,2,3,4-tetrahydro-1-napthol indicated that the stereospecificity of the sulfotransferase was altered by the substitutions of alanine for either Phe77 or Phe138, but stereoselectivity was maintained by alanine substitution at Tyr236. Molecular models of the mutant enzymes interacting with enantiomers of 1,2,3,4-tetrahydro-1-napthols and with 2-napthol indicate that Phe77 and Phe138 provide significant steric interactions at the active site that both enhance catalytic efficiency and impart stereoselectivity in molecular recognition of substrates and inhibitors.

The mammalian cytosolic aryl (phenol) sulfotransferases constitute a family of enzymes that are important in the detoxication of drugs, carcinogens, and other xenobiotics containing organic functional groups as diverse as phenols, primary and secondary alcohols, \(N\)-hydroxyarylamines, arylamines, oximes, and nitroalkanes (Jakoby et al., 1980; Weinsilboum and Otterness, 1994; Falany, 1997; Duffel et al., 2001). Additionally, these enzymes catalyze the sulfation of endogenous hormones, such as thyroid hormone T\(3\) (3,5,3\'-triiodothyronine) and related metabolites (Sekura et al., 1981), as well as neurotransmitters such as epinephrine and dopamine (Jakoby et al., 1980). In all cases, the aryl sulfotransferases catalyze the transfer of a sulfuryl group from the donor substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS\(^1\)) to an acceptor substrate. Although sulfotransferase-catalyzed reactions with most endogenous and xenobiotic substrates yield sulfuric acid esters that are less biologically active and more readily excreted, there are significant exceptions with important implications for carcinogenesis and other toxic responses to xenobiotics (Miller, 1994; Duffel, 1997; Glatt et al., 2001).

The primary sequences of many aryl sulfotransferases have now been deduced from cDNA analysis, and it has therefore been possible to develop classification schemes for these enzymes in relation to other cytosolic sulfotransferases (Weinsilboum et al., 1997; Nagata and Yamazoe, 2000; Honma et al., 2001). The human cytosolic sulfotransferases have been classified according to families and subfamilies preceded by the prefix “SULT” (Weinsilboum et al., 1997), and a nomenclature for isoforms of these enzymes in all mammalian species has been proposed wherein the prefix “ST” precedes family and subfamily designation (Nagata and Yamazoe, 2000). Crystallographic analyses of several aryl sulfotransferases have greatly contributed to our understanding of the structure and function of these enzymes (Kakuta et al., 1997, 1998; Bidwell et al., 1999; Dajani et al., 1999; Negishi et al., 2001; Yoshinari et al., 2001; Pedersen et al., 2002; Gamage et al., 2003). In addition to important advances in structural details of the mechanism of sulfuryl transfer, it is also clear that the three-dimensional structures of sulfotransferases retain a high degree of homology, even as the primary sequences exhibit decreasing identity (Negishi et al., 2001). Thus, it becomes increasingly important to discern the roles of individual amino acid residues that determine molecular recognition of substrates and inhibitors of these enzymes.

The major form of cytosolic aryl sulfotransferase present in rat liver is AST IV (also known as tyrosine-ester sulfotransferase or ST1A1)
Materials and Methods

Chemicals and Biochemicals. Dithiothreitol, sucrose, 2-mercaptoethanol, glycerol, methanol, and acetone were purchased from Fisher Scientific Co. (Pittsburgh, PA). Isopropyl-1-thio-D-galactopyranoside was purchased from Amresco Inc. (Solon, OH). The \((R)\)(−) and \((S)\)(+) enantiomers of 1,2,3,4-tetrahydro-1-naphthol (structures shown in Fig. 1) are used as chiral probes to examine the potential role(s) of three conserved aromatic residues in enzyme-substrate interactions at the sulfuryl acceptor binding site of AST IV. Furthermore, results are analyzed within the context of molecular modeling studies using a homology model of AST IV.

Site-Directed Mutagenesis. The bacterial expression vector PET-3c containing AST IV cDNA obtained from previous studies (Chen et al., 1992) was used as a template to generate mutant constructs. The mutant oligonucleotide primers (5'-CCGGTCTACCGGCTTTCGGTCA-3' for F77A, 5'-TCCTAT-TATAACGGCTTACACATGGCC-3' for F138A, and 5'-CATGACTAACGC-CACAACAATGC-3' for Y216A) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The mutations were performed with a Transformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA). In each case, the oligonucleotide primer was annealed to one strand of the wild-type template DNA along with another oligonucleotide (5'-TTT-GCTCAGCTGCCTTCTCAGG-3') to generate a mutant strand. Subsequently, synthesis and ligation were performed to link them. The DNA was then transformed into a recipient strain of E. coli (DH5α). When the culture reached an absorbance of 0.6 at 600 nm, the cells were harvested by centrifugation at 10,000g for 30 min. The cell pellets were stored at −70°C until used.

Affinity Purification of the Recombinant AST IV. Chromatography on PAP-agarose (Sigma-Aldrich) was used to purify the wild-type and mutant AST IV enzymes. The cell extract was charged onto a column of PAP-agarose (5 ml) that had been previously equilibrated with buffer A. The direction of flow for the column was reversed after the column was washed with 100 ml of buffer A. The column was then washed with an additional 30 ml of buffer A, and the recombinant protein was eluted from the column with 15 ml of buffer A containing 500 nM PAP. The effluent was then concentrated to approximately 2 ml by ultrafiltration (PM-10 membrane, Millipore Corporation, Billerica, MA). Excess PAP remaining in the enzyme solution was extracted by chromatography on a disposable PD-10 column (Amersham Biosciences, Inc., Piscataway, NJ) that was pre-equilibrated with buffer A. The PD-10 column was eluted with 10 ml of buffer A. The fractions containing the AST IV enzymes were collected, concentrated to about 1 ml, and stored at −70°C. The protein concentration was determined using a Bio-Rad protein assay as described by Lowry et al. (1951). Before SDS-PAGE, the purified proteins were dialyzed to remove the excess sucrose and glycerol in the enzyme solution using a YM-10 ultrafiltration membrane (Millipore) and a pH 7.4 buffer containing 140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, and 1.8 mM potassium phosphate. After SDS-PAGE, the protein bands were either stained by Coomassie Blue R-250 or analyzed by Western blotting as described previously (Sheng et al., 1995).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. For protein analyses, 2 μg of the wild-type or mutant AST IV proteins was purified by polyacrylamide gel and digested with 7 ng of trypsin (Promega, Madison, WI) in 10 mM NH₂HCO₃, pH 8.0 for 18 to 20 h at 37°C. Samples were diluted 1:1 in 50% acetonitrile/0.1% (v/v) trifluoroacetic acid and air-dehydrated with α-cyano-4-hydroxy-cinnamic acid (matrix) before analysis. Mass determination was performed with a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA). All spectra were taken in the reflection positive ion mode. Spectra were calibrated using the external standards angiotensin II (1046.5 [M+H]+) and ACTH (2465.2 [M+H]+). The MALDI-TOF mass spectrometry data were acquired and analyzed using the Bruker XMass software.

Assay Procedures for the Wild-Type and Mutant AST IVs. The enzyme activity during the expression and purification process was monitored using a methylene blue assay procedure described previously (Sheng et al., 2001), with 2-naphthol as substrate at pH 5.5. For determination of kinetic constants, the reaction initial rates were performed in a 200-μl total volume containing 0.25 M potassium phosphate buffer (pH 7.0), 8.3 mM 2-mercaptoethanol, 0.2 mM PAPS, and various concentrations of the substrates dissolved in acetone (the final concentration of acetone in the assay was 5% v/v). Reactions were initiated by addition of enzyme (1.0–5.5 μg), incubated at 37°C for 15 min, and terminated by addition of 30 μl of methanol. Aliquots (1.5–3.0 μl) of enzyme solutions containing 0.7 to 4.5 mg/ml of wild-type or mutant AST IV were added to glass microtiter syringe to each assay mixture. Concentrations of enzyme in the stock solutions of AST IV and the mutant enzymes were determined by the

Preparation of Crude Cell Extract. The cell extract was centrifuged at 4°C for 30 min. The cells were then suspended in 15 ml of ice-cold buffer A [25 mM Tris HCl, pH 7.4, containing 0.25 M sucrose, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 1 mM dithiothreitol]. Cells were then disrupted at 4°C with a Sonifier cell disruptor model 350 (Branson Sonic Power Company, Danbury, CT). The sonicator was programmed to provide two periods of 90 s (2-s cycle pulses at 50% power output during each 90-s period) with 30 s of “off-time” between the periods. The supernatant fluid was collected after centrifugation at 24,000g for 30 min.

Prokaryotic Expression of AST IV. The bacterial expression vector PET-3c containing AST IV cDNA obtained from previous studies (Chen et al., 1992) was used as a template to generate mutant constructs. The mutant oligonucleotide primers (5'-CCGGTCTACCGGCTTTCGGTCA-3' for F77A, 5'-TCCTAT-TATAACGGCTTACACATGGCC-3' for F138A, and 5'-CATGACTAACGC-CACAACAATGC-3' for Y216A) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The mutations were performed with a Transformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA). In each case, the oligonucleotide primer was annealed to one strand of the wild-type template DNA along with another oligonucleotide (5'-TTT-GCTCAGCTGCCTTCTCAGG-3') to generate a mutant strand. Subsequently, synthesis and ligation were performed to link them. The DNA was then transformed into a recipient strain of E. coli (DH5α). When the culture reached an absorbance of 0.6 at 600 nm, the cells were harvested by centrifugation at 10,000g for 30 min. The cell pellets were stored at −70°C until used.

Affinity Purification of the Recombinant AST IV. Chromatography on PAP-agarose (Sigma-Aldrich) was used to purify the wild-type and mutant AST IV enzymes. The cell extract was charged onto a column of PAP-agarose (5 ml) that had been previously equilibrated with buffer A. The direction of flow for the column was reversed after the column was washed with 100 ml of buffer A. The column was then washed with an additional 30 ml of buffer A, and the recombinant protein was eluted from the column with 15 ml of buffer A containing 500 nM PAP. The effluent was then concentrated to approximately 2 ml by ultrafiltration (PM-10 membrane, Millipore Corporation, Billerica, MA). Excess PAP remaining in the enzyme solution was extracted by chromatography on a disposable PD-10 column (Amersham Biosciences, Inc., Piscataway, NJ) that was pre-equilibrated with buffer A. The PD-10 column was eluted with 10 ml of buffer A. The fractions containing the AST IV enzymes were collected, concentrated to about 1 ml, and stored at −70°C. The protein concentration was determined using a Bio-Rad protein assay as described by Lowry et al. (1951). Before SDS-PAGE, the purified proteins were dialyzed to remove the excess sucrose and glycerol in the enzyme solution using a YM-10 ultrafiltration membrane (Millipore) and a pH 7.4 buffer containing 140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, and 1.8 mM potassium phosphate. After SDS-PAGE, the protein bands were either stained by Coomassie Blue R-250 or analyzed by Western blotting as described previously (Chen et al., 1995).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. For protein analyses, 2 μg of the wild-type or mutant AST IV proteins was purified by polyacrylamide gel and digested with 7 ng of trypsin (Promega, Madison, WI) in 10 mM NH₂HCO₃, pH 8.0 for 18 to 20 h at 37°C. Samples were diluted 1:1 in 50% acetonitrile/0.1% (v/v) trifluoroacetic acid and air-dehydrated with α-cyano-4-hydroxy-cinnamic acid (matrix) before analysis. Mass determination was performed with a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA). All spectra were taken in the reflection positive ion mode. Spectra were calibrated using the external standards angiotensin II (1046.5 [M+H]+) and ACTH (2465.2 [M+H]+). The MALDI-TOF mass spectrometry data were acquired and analyzed using the Bruker XMass software.

Assay Procedures for the Wild-Type and Mutant AST IVs. The enzyme activity during the expression and purification process was monitored using a methylene blue assay procedure described previously (Sheng et al., 2001), with 2-naphthol as substrate at pH 5.5. For determination of kinetic constants, the reaction initial rates were performed in a 200-μl total volume containing 0.25 M potassium phosphate buffer (pH 7.0), 8.3 mM 2-mercaptoethanol, 0.2 mM PAPS, and various concentrations of the substrates dissolved in acetone (the final concentration of acetone in the assay was 5% v/v). Reactions were initiated by addition of enzyme (1.0–5.5 μg), incubated at 37°C for 15 min, and terminated by addition of 30 μl of methanol. Aliquots (1.5–3.0 μl) of enzyme solutions containing 0.7 to 4.5 mg/ml of wild-type or mutant AST IV were added to glass microtiter syringe to each assay mixture. Concentrations of enzyme in the stock solutions of AST IV and the mutant enzymes were determined by the
modified Lowry procedure (Bensadoun and Weinstein, 1976). The concentration of PAP formed in each reaction was determined by HPLC on an Econosphere C18 column (5 μ, 4.6 mm × 250 mm; Alltech Associates, Deerfield, IL). The mobile phase consisted of water/methanol (88:12, v/v) containing 65 mM potassium phosphate, 1.0 mM 1-octylamine, and 65 mM ammonium chloride, at pH of 5.45 before addition of methanol. A flow rate of 2 ml/min and detection at 259 nm were used for all determinations of PAP. The concentration of PAP formed in a reaction mixture was determined from the HPLC peak area using a linear standard curve relating peak area to the concentration of PAP. Results from control reaction mixtures (all components present except substrate) were subtracted from the concentrations of PAP formed in complete reaction mixtures to obtain the substrate-dependent formation of PAP.

Calculation of Kinetic Constants. At least six concentrations of each substrate were used, and these included concentrations both greater than and less than the apparent $K_m$ value. Apparent $K_m$ and $V_{max}$ values were obtained by nonlinear least-squares fitting of initial rate data to the equation: $V = V_{max} \cdot [S]/(K_m + [S])$. The apparent $K_m$ and $V_{max}$ values are presented as the mean ± the standard error. The molecular mass of AST IV calculated from the deduced amino acid sequence (33,909) was used for determination of $K_m$.

Molecular Modeling. Computational studies on 1,2,3,4-tetrahydro-1-naphthol and 2-naphthol in the active site of the rat AST IV were performed using the SYBYL molecular modeling package (SYBYL version 6.7; Tripos Inc., St. Louis, MO) running on a Silicon Graphics O2 workstation (SGI, Mountain View, CA). A previously developed homology model of AST IV (King et al., 2000) was used as the basis for all docking and energy minimization calculations. Coordinates for modeling an estradiol molecule at the sulfuryl acceptor site were obtained from the structure of a mouse estrogen sulfotransferase-17β-estradiol-PAP complex (PDB crystal structure code: 1AQK (Kakuta et al., 1997). The three-dimensional structures of 2-naphthol and each of the enantiomers of 1,2,3,4-tetrahydro-1-naphthol were created with the Build/Edit module of SYBYL and optimized by energy minimization using the Tripos force field. Correct atom types, bond types, and chiral centers were defined with respect to the SYBYL mol2 file format. The structure of each substrate was then prepositioned into the sulfuryl acceptor binding site by aligning the oxygen atom of the hydroxyl group with the position of the 3α oxygen of the 17β-estradiol that had been determined by homology with the crystal structure of mouse estrogen sulfotransferase. The docking experiments between substrate and protein were conducted manually using the dock module of SYBYL. Calculations of the total energy of each docked substrate/enzyme pair were performed using the SYBYL program at 10-degree intervals of rotation about the carbon-oxygen bond.

Results

Homology Modeling of AST IV. A homology model for AST IV has been previously developed (King et al., 2000) based on the electron density map of mouse estrogen sulfotransferase (Kakuta et al., 1997). Mouse estrogen sulfotransferase serves as a particularly good structure for homology modeling of AST IV due to the availability of crystal structures that include enzyme-substrate and enzyme-product complexes as well as the structure of an enzyme-PAP-vanadate complex that serves as a transition state model (Kakuta et al., 1998). Moreover, the coordinates of the mouse estrogen sulfotransferase have also been used as the model for determining structure factor phases by molecular replacement for crystal structures of other aryl/phenol sulfotransferases, including human phenol sulfotransferases SULT1A1 (Gamage et al., 2003) and SULT1A3 (Bidwell et al., 1999; Dajani et al., 1999), as well as human estrogen sulfotransferase SULT1E1 (Pedersen et al., 2002). The structure of (R)-(−)-1,2,3,4-tetrahydro-1-naphthol (Figs. 1 and 2) was docked into the homology model for AST IV with the oxygen placed in the position analogous to the oxygen of estradiol in the crystal structure of mouse estrogen sulfotransferase. Although the homology model revealed several amino acid residues in the vicinity of (R)-(−)-1,2,3,4-tetrahydro-1-naphthol, three amino acid residues, Phe77, Phe138, and Tyr236, were identified as occupying positions that could potentially provide steric interactions near the benzylic carbon and aromatic ring of the substrate that might be important to the stereospecificity of the enzyme with the enantiomers of 1,2,3,4-tetrahydro-1-naphthol (Fig. 2). Tyr236, for example, was identified as an interaction residue for the (R)-(−)-1,2,3,4-tetrahydro-1-naphthol enantiomer. The 3α oxygen of the enantiomer is located within 8 Å of Tyr236. The (R)-(−)-1,2,3,4-tetrahydro-1-naphthol enantiomer that had been determined by homology with the crystal structure of mouse estrogen sulfotransferase-17β-estradiol-PAP complex (PDB crystal structure code: 1AQK (Kakuta et al., 1997) was docked into the homology model of the active site of AST IV: and the proximity of the side chains of Phe77, Phe138, and Tyr236 to the position of (R)-(−)-1,2,3,4-tetrahydro-1-naphthol (Fig. 2).

Expression and Purification of the Wild-Type and Mutant AST IV Enzymes. We tested the hypothesis generated by the homology modeling studies described above by alanine substitution at residues Phe77, Phe138, and Tyr236. Three bacterial expression vectors for the mutant AST IV sequences, F77A, F138A, and Y236A, were constructed as described under Materials and Methods, and the wild-type and mutant sulfotransferases were overexpressed in E. coli BL21 (DE3) cells. SDS-PAGE and Western blot analysis of cell extracts revealed that the wild type and mutant exhibited identical mobilities and reactivity with antiserum to rat hepatic AST IV (Fig. 3).

Although a method for bacterial expression and purification of AST IV has been previously described (Chen et al., 1992), a procedure adaptable to expression and rapid purification of sufficient amounts of the three mutant AST IV proteins was required. Therefore, we developed a rapid purification procedure based on PAP-agarose affinity chromatography with subsequent separation of the excess PAP from the purified proteins by gel permeation chromatography. Purifications of wild-type and mutant AST IV proteins are summarized in Table 1. For wild-type AST IV, the yield of purified enzyme from this procedure was 23% based on enzyme units in the cell extract, and yields of the purified F77A, F138A, and Y236A enzymes from cell extracts were 15, 45, and 36%, respectively. Although most of the PAP in the eluting buffer was removed from the sulfotransferase by passing the protein solution through a PD-10 column, a small amount of the PAP (59 μM PAP in a 44 μM solution of AST IV) was not removed by this gel permeation method. This low concentration of PAP did not, however, interfere with the determination of reaction kinetics when the enzyme was diluted in the assay mixture in the presence of saturating concentrations of PAPS.

Characterization of the Wild-Type and Mutant AST IV Enzymes. As seen in Table 1, all three mutant enzymes catalyzed the sulfation of the standard phenol sulfotransferase substrate, 2-naphthol,
at pH 5.5, although the specific activities of the purified mutant enzymes were lower than that seen with the wild-type AST IV. Verification of the expressed protein sequences of these sulfotransferases was accomplished by tryptic digestion and analysis of the peptide fragments by MALDI-TOF mass spectrometry. Mass values of tryptic peptides were matched with the values for the expected sequences of the wild-type and mutant enzymes (11 peptides analyzed from wild-type AST IV represented 50% of the protein sequence; 15 peptides analyzed from F77A represented 77% of the protein sequence; 14 peptides analyzed from F138A represented 64% of the protein sequence; and 15 peptides analyzed from Y236A represented 50% of the protein sequence). The peptides incorporating the regions of site-directed mutation, and their corresponding masses are pre-

### TABLE 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Cell extract</th>
<th>F77A</th>
<th>F138A</th>
<th>Y236A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme units</td>
<td>39</td>
<td>0.6</td>
<td>0.6</td>
<td>3.7</td>
</tr>
<tr>
<td>(nmol/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAP-Agarose</td>
<td>382</td>
<td>6.7</td>
<td>31</td>
<td>134</td>
</tr>
<tr>
<td>(NIH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-10 gel filtration</td>
<td>515</td>
<td>13</td>
<td>40</td>
<td>348</td>
</tr>
</tbody>
</table>

(Enzyme units are expressed as nanomoles of 2-naphthylsulfate formed per minute, following assay with 250 μM 2-naphthol at pH 5.5.)

As a further investigation of the loss of catalytic efficiency as a function of removal of steric barriers at the active site of the enzyme, we examined the standard phenol sulfotransferase substrate, 2-naphthol, with the same computational techniques applied to our homology model of AST IV. As seen in Fig. 5, both Phe77 and Phe138 appear to provide barriers to rotation of 2-naphthol in the homology model. Removal of the phenyl ring in either F77A or F138A results in both decreased catalytic function and decreased barriers to substrate rotation (Table 1, Fig. 5). In contrast, Tyr236 does not appear to affect rotation of 2-naphthol in the active site model for the enzyme and has only a minimal effect on catalytic function.

### Discussion

The advent of crystallographic structural information on various sulfotransferases has provided opportunities for detailed study of relationships between enzyme structure and catalytic function. Whereas structural studies have been coupled with kinetic data to provide greatly increased understanding of the mechanistic aspects of sulfuryl group transfer in these enzymes, the relationships between sulfotransferase structure and molecular recognition of substrates and inhibitors have been somewhat more elusive. Nonetheless, combinations of crystal structures, homology modeling and other variations of computational biology, site-directed mutagenesis, and quantitative structure-activity relationships are beginning to yield important infor-

### TABLE 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tryptic Fragment</th>
<th>Calculated Mass</th>
<th>Observed Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>F77A</td>
<td>VPALF</td>
<td>803.0</td>
<td>803.2</td>
</tr>
<tr>
<td>(Wild-type)</td>
<td>(VPELEFK)</td>
<td>(879.1)</td>
<td>(879.4)</td>
</tr>
<tr>
<td>F138A</td>
<td>DVVVSYYNAYNMAK</td>
<td>1636.9</td>
<td>1636.7</td>
</tr>
<tr>
<td>(Wild-type)</td>
<td>(DVVSYYNAYNMAK)</td>
<td>(1713.0)</td>
<td>(1712.6)</td>
</tr>
<tr>
<td>Y236A</td>
<td>ENCMTNATTPTEDMDHNVSPFM</td>
<td>2756.2</td>
<td>2753.2</td>
</tr>
<tr>
<td>(Wild-type)</td>
<td>(ENCMTNTPTPTEDMDHNVSPFM)</td>
<td>(2845.3)</td>
<td>(2845.7)</td>
</tr>
</tbody>
</table>
mation on the sometimes subtle differences that dictate specificity of these enzymes.

One example of investigations where structural information has guided studies on relationships between sulfotransferase structure and substrate specificity involves the catalytic specificity of the mouse estrogen sulfotransferase (mEST or St1e5) for estradiol as opposed to dehydroepiandrosterone (DHEA), a substrate for hydroxysteroid sulfotransferase (Petrotchenko et al., 1999). A combination of crystallographic and mutagenesis techniques was used to determine that tyrosine-81 in mEST prevents efficient binding of dehydroepiandrosterone in the sulfuryl acceptor site of mEST due to steric interactions with C-19 methyl of DHEA (Petrotchenko et al., 1999). These studies showed that mutation of Tyr81 in mEST to a leucine rendered the enzyme capable of catalyzing sulfation of DHEA with kinetic constants similar to those of a wild-type hydroxysteroid sulfotransferase. Moreover, the results of these and further mutational studies on the mEST led to the proposal that Tyr81 and Phe142 in this enzyme form a gate-like structural feature that is important in discrimination between estradiol and DHEA (Petrotchenko et al., 1999).

Structural information has also been coupled with modeling and mutagenesis studies in the investigation of the differences in specificity for catecholamine substrates and 4-nitrophenol that are seen between the human aryl (phenol) sulfotransferase isoforms SULT1A1 (also known as ST1A3 or P-PST) and SULT1A3 (also known as

<table>
<thead>
<tr>
<th>AST IV</th>
<th>(R)-(−)-1,2,3,4-Tetrahydro-1-naphthol</th>
<th>(S)-(−)-1,2,3,4-Tetrahydro-1-naphthol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_{\text{m(app)}}$</td>
</tr>
<tr>
<td></td>
<td>nmol/min/mg</td>
<td>mM</td>
</tr>
<tr>
<td>Wild-type</td>
<td>38.0 ± 2.0</td>
<td>0.012 ± 0.003</td>
</tr>
<tr>
<td>F77A</td>
<td>6.0 ± 0.7</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>F138A</td>
<td>5.0 ± 0.4</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Y236A</td>
<td>5.0 ± 0.8</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

$V_{\text{max}}$, $K_{\text{m(app)}}$, and $k_{\text{cat}}/K_{\text{m}}$ values are derived from the homology models of the active site of wild-type and mutant AST IV enzymes with the docked substrates. Total energy of the system was calculated for each 10-degree rotation of the ligand around the C-O bond fixed as shown in Fig. 2. Log (kcal/mol) values greater than 9 are not shown. The wide dark lines on the horizontal axes mark regions where there are differences from the wild-type AST IV.

$^a$ The specific activities were determined with six substrate concentrations at pH 7.0 by the HPLC method for substrate-dependent PAP formation as described under Materials and Methods.

$^b$ The molecular mass for AST IV was 33,909 as calculated from the deduced amino acid sequence.

$^c$ Not detectable.
are implicated in the stereospecific sulfation of the D-isomers of DOPA and tyrosine (Pai et al., 2003). Additional investigations on SULT1A1 SULT1A3 that corresponds to an alanine in SULT1A1 (Dajani et al., 2000), and preliminary molecular docking experiments led to the three-dimensional structure of this phenol sulfotransferase (King et al., 1997, 1998). While holding the coordinates of those two atoms fixed in the model of the active site, the total energy of the system was calculated after each 10-degree rotation around the carbon-oxygen bond of the sulfuryl acceptor. This produced a map of interactions at the active site, with the changes due to site-directed mutagenesis showing up as a difference from the wild-type enzyme in calculated energy profile.

Comparison of the overall catalytic efficiencies of the wild-type and mutant enzymes with the relative profiles of interactions obtained from the models clearly shows that removal of aromatic side chains from F77A and F138A at the sulfuryl acceptor site (i.e., mutations of phenylalanine to alanine) results in lower catalytic efficiency for the enantiomeric 1,2,3,4-tetrahydronaphthol docked in the active site model. For this study, the hydroxyl group and adjacent carbon atom in the sulfuryl acceptor were positioned in the homology model by analogy to the crystal structures of catalytic complexes that were previously determined for 17β-estradiol and estrogen sulfotransferase (Kakuta et al., 1997, 1998). When holding the coordinates of those two atoms fixed in the model of the active site, the total energy of the system was calculated after each 10-degree rotation around the carbon-oxygen bond of the sulfuryl acceptor. This produced a map of interactions at the active site, with the changes due to site-directed mutagenesis showing up as a difference from the wild-type enzyme in calculated energy profile.

Comparison of the overall catalytic efficiencies of the wild-type and mutant enzymes with the relative profiles of interactions obtained from the models clearly shows that removal of aromatic side chains from F77A and F138A at the sulfuryl acceptor site (i.e., mutations of phenylalanine to alanine) results in lower catalytic efficiency for the enantiomeric 1,2,3,4-tetrahydronaphthols. Although one might interpret this as due to a lowered ability to facilitate the initial binding of

---

**Fig. 5.** Model computation of steric interactions of 2-naphthol with AST IV.

Values shown are derived from the homology models of the active sites of wild-type and mutant AST IV enzymes with the docked substrate. The total steric energy of enzyme and substrate was calculated for each 10-degree rotation around the C-O bond fixed as in Fig. 2. Log (kcal/mol) values greater than 9 are not shown. Highlighted regions mark differences from wild-type AST IV. Units of enzyme activity are expressed as nanomoles of 2-naphthyl sulfate formed per minute after assay with 250 μM 2-naphthol at pH 5.5.
the molecule in a catalytically competent enzyme-substrate complex, it may also be a result of the hindered conformational rotation of the molecule in the active site to ultimately reach an orientation of the molecule such that sulfuryl transfer may occur. Thus, removal of barriers either to initial binding or to subsequent conformational change of the substrate, as seen with the F77A and F138A mutants, leads to the ability of the enzyme to accept both enantiomers of 1,2,3,4-tetrahydronaphthol as substrates, but this comes at the expense of decreased catalytic efficiency.

Although these results on interactions at the active site of AST IV based on homology models and calculated relative energies are consistent with interpretations of the importance of active site geometry in substrate recognition, they clearly represent an early attempt at modeling these interactions. In addition to providing a basis for further experimental probes of specificity in aryl (phenol) sulfotransferases, they also serve as a starting point for the more extensive modeling studies necessary for exploration of the role of conformational changes by both substrate and enzyme in determining molecular specificity of the sulfotransferases. Indeed, since recent studies suggest that the human phenol sulfotransferase SULT1A1 may adopt structural conformations not seen in the crystal structure to accommodate estradiol as substrate (Gamage et al., 2003), it will be important to learn the extent to which the rat AST IV might exhibit similar conformational changes. Additionally, the possibility that these mutant forms of AST IV might actually facilitate binding and catalysis with larger substrates also remains to be explored.

References


