A Three-dimensional Model of Human Organic Anion Transporter 1

AROMATIC AMINO ACIDS REQUIRED FOR SUBSTRATE TRANSPORT

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Organic anion transporters (OATs) play a critical role in the handling of endogenous and exogenous organic anions by excretory and barrier tissues. Little is known about the OAT three-dimensional structure or substrate/protein interactions involved in transport. In this investigation, a theoretical three-dimensional model was generated for human OAT1 (hOAT1) based on fold recognition to the crystal structure of the glycerol 3-phosphate transporter (GlpT) from Escherichia coli. GlpT and hOAT1 share several sequence motifs as major facilitator superfamily members. The structural hOAT1 model shows that helices 5, 7, 8, 10, and 11 surround an electronegative putative active site (~830 Å^2). The site opens to the cytoplasm and is surrounded by three residues not previously examined for function (Tyr^{230} (domain 5) and Lys^{431} and Phe^{438} (domain 10)). Effects of these residues on p-aminohippurate (PAH) and cidofovir transport were assessed by point mutations in a Xenopus oocyte expression system. Membrane protein expression was severely limited for the Y230A mutant. For the K431A and F438A mutants, [^3H]PAH uptake was less than 30% of wild-type hOAT1 uptake after protein expression correction. Reduced V^{max} values for the F438A mutant confirmed lower protein expression. In addition, the F438A mutant exhibited an increased affinity for cidofovir but was not significantly different for PAH. Differences in handling of PAH and cidofovir were also observed for the Y230F mutant. Little uptake was determined for cidofovir, whereas PAH uptake was similar to wild-type hOAT1. Therefore, the hOAT1 structural model has identified two new residues, Tyr^{230} and Phe^{438}, which are important for substrate/protein interactions.

Members of the organic anion transporter (OAT)^2 family mediate transport of anionic drugs, toxins, and other xenobiotics across excretory and barrier tissues. In kidney, OATs are responsible for extracting potentially toxic endogenous and exogenous molecules from plasma and delivering them to the tubular lumen for excretion in urine. Organic anion transport in the kidney occurs via two steps: 1) anions are taken up by basolateral membrane transporters and 2) anions are then excreted into the tubular lumen by brush-border membrane transporters (1). Reabsorption may also occur at the luminal membrane with apical OATs. Cloning of OAT isoforms has advanced our knowledge of tissue distribution, energy dependence, function and regulation within the OAT family (2–5). For example, human OAT1 (hOAT1, SLC22A6) transports organic anions across the basolateral membrane into renal proximal tubule cells against an electrochemical gradient in exchange for intracellular dicarboxylates. The outwardly directed dicarboxylate gradient is maintained by the basolateral sodium-dicarboxylate cotransporter, which is indirectly dependent on an inward directed sodium gradient provided by sodium, potassium-ATPase (Na,K-ATPase) (4).

Substrate specificity studies have provided some insight into OAT structure. Experiments targeting the PAH transporter in rat kidney (later described as rOAT1) suggest ideal substrates are hydrophobic, may form hydrogen bonds, and have increased affinity with increased negative charge strength (6). Furthermore, for optimal transport, a distance of 6–7 Å should exist between the two carboxylate moieties (7, 8). In addition to the classical substrate p-aminohippurate (PAH), OAT1 substrates include nonsteroidal anti-inflammatory drugs, β-lactam antibiotics, N-acetyl-L-cysteine conjugates, antiviral drugs, diuretics, vitamins, hormones, and uremic toxins (5). Probencid, a uricosuric drug, used in the prevention of gout, inhibits OAT1 transport of many organic anions (5). The isoform OAT3 shares many of these same substrates and inhibitors in addition to transporting more bulky compounds (5). Thus, a molecular level structural model of OAT1 would greatly facilitate understanding substrate similarities and differences between these isoforms.

Like other membrane proteins, there is a lack of structural information on OATs. Membrane proteins are encoded by almost 30% of gene sequences (9), but only 89 unique crystal structures exist for these proteins. Emerging methods to overcome problems with protein crystallization include the development of computational models based on ab initio techniques (building the structure amino acid by amino acid), sequence homology, or fold assignment (10). If sequence homology is low...
(<50%), fold recognition offers a viable alternative for model building (10, 11). Threading, or fold recognition, predicts structure based upon two-dimensional fold recognition between the target (a protein of unknown structure) and the template (a protein of known structure).

OATs share many structural characteristics with other major facilitator superfamilies (MFS) proteins despite weak sequence similarities. In particular, OATs possess MFS hallmark domains including 12 transmembrane α-helices with cytosolic N and C termini, a long intracellular loop that connects the two 6-helix halves, a sequence length between 400 and 600 amino acids, and a RXXXR signature sequence conserved between loop 2–3 (12–14). X-ray crystal structures of two MFS proteins, the glycerol 3-phosphate transporter (GlpT, SLC37A2) (15) and the lactose permease (LacY) (16) of *Escherichia coli*, are available and provide templates for OAT structure. GlpT transports substrate as an antiporter similar to the hOATs, whereas LacY is a proton/sugar symporter. From these studies and comparison to transporters within the same family, transmembrane domains are assumed to form a binding site that is compatible with anionic features of substrates to facilitate translocation (17).

A fold recognition model of hOAT1 based on the structure of the GlpT is proposed here. Residues that line a putative active cavity and may have potential interactions with hOAT1 substrates are identified. Alanine scanning illustrated that two residues (Lys431, Phe438, domain 10) result in significant functional losses independent of membrane protein expression differences in a *Xenopus* oocyte expression system. Kinetic analysis on F438A and the conservative mutant Y230F exhibit distinctions between two hOAT1 substrates, PAH and cidofovir, a nucleoside phosphonate, in addition to confirming the loss of membrane protein expression of F438A. Therefore, the hOAT1 structural model offers the ability to perform hypothesis-driven research, testing substrate/protein interactions experimentally with point mutations, and refining the model by experimental outcomes.

**EXPERIMENTAL PROCEDURES**

**Computational Methods**

hOAT1 Template Identification and Model—Sequence accession number AB009698 for hOAT1 was submitted to the metaserver 3D-Jury (18) for protein template identification. Templates corresponding to GlpT (Protein Data Bank code 1pw4) (15) and LacY (1p6v chain A) (16) were identified. Shorter loops were present in template proteins; therefore the hOAT1 sequence was modified by deletion of residues Asn56–His130 in loop 1–2 and residues Glu300–Arg336 in loop 6–7. hOAT1 loop assignments were based on hydropathy analysis of primary sequences from PHDsec (19). Based on significant 3D-Jury (18) metaserver scores with consensus between fold recognition (yielding prediction accuracy of 90%) (18), GlpT was utilized as a structure template.

Structure models were calculated by alignment of the hOAT1 sequence with the template from 3D-Jury (18) followed by satisfaction of spatial constraints in MODELLER (10, 20, 21) software. Residues 1–5 and 227–239 in loop 6–7 of GlpT were not resolved in the crystal structure and therefore are replaced with gaps in the model structure. Hydrogens were added and the structures were energy minimized with the OLPS-2001 force field in MacroModel (22) software with the Maestro (Schrodinger, Inc., New York, NY) interface on a Dell Xenon work station with Fedora Core 2. Minimization parameters included: Polak-Ribiere Conjugate Gradient with a convergence threshold of 0.05 kJ/mol, normal cutoffs, water solvation, and a constant dielectric of 1.0.

**Structure Validation**—The energy minimized hOAT1 model was evaluated for structural errors and stereochemistry quality. Structure verification was determined by the UCLA Structure Analysis and Verification Server with the PROCHECK and ERRAT programs.3

**Electrostatic Potential and Solvent Accessibility Maps**—Partial atomic charges were obtained in DeepView/Swiss PDB Viewer (24) with the GRAMOS43A1 force field (25) at pH 7. The electrostatic potential map was calculated with the Poisson-Boltzmann method utilizing the following parameters: solvent dielectric constant of 80 and protein dielectric constant of 4. The electrostatic potential was then mapped to the molecular surface with a solvent probe radius of 1.4 Å. Solvent accessibility images were also generated with utilities in DeepView/Swiss PDB Viewer (24).

**Putative Active Sites of hOAT1 Model**—Active site(s) that lie within the central cavity of hOAT1 were predicted by protein void volumes with Putative Active Sites with Spheres (PASS) software (26). Positions favorable for binding sites were calculated based on size, shape, and burial extent of protein void volumes by the PASS program (26). The computational tool was executed on minimized protein models on a SGI Octane with IRIX 6.5 utilizing the “more” and “volumes” features.

**Experimental Methods**

**Chemicals**—[^H]PAH (3.47 or 4.18 Ci/mmol) was obtained from PerkinElmer Life Sciences. Probenecid and unlabeled PAH were purchased from Sigma. [^H]Cidofovir (15.0 or 28.0 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled cidofovir was a gift from Gilead Sciences (Foster City, CA). All other chemicals were of analytical grade and obtained commercially.

**Site-directed Mutagenesis and cRNA Synthesis**—Specific mutant amino acids were generated by site-directed mutagenesis using the QuikChange® Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotide sequences used in the mutagenesis procedure are shown in Table 1. Mutations were confirmed with automatic sequencing using the 3100 Genetic Analyzer and the BigDye® Terminator Kit (Applied Biosystems, Foster City, CA). Capped cRNA was synthesized from hOAT1 (pcDNA3.1 and pcDNA3.1/V5-His-Tope, Invitrogen) linearized plasmids using the T7 and T7 Ultra mMessage mMachine in vitro transcription kits (Ambion, Inc., Austin, TX). cRNA concentrations were determined by extinction coefficients.

**Xenopus Oocyte Uptake Assays**—Oocytes from *Xenopus laevis* (Xenopus One, Ann Arbor, MI) were isolated as previously described (28). One day after isolation, stage V and VI eggs were
selected and injected with 30–35 ng of hOAT1/pcDNA3.1 or hOAT1/pcDNA3.1.V5-His-Topo cRNA. Three days post-injection, 10 μM [3H]PAH (4 μCi/ml) or [3H]cidofovir (2–4 μCi/ml) were incubated with oocytes for 1 h at room temperature in oocyte ringer buffer (OR-2, containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM NaHPO₄, 3 mM NaOH, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM pyruvic acid, 5 mM HEPES, pH 7.6). The organic anion transport inhibitor probenecid (1 mM) was used to specify the mediated uptake component of hOAT1. Uptake was terminated by aspiration of substrate and washing oocytes (3 times) with ice-cold OR-2. Individual oocytes were placed in scintillation vials, lysed with 300 μl of 1 N NaOH, heated (65 °C) for 5–10 min, shaken for ~1 h, and then neutralized with an equivalent volume of 1 N HCl. Scintillation fluid (4.4 ml; Ecolume, ICN Biomedical, Costa Mesa, CA) was added to the vials and radioactivity was measured with a Packard Tri-Carb 1600TR (PerkinElmer Life Sciences) liquid scintillation counter with external quench correction. [3H]PAH or [3H]cidofovir uptake was calculated in pmol/oocyte/h from disintegrations per min/oocyte and medium specific activity. Uptake data were corrected for uptake in uninjected oocytes and were expressed as a percentage of control (wild-type hOAT1) uptake.

Xenopus Oocyte Membrane Preparation—Approximately 80 oocytes were injected as described above for each hOAT1 clone. Three days after injection, oocytes were rinsed with homogenization buffer (83 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, pH 7.9, with NaOH). Plasma membranes were prepared by homogenization and centrifugation as previously described with minor alterations (29). Protease inhibitors (Complete EDTA-free, Roche Applied Science) were added to the homogenization buffer. Homogenates were centrifuged at 1,000 × g at 4°C for 10 min (2 times) to separate the yolk fractions. The supernatant directly below the lipid layer was removed and the remaining lipid and pellet were discarded. The supernatant was transferred to new tubes and then centrifuged at 10,000 × g at 4°C for 20 min. Lipid and supernatant fractions were removed, leaving a pellet enriched with plasma membranes. The pellet was resuspended in homogenization buffer with protease inhibitors (0.5–1 μl/oocyte) and a microgrinder (Research Products International, Mt. Prospect, IL) was used to solubilize the pellet on ice. A Bradford assay (Bio-Rad) was completed to determine protein concentrations.

Western Blotting—The Invitrogen NuPage BisTris electrophoresis system was utilized for Western blotting. Wild-type hOAT1 and the three hOAT1 mutant oocyte membrane preparations (50 μg/lane) from two membrane preparations were electrophoresed on 4–12% NuPage BisTris gradient gels and transferred to Invitrolon polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with Starting Block phosphate-buffered saline T20 buffer (Pierce, Rockford, IL) for 1 h followed by overnight incubation with a 1:1000 dilution of primary monoclonal mouse antibody V5 (Invitrogen), 1:1000 dilution of primary monoclonal mouse penta-His antibody (Qiagen, Valencia, CA), or with a 1:1000 dilution of primary monoclonal mouse antibody to the Na,K-ATPase α1-subunit (Novus Biologicals Inc., Littleton, CO). Membranes were washed with phosphate-buffered saline/Tween and incubated with horseradish peroxidase-conjugated ImmunoPure secondary goat anti-mouse IgG (1:10,000; Pierce) for 1 h. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was utilized to detect antibodies. A Gel Doc 2000 gel documentation system (Bio-Rad) was utilized to detect proteins. Band densities from a total of 4 blots were quantified with the Image J program (version 1.34s).

Kinetics—Oocytes were injected with 35 ng of hOAT1/pcDNA3.1 for wild-type, Y230F and F438A clones for kinetic experiments. Three days post-injection ~10 oocytes were incubated in OR-2 at room temperature with varying concentrations of [3H]cidofovir (4–5 μCi/ml) or [3H]PAH (3.5–4 μCi/ml) for 20 min. Previously published experiments (30) indicated 20 min fell within the linear range of hOAT1 transport. Diffusion components were assessed by determining the amount of substrate uptake in the presence of 1 mM probenecid at 10 and 200 μM cidofovir and 2.5 and 150 μM PAH. The transporter-mediated component was determined by subtracting the diffusion component from the total uptake for cidofovir and PAH, respectively. Radioactivity was measured as described under “Uptake Assay.” Kinetic data were determined from Lineweaver-Burk plots. Kₘ and Vₘₐₓ values tabulated in Table 3 are means from at least four independent experiments.

Statistics—Percent of control uptake data was calculated from mean ± S.E. p values were calculated with the paired, two-tailed Student’s t test in OriginPro 7.5 (Origin Lab, Northampton, MA).

RESULTS

Identification of an hOAT1 Model—GlpT was used as a template for the hOAT1 model. Fold recognition software (see “Experimental Materials”) demonstrates similar two- and three-dimensional folds between GlpT and hOAT1 despite possessing low sequence identity (14%). The hOAT1 model open in the cytoplasmic conformation is illustrated in Fig. 1A. The two-dimensional hydropathy model is shown for comparison (Fig. 1B). As in GlpT, HOAT1 contains 12 transmembrane domains arranged with 2-fold symmetry to form a central cavity. From the crystal structure of GlpT, the medium gray helices are believed to provide support, whereas the black and light gray helices line the internal cavity. The model contains residues within short loops but lacks residues within the extended loops present between transmembrane domains 1 and 2 and 6 and 7 (see “Experimental Design” and Fig. 2 for details). Fig. 2 depicts the structural alignment and location of conserved motifs between GlpT and hOAT1.
**Model Validation**—Location of conserved structural motifs provides a means to assess model structure. Primary structure analysis indicates OAT1 shares at least five motifs (with some amino acid alterations) with GlpT and the MFS family. The location of conserved motifs \( \{G(X_1)_X(D/R/K)13\} \) and \( \{X_1)_X(D/R/K)13\} \) are thought to provide support for the transporter.

**Model Evaluation**—Location of conserved structural motifs provides a means to assess model structure. Primary structure analysis indicates OAT1 shares at least five motifs (with some amino acid alterations) with GlpT and the MFS family. The location of conserved motifs \( \{G(X_1)_X(D/R/K)13\} \) and \( \{X_1)_X(D/R/K)13\} \) are thought to provide support for the transporter.

**Structural Analysis and Verification Server for structure verification (24).** Evaluation of the hOAT1 structure shows the membrane protein exists largely as a non-solvent accessible structure. This suggests that the binding site is a hydrophobic lipid bilayer surrounded by basic amino acids. This is concurrent with the presence of 41 positive amino acids (Lys, Arg) for hOAT1. A large positive charge surrounds the center cavity of hOAT1 suggesting the possibility of a binding site for anionic substrates. Acidic amino acids are present in the C-terminal as shown in the molecular surface map. In addition, solvent accessibility images were generated with utilities in DeepView/Swiss PDB Viewer (24). Evaluation of the hOAT1 structure shows the membrane protein exists largely as a non-solvent accessible structure. This property corresponds to helices that span a hydrophobic lipid bilayer.

**Putative Active Site within hOAT1**—The putative active site for hOAT1 was predicted by protein void volumes with PASS software and is located in the cavity formed by the 12 transmembrane domains (26). The active site has a volume of 830 Å³, as depicted in Fig. 3C. Arg⁶¹⁶ sits at the opening of the pocket, whereas aromatic amino acids such as tryptophan and phenylalanine surround the site. Many of these residues have been previously confirmed to affect substrate uptake and are described under “Discussion.” In addition, residues Tyr2³⁰⁰ (domain 5), Lys⁴³¹ (domain 10), and Phe⁴³⁸ (domain 10) provide new aromatic and basic residues to test for model validation and potential impact on substrate transport. Tyr²³⁰ and Phe⁴³⁸ are within ~7 Å of the center of the binding site, whereas Lys⁴³¹ sits just below the site (<12 Å from the center). Given the location of Lys⁴³¹, it may be important in anionic substrate recognition from the extracellular side.

The active site of the crystalized structure of apo (no substrate present) GlpT was also calculated as a control. The putative active site was identified within the central cavity with a volume of 915 Å³. Amino acids within this region have been shown experimentally to affect substrate binding of GlpT (15).

**Effect of Alanine Mutations in hOAT1 on \[^3H\]PAH Transport**—Transport of 10 μM \[^3H\]PAH in oocytes expressing hOAT1 and hOAT1 mutants (Y230A, K431A, F438A) is shown in Fig. 4A as percent control of wild-type hOAT1 values. Uptake was probenecid sensitive, suggesting hOAT1-mediated transport of PAH. In addition, significant reduction (>70%) of \[^3H\]PAH uptake was apparent in every mutant, suggesting the importance of these residues in substrate transport. However, differences in membrane expression could account
for differences in the observed uptake data. To assess this possibility, hOAT1 and hOAT1 mutants (Y230A, K431A, F438A) with a V5/His tag were expressed in Xenopus oocytes. Following preparation of oocyte membranes, membrane proteins were electrophoresed and detected by Western blotting with a V5 antibody. Fig. 4B shows a band for wild-type hOAT1 at ~75 kDa. No staining was evident in the un.injected oocytes. Bands for hOAT1 could also be detected with a penta-His antibody (data not shown). The blot in Fig. 4B indicates little to no membrane expression of Y230A exists. However, protein expression for K431A and F438A was detected, quantified, and compared with that of wild-type hOAT1. To determine whether variations in membrane preparation influenced mutant detection, we re-probed the blots with an antibody for Na,K-ATPase, a plasma membrane marker. No difference in the levels of Na,K-ATPase was observed, confirming that the preparation method had no effect on detection of mutant expression quantification.

We have previously seen that the wild-type hOAT1 V5/His tag construct results in reduced expression levels in oocytes when compared with the untagged wild-type hOAT1. Therefore, we repeated 10 μM [3H]PAH uptake experiments in the presence of the V5/His tag and noted that uptake of 10 μM [3H]PAH was 27 pmol less than that of untagged hOAT1. The hOAT1 V5/His tag uptake data were normalized to account for differences present in membrane expression. As shown in Fig. 4C, the effect of each mutation on 10 μM [3H]PAH uptake was significant. Uptake values for K431A and F438A were less than 30% of wild-type hOAT1 uptake values, indicating that the residues are required for normal hOAT1 function.

**Effect of More Conservative hOAT1 Mutations on Substrate Transport**—Alanine scanning suggested Tyr230, Lys431, and Phe438 might be important in hOAT1 function. Reduced membrane expression accounted for a portion of the loss of transport function, but changes in substrate affinity might also explain the reductions in PAH uptake levels. Unfortunately, kinetic analysis of Y230A and K431A mutants was not possible given the low uptake levels in oocytes (<1 pmol). Therefore, more conservative mutations (Y230F, K431R, and F438Y) were made. Fig. 5A depicts uptake of 10 μM [3H]PAH values as percent control of wild-type hOAT1 uptake. Levels of PAH uptake in K431R and F438Y expressing oocytes were significantly lower (<70%) than that of wild-type hOAT1, similar to alanine mutations at those residues. However, Y230F exhibited no significant difference in 10 μM [3H]PAH uptake when compared with wild-type hOAT1. For all mutants, PAH uptake was sensitive to probenecid.

Uptake of 10 μM [3H]PAH in hOAT1 mutants Y230F and F438A was large enough to attempt kinetic analysis. However, prior to kinetic experiments, uptake of 10 μM [3H]cidofovir was determined for all hOAT1 mutants (Y230A, Y230F, K431A, K431R, F438A, F438Y). Cidofovir is an hOAT1 substrate with a Km that differs from PAH by an order of magnitude (30). Fig. 5B illustrates uptake of cidofovir in oocytes expressing hOAT1 mutants as percent control of wild-type hOAT1 uptake. All hOAT1 mutants examined had significantly less uptake than wild-type hOAT1 (<70%). In addition, cidofovir uptake values were sensitive to pro-

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**TABLE 2**

| Protein | ERRAT score | Ramachandran plot (ProCheck) |
|---------|-------------|-----------------------------|
|         |             | Core | Disallowed | General |
| GlpT*   | 85.2        | 85.4 | 13.5       | 1.1      |
| hOAT1   | 91.7        | 90.9 | 7.7        | 0.6      |

* Not minimized.

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**FIGURE 3.** A, electrostatic potential map of the hOAT1 model. Two perspectives are shown, the first image depicts a horizontal view of the protein and the second image looks down on the cavity between the helices from the cytoplasm. Blue shading indicates large positive areas, whereas red shading indicates large negative regions. B, solvent accessibility for the hOAT1 model. Blue depicts completely buried residues, whereas orange and yellow residues (mostly in loops) have greater than 50% of their surfaces accessible. C, putative active site of hOAT1 surrounded by helices 5, 7, 10, and 11. The inset depicts residues examined within this study along with residues 9 Å from the center of the predicted binding cavity for hOAT1. Basic, Lys431, Arg466 (red); polar, Ser462 (orange); aromatic, Tyr230, Phe438 (green); aliphatic, Leu22, Met1, Ala32, Met207, Ile226, Gly227, Val229, Ala465 (yellow). Images were generated in Accelrys DS ViewerLite 5.0 (27).

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**FIGURE 4.** A, Western blot showing a band for wild-type hOAT1 at ~75 kDa. B, the blots with an antibody for Na,K-ATPase, a plasma membrane preparation influenced mutant detection, we re-probed wild-type hOAT1. To determine whether variations in membrane preparation influenced mutant detection, we re-probed the blots with an antibody for Na,K-ATPase, a plasma membrane marker. No difference in the levels of Na,K-ATPase was observed, confirming that the preparation method had no effect on detection of mutant expression quantification.

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**FIGURE 5.** A, the effect of each mutation on 10 μM [3H]PAH uptake was significant. Uptake values for K431A and F438A were less than 30% of wild-type hOAT1 uptake values, indicating that the residues are required for normal hOAT1 function.

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**FIGURE 6.** A, the effect of each mutation on 10 μM [3H]PAH uptake was significant. Uptake values for K431A and F438A were less than 30% of wild-type hOAT1 uptake values, indicating that the residues are required for normal hOAT1 function.

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**FIGURE 7.** A, the effect of each mutation on 10 μM [3H]PAH uptake was significant. Uptake values for K431A and F438A were less than 30% of wild-type hOAT1 uptake values, indicating that the residues are required for normal hOAT1 function.

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**FIGURE 8.** A, the effect of each mutation on 10 μM [3H]PAH uptake was significant. Uptake values for K431A and F438A were less than 30% of wild-type hOAT1 uptake values, indicating that the residues are required for normal hOAT1 function.

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**FIGURE 9.** A, the effect of each mutation on 10 μM [3H]PAH uptake was significant. Uptake values for K431A and F438A were less than 30% of wild-type hOAT1 uptake values, indicating that the residues are required for normal hOAT1 function.
FIGURE 4. A, oocytes expressing hOAT1 and hOAT1 mutants were incubated with OR-2 buffer containing 10 μM [3H]PAH in the absence (gray) or presence (black) of 1 mM probenecid for 1 h. The results are expressed as a mean percentage of control from four separate experiments ± S.E. Mean control PAH was 42.2 ± 5.2 pmol/oocyte/h, respectively. **, p < 0.01; ***, p < 0.001). B, representative blot from plasma membrane fractions of oocytes expressing V5/His-tagged wild-type hOAT1 mutants were prepared to quantify protein expression with a V5 antibody. Expression was not present for the Y230A mutant, and was reduced in the K431A and F438A mutants. Normalized band densities from four independent experiments were hOAT1 wild-type, 1; Y230A, 0.03 ± 0.01; K431A, 0.25 ± 0.04; F438A, 0.06 ± 0.03. Staining with the Na,K-ATPase α1-subunit antibody (112 kDa band) indicates the oocyte membrane preparations did not affect expression levels. C, oocytes expressing V5/His-tagged hOAT1 and hOAT1 mutants were incubated with OR-2 buffer containing 10 μM [3H]PAH in the absence (gray) or presence (black) of 1 mM probenecid for 1 h. The results are normalized for protein expression (normalization factors: Y230A (32); K431A (4); F438A (15)) and are expressed as a mean percentage of control from three separate uptake experiments ± S.E. Mean control PAH was 14.9 ± 2.5 pmol/oocyte/h, respectively. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 5. Oocytes expressing hOAT1 and various hOAT1 mutants were incubated with OR-2 buffer containing 10 μM [3H]PAH (A) or 10 μM [3H]cidofovir (B) in the absence (gray) or presence (black) of 1 mM probenecid for 1 h. The results are expressed as a mean percentage of control from at least three separate uptake experiments ± S.E. Mean control PAH was 33.01 pmol ± 7.83 pmol/oocyte/h and mean control cidofovir was 11.62 ± 2.82 pmol/oocyte/h. **, p < 0.01; ***, p < 0.001.
TABLE 3  
Mean $K_m$ ($\mu M$) values and $V_{\text{max}}$ (pmol/oocyte/20 min) for hOAT1 substrates

|          | PAH    | Cidofovir |
|----------|--------|-----------|
| **hOAT1** |        |           |
| $K_m$    | 6.5 ± 1.45 | 63.36 ± 10.41 |
| $V_{\text{max}}$ | 8.25 ± 1.35 | 14.49 ± 1.57 |
| **hOAT1 Y230F** | 4.70 ± 0.78 | 7.26 ± 1.13 |
| **hOAT1 F438A** | 3.69 ± 1.30 | 1.26 ± 0.35 |

*NA, not applicable.

Note: Significantly different at $p < 0.01$.

**DISCUSSION**

Crystal structures of GlpT and LacY have prompted the models of several MFS transporters. For example, a structure of the rat organic cation transporter 1 (rOCT1) is based on LacY, whereas models of the rabbit organic cation transporter 2 (rbOCT2) and the glucose transporter 1 (glut1) are formed from GlpT (34–36). Whereas none of these model structures fully explain substrate specificity and conformational changes (all are positioned with the binding cavity open to the cytoplasm and the central cavity may be expanded due to truncation of the long internal loop between domains 6 and 7), they do provide structural details not previously available. The structural information yields the prospect of designing experiments and developing more predictive models. The rOCT1 model depicts a large binding cleft that is accessible from either side of the plasma membrane that may be capable of binding more than one ligand (36). Both the rOCT1 and rbOCT2 models share many of the same substrate interacting residues that are directed toward the central binding cavity. Furthermore, the models demonstrate selectivity differences between OCT1 and OCT2. Selectivity is dependent on a glutamate residue (Glu$^{447}$) that resides in the binding cavity of OCT2 and is replaced by an uncharged glutamine residue (Gln$^{448}$) in rOCT1 (35).

Similar to the OCT models, the hOAT1 model provides a framework to project data collected from substrate affinities and substrate inhibitors and from the consequences of various mutations. Support for the hOAT1 model originates from overlap of position with MFS transporters, along with descriptive data including residues solvent accessibility and electrostatics. Structural comparisons to the OCT models provide additional confidence in the hOAT1 model. All models exhibit a potential binding cavity located within the center of the 12-transmembrane domains that are arranged with 2-fold symmetry. Superimposing the rbOCT2 and hOAT1 models shows similar structural alignment as the backbone atoms show a root mean square deviation less than 2 Å. Deviations occur within N- and C-terminal regions, in several loop locations, and in domain 2. These deviations likely result from slight differences in alignment of the long extracellular loop and differences in the energy minimization procedures. The rbOCT2 model also takes into account that residue Asn$^{39}$ is a glycosylation site in loop 1–2 and not present in domain 1 as based on the GlpT template (35, 37). In the hOAT1 model, the Asp$^{39}$ was left in domain 1 as it did not affect the remaining alignment. More information is obtained from the sequence alignment of the models. A large number of prolines and glycines were conserved between template and model proteins, indicative of helix formation and potential helix bends. In addition, residues surrounding the putative active cavity of hOAT1 align with residues present in the center cavity of the OCT1 and OCT2 models. A sequence alignment of the models in Fig. 6 shows residues that surround the putative active cavity of hOAT1 (Met$^{207}$, Phe$^{438}$, and Arg$^{466}$) correspond to OCT1/OCT2 residues (Tyr$^{222}$/Tyr$^{221}$, Leu$^{447}$/Tyr$^{446}$, Asp$^{477}$/Asp$^{474}$). Site-directed mutagenesis at these locations (except OCT2 residue Tyr$^{446}$) was experimentally shown to contribute to OCT substrate interactions (36, 38). Although these residues surround the putative binding sites in all models, their location in the site may differ. Differences between the alignment of loops and transmembrane domains generate shifts in the location of the residue within the transmembrane domain. For example, residues Asp$^{466}$/Asp$^{477}$/Asp$^{474}$ of hOAT1/rOCT1/rbOCT2 remain in the middle of transmembrane domain 11 for all models. The location of residues Met$^{207}$/Tyr$^{222}$/Tyr$^{221}$ varies from residing close to the extracellular region of hOAT1 to residing near the middle of transmembrane domain 4 in the OCT models.

OAT structural information is also derived from uptake experiments in point mutated sequences performed on four OAT isoforms. Fig. 7 depicts at least 43 residues that have been mutated in OAT transmembrane domains (39–45). Eleven residues from the different isoforms (shown in squares) have been demonstrated to affect substrate or inhibitor interaction with the transporter and seven of those residues are within 10 Å of the center of the putative active site of the hOAT1 model. Eight residues that have been mutated have had no effect on substrate transport or surface expression. None of these residues are near the putative active site for the hOAT1 model. Mutations at 25 additional residues resulted in reduced surface expression (depicted in circles) and only residues Leu$^{24}$, Met$^{27}$, Ala$^{28}$, Met$^{31}$, and Ala$^{32}$ (domain 1) were of close proximity to the
At least 16 additional mutations have been examined in loop regions (primarily loop 1–2) of the OATs, but do not provide insight into the hOAT1 structural model (23, 30, 37, 41, 45, 46).

Furthermore, a model of the substrate recognition site of rat OAT3 (rOAT3) and mutational experiments in the flounder OAT (fOAT) provide support for the hOAT1 structural model (39, 42). Mutations in conserved residues between rOAT3 and fOAT correspond to hOAT1 residues Lys382 (domain 8) and Arg466 (domain 11). Uptake of organic anions was severely reduced in these mutants, demonstrating the importance of basic amino acids in charge recognition and substrate translocation. The rOAT3 substrate recognition site model provides evidence for different binding domains within the same binding site. Although the basic amino acids were important in transport of negatively charged substrates, five aromatic amino acids were required for transport of hydrophilic substrates such as cimetidine or PAH. In addition, only four of the six aromatic residues conserved between rOAT3 and hOAT1 surround the hOAT1 binding site (43). This suggests possible structural differences in the binding site of OAT1 and OAT3 that may impact substrate specificity.

The model of hOAT1 structure prompted Xenopus oocyte uptake studies with additional hOAT1 mutants to assist with understanding substrate-protein interactions and to yield more support to the model. Based upon the hOAT1 putative active site, residues including Tyr230 (domain 5), Lys 431 (domain 10), and Phe438 (domain 10) should impact the translocation of OAT substrates. The mutagenesis experiments suggested that all residues had an impact on hOAT1 function. Initial experiments with Y230A indicated a lack of membrane protein expression; however, function was retained for PAH when the tyrosine was mutated to phenylalanine (Y230F). As no changes in protein expression were present (implied by constant $V_{max}$), the data suggest that an aromatic amino acid is necessary for PAH transport at residue 230. Surprisingly, uptake values for the additional hOAT1 substrate cidofovir were not different between Y230A and Y230F. Because the lack of uptake does not seem to be dependent on the aromatic ring,
one may conclude that the hydroxyl group of tyrosine may be important for interaction with cidofovir. Given the minimal transport of PAH and cidofovir by Lys431 mutants (K431A and K431R) no assessment on the differences in substrate affinity was possible. Thus, the importance of Lys431 in hOAT1 function remains unclear. Membrane protein expression of K431A is reduced, but the reduction does not fully explain decreases in PAH uptake. Finally, the mutations at F438A appear to affect substrate affinity for cidofovir and membrane protein expression levels as indicated by $V_{\text{max}}$ levels for both PAH and cidofovir. $K_m$ values decreased for both PAH and cidofovir in the F438A mutant. For the F438A mutant, the loss of the bulky phenylalanine could allow for more favorable interaction with PAH and cidofovir.

The presence of a single binding cavity with multiple binding domains in hOAT1 is advantageous for interaction with xenobiotics that possess varied structures. However, such a diverse site makes it difficult to identify specific recognition sites and to understand the need for multiple transporters with overlapping substrates. The development of a model of hOAT1 based upon the crystal structure of GlpT in this work gives us an opportunity to explore how binding of different substrates may occur. We have identified several structural characteristics shared between the hOAT1 and OCT models. In addition, the putative binding site.

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