# Role of Aromatic Transmembrane Residues of the Organic Anion Transporter, rOAT3, in Substrate Recognition†

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ABSTRACT: Organic anion transporters (OATs, SLC21) are important in the excretion of endogenous and exogenous compounds in the kidney. The rat organic anion transporter, rOAT3, mediates the transport of organic anions such as *p*-aminohippurate (PAH) and estrone sulfate as well as the basic compound, cimetidine. In the present study, we examined the role of conserved transmembrane aromatic amino acid residues of rOAT3 in substrate recognition and transport. Alanine scanning followed by amino acid replacements was used to construct mutants of rOAT3. The uptake of model compounds was studied in *Xenopus laevis* oocytes expressing the mutant transporters. We observed that four mutants in transmembrane domain 7 (TMD 7), W334A, F335A, Y341A, and Y342Q, and one mutant in transmembrane domain 8 (TMD 8), F362S, exhibited a less than 2-fold enhanced uptake of PAH and cimetidine in comparison to wild-type rOAT3, which exhibited a 16-fold enhanced uptake of PAH and an 8-fold enhanced uptake of cimetidine. Estrone sulfate uptake in oocytes expressing any one of these five mutants remained at least 8-fold enhanced. The data suggest that the five residues, W334, F335, Y341, Y342, and F362, contribute differently to the transport of the small hydrophilic organic substrates PAH and cimetidine in comparison to the large hydrophobic organic substrate estrone sulfate. The effects of side chains of these five residues on transporter functions were also evaluated by constructing conservative mutations. We observed that the residues contribute to PAH and cimetidine transport in different ways: the −OH group of Y342, the indole ring of W334, and the aromatic rings of F335, Y341, and F362 are important for PAH and cimetidine transport by rOAT3. These data suggest that there is an aromatic pocket composed mainly of residues in TMD 7 in the translocation pathway of rOAT3, which is important for the transport of PAH and cimetidine. Aromatic residues in this pocket may interact directly with substrates of rOAT3 through hydrogen bonds and $\pi-\pi$ interactions.

Organic anion transporters (OATs, SLC21)[1] in the kidney play important roles in the excretion of a variety of foreign and endogenous substances (*1*, *2*). In particular, OAT1 and OAT3 are present in abundance in the kidney and are thought to be the primary transporters responsible for the uptake of many organic anions from the blood into the renal tubule epithelium (*3*). Although both rOAT1 and rOAT3 transport small molecular weight, hydrophilic organic anions such as *p*-aminohippurate (PAH), rOAT3 also transports more hydrophobic organic anions (e.g., estrone sulfate) and the organic cation cimetidine (*3*, *4*). Unlike rOAT1, rOAT3 does not appear to be an exchanger.

A key question in the study of organic anion transporters is "What are the structural determinants of specificity?" This question is particularly interesting in the study of xenobiotic transporters because these proteins are promiscuous, interacting with a multitude of structurally diverse molecules. rOAT3 is of special interest because it is capable of transporting a range of substrates, including both organic cations and anions, and substrates of diverse structures and hydrophobicities (*4*). The key structural elements of substrates of organic anion transporters are (a) the charge and (b) the organic moiety, which depending upon its structure is capable of forming hydrogen bonds or hydrophobic interactions. Thus, one can hypothesize that there are distinct amino acid residues in rOAT3 responsible for recognition of charge, which interact electrostatically with the substrate, and residues that are capable of interacting with the organic moiety. These residues may form hydrogen bonds, $\pi-\pi$ interactions, or hydrophobic interactions with the substrates.

In previous studies from our laboratory, we focused on the role of conserved basic amino acids in the charge specificity of rOAT3 (*5*). We determined that arginine 454 and lysine 370 are critical in the anion specificity of the transporter. That is, mutations of each of these residues resulted in transporters that lost the specificity for the organic anions PAH and estrone sulfate. The double mutant R454DK370A exhibited a reversal in its charge specificity. That is, the mutant transporter preferred the organic cation,

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[1] Abbreviations: $K_m$, Michaelis−Menten constant; $MPP^+$, 1-methyl-4-phenylpyridinium; OAT, organic anion transporter; OCT, organic cation transporter; PAH, *p*-aminohippuric acid; TMD, transmembrane domain; $V_{max}$, maximal uptake rate.

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$MPP^+$, to the organic anion, PAH. Recent studies also support that cationic amino acids are involved in the substrate specificity of a flounder OAT (*6*).

In this study, we investigated the role of conserved aromatic amino acid residues in maintaining substrate specificity of rOAT3. Our hypothesis was that the conserved aromatic amino acid residues in rOAT3 are essential for the interaction of the transporter with the organic moieties of its substrates. By studying the interactions of three substrates of rOAT3, PAH, cimetidine, and estrone sulfate, which differ in terms of charge, size, hydrophobicity, and the ability to form hydrogen bonds, we identified residues that are important in the interaction of the smaller, more hydrophilic compounds cimetidine and PAH. These residues are distinct from arginine 454 and lysine 370 in rOAT3 which recognize the charged anionic moiety of the substrates. Our results together with results from our previous studies suggest that multiple amino acid residues in rOAT3 contribute distinctly to charge specificity and specificity for the organic moiety of the diverse substrates of rOAT3.

## EXPERIMENTAL PROCEDURES

*Site-Directed Mutagenesis of the rOAT3 cDNA.* The cDNA of wild-type rOAT3 (GenBank accession number AB017446) was amplified by RT-PCR using an oligo (dT) primer (Gibcol-BRL, Gaithersburg, MD) with total RNA isolated from rat brain (Clontech, Palo Alto, CA).

The Stratagene Quikchange site-directed mutagenesis kit (La Jolla, CA) was used to construct mutant cDNA, following the manufacturer's protocols. Mutants with single amino acid substitution [Y230A (tyrosine 230 to alanine), W254A (tryptophan 254 to alanine), W255A (tryptophan 255 to alanine), F328A (phenylalanine 328 to alanine), W334A (tryptophan 334 to alanine), W334F (tryptophan 334 to phenylalanine), F335A (phenylalanine 335 to alanine), F335Y (phenylalanine 335 to tyrosine), F339V (phenylalanine 339 to valine), Y341A (tyrosine 341 to alanine), Y341F (tyrosine 341 to phenylalanine), Y342Q (tyrosine 342 to glutamine), Y342F (tyrosine 342 to phenylalanine), F362S (phenylalanine 362 to serine), F362A (phenylalanine 362 to alanine), and F478A (phenylalanine 478 to alanine)] were prepared using the cDNA of wild-type rOAT3 as the template. The sequences of all mutants were confirmed by directed DNA sequencing.

*cRNA Transcription and Expression in Xenopus laevis Oocytes.* Oocytes were harvested from oocyte positive *Xenopus laevis* (Nasco, Fort Atkinson, WI) and were dissected and treated with collagenase D (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in a calcium-free ORII solution, as previously described (*7*). Oocytes were maintained at 18 °C in modified Barth's medium. Healthy stage V and VI oocytes were injected with capped cRNA (1 $\mu$g/$\mu$L) that was transcribed in vitro with T3 polymerase (mCAP RNA Capping kit; Stratagene) from Not I linearized plasmids containing transporter cDNA inserts.

*Tracer Uptake Measurements.* Transport of radiolabeled compounds in oocytes was measured 3−4 days after cRNA injection, as described previously (*7*). The compounds were used at the following concentrations: $^3$H-cimetidine (1 $\mu$M) (15 Ci/mmol), $^3$H-PAH (10 $\mu$M) (4 Ci/mmol), and $^3$H-estrone sulfate (150 nM) (53 Ci/mmol). Uptake experiments were carried out as follows: groups of seven to nine oocytes were incubated in 100 $\mu$L $Na^+$ buffer (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES/Tris (pH 7.2)) containing a radiolabeled compound at 25 °C for 1 h. Uptake was stopped by washing the oocytes 5 times with 3 mL of ice-cold $Na^+$ buffer. The oocytes were then lysed with 100 $\mu$L 10% sodium dodecyl sulfate (SDS) individually, and the amount of radiolabeled substrates associated with each oocyte was determined by liquid scintillation counting. For inhibition studies, unlabeled compounds (1 mM) were added to the reaction solutions as needed.

*Data Analysis.* Values are expressed as mean ± standard error (SE) or mean ± standard deviation (SD) as indicated in the legends. Six to nine oocytes were used to generate a data point in each experiment. Because of the intrinsic variability in the expression levels of the transporters between batches of oocytes, the data shown in the figures are generally from a representative experiment performed in the same batch of oocytes. However, replicate experiments performed using oocytes from different donor frogs produced qualitatively similar results. The kinetic parameters (apparent $K_m$ and $V_{max}$) were determined by nonlinear least-squares fits of substrate/velocity profiles to the Michaelis−Menten equation using Kaleidagraph (version 3.0, Synergy Software). Statistical analysis of the data was carried out by the unpaired *t* test where $P < 0.05$ was considered significant.

*Materials.* All unlabeled compounds (inhibitors) and buffer components were purchased from Sigma (St. Louis, MO). Radiolabeled compounds were from the following suppliers: $^3$H-cimetidine (15 Ci/mmol) (Amersham Life Sciences, Arlington Heights, IL), $^3$H-PAH (4 Ci/mmol), and $^3$H-estrone sulfate (53 Ci/mmol) (NEN Life Science Products, Boston, MA). Oligonucleotide primers were synthesized by Gibco-BRL (Gaithersburg, MD).

## RESULTS

*Alanine Scanning of the Conserved Aromatic Transmembrane Residues in rOAT3.* To probe the contribution of the conserved aromatic residues to the formation of a possible substrate binding site of rOAT3, we systematically mutated each conserved aromatic residue to alanine. Alignment of the predicted transmembrane domains of OCTs and OATs indicated that there are six conserved aromatic amino acids in the transmembrane domains of rOAT3: Y230 (TMD 5); W254 (TMD 6); W334, F335, and Y341 (TMD 7); and F478 (TMD 12). A summary of data obtained for all mutants is shown in Table 1. Alanine scanning was used to substitute each conserved aromatic amino acid with alanine independently (Figure 1). The mutants W334A, F335A, and Y341A exhibited a less than 2-fold enhanced uptake of PAH and a less than 2-fold enhanced uptake of cimetidine in comparison to the wild-type rOAT3, which exhibited a 16-fold enhanced uptake of PAH and an 8-fold enhanced uptake of cimetidine (Figure 2). However, all mutants exhibited a greater than 8-fold enhanced uptake of estrone sulfate (data not shown). The data suggest that W334, F335, and Y341 in TMD 7 are important for PAH and cimetidine transport but are not important for estrone sulfate transport.

In contrast, Y230A, W254A, and F478A exhibited modest or no reductions in the uptake of the PAH and cimetidine in comparison to the wild-type rOAT3 (Figure 2). Replacements

Table 1: Summary of the Substrate Specificity Data for Wild-Type and the Mutant rOAT3[a]

| | TM | hydrophobic or hydrophilic | PAH uptake | cimetidine uptake | estrone sulfate uptake | side chains important for PAH and cimetidine uptake |
|---|---|---|---|---|---|---|
| rOAT3 | | | +++ | +++ | +++ | |
| Y230A | 5 | hydrophilic | +++ | +++ | +++ | − |
| W254A | 6 | hydrophobic | ++ | ++ | ++ | − |
| **W334A** | 7 | hydrophobic | − | − | + | −NH |
| **W334F** | 7 | hydrophobic | − | − | ++ | −NH |
| **F335A** | 7 | hydrophobic | − | − | + | aromatic ring |
| **F335Y** | 7 | hydrophobic | ++ | ++ | +++ | aromatic ring |
| F339V | 7 | hydrophobic | +++ | +++ | +++ | − |
| **Y341A** | 7 | hydrophilic | − | − | +++ | aromatic ring |
| **Y341F** | 7 | hydrophilic | +++ | ++ | +++ | aromatic ring |
| **Y342Q** | 7 | hydrophilic | − | − | +++ | −OH |
| **Y342F** | 7 | hydrophilic | − | − | + | −OH |
| **F362S** | 8 | hydrophobic | − | − | ++ | aromatic ring |
| **F362A** | 8 | hydrophobic | − | − | + | aromatic ring |
| F478A | 12 | hydrophobic | +++ | +++ | +++ | − |
| F328A | 7 | hydrophobic | +++ | +++ | +++ | − |
| W255A | 6 | hydrophobic | +++ | +++ | +++ | − |

[a] Y230, W254, W334, F335, Y341, and F478 are conserved in all OATs and OCTs. F339, Y342, and F362 are conserved in OATs only. F328 and W255 are nonconserved aromatic residues as negative control. The boldfaced residues of rOAT3 contribute to the PAH and cimetidine transport. For PAH, (+++) ≥15-fold over controls, (++) 5−14-fold over controls, (−) ≤2-fold over controls. For cimetidine, (+++) ≥8-fold over controls, (++) 5−7-fold over controls, (−) ≤2-fold over controls. For estrone sulfate, (+++) ≥25-fold over controls, (++) 15−24-fold over controls, (+) 8−14-fold over controls, (−) ≤2-fold over controls.

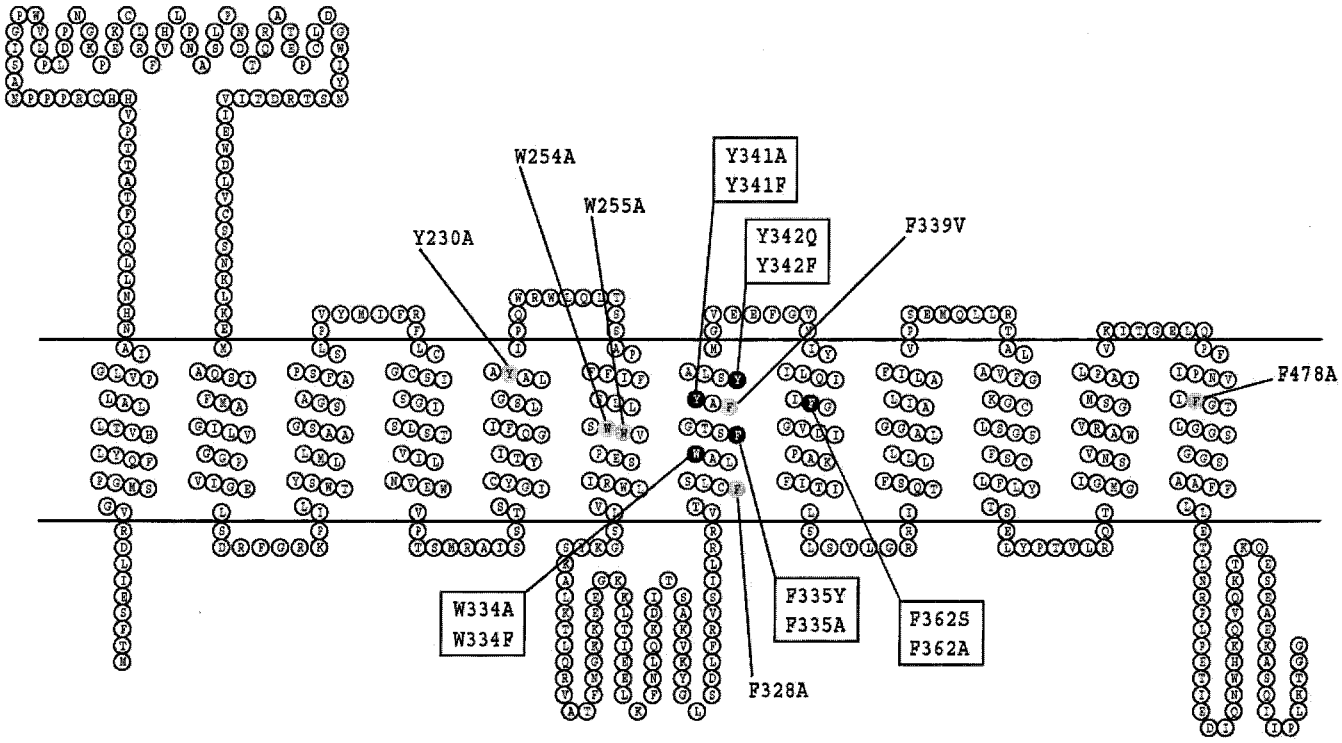


FIGURE 1: Site-directed mutagenesis of aromatic amino acid residues of rOAT3. A schematic representation of the putative 12 transmembrane domain topology of the rOAT3 is shown and residues mutated in this study are highlighted. The black residues are important for PAH and cimetidine transport, and the gray residues are not essential for substrate transport. The single-letter amino acid code is used. The numbers next to the mutated amino acids refer to their positions within the rOAT3 sequence.

of the nonconserved residues, W255 and F328, by alanine produced mutants which also showed no significant reductions in the uptake of PAH and cimetidine (data not shown).

Because of the pronounced reductions in the uptakes of PAH and cimetidine by W334A, F335A, and Y341A, we focused on these residues. To further investigate the distinct contribution of the side chains of these aromatic residues (the indole ring of W334, the −OH group of Y341, or the aromatic rings of W334, F335, and Y341), each of the three conserved aromatic residues of rOAT3 was independently mutated to another aromatic residue. Therefore, single amino acid replacement mutants, W334F, F335Y, and Y341F, were constructed and assessed (Figure 1). Functional studies of the mutants showed that W334F functions as W334A, losing PAH and cimetidine uptake (Figure 3) but still mediating estrone sulfate uptake (data not shown). The data suggest that the indole ring of W334 and possibly the size of the lateral side chain of W334 are important for PAH and

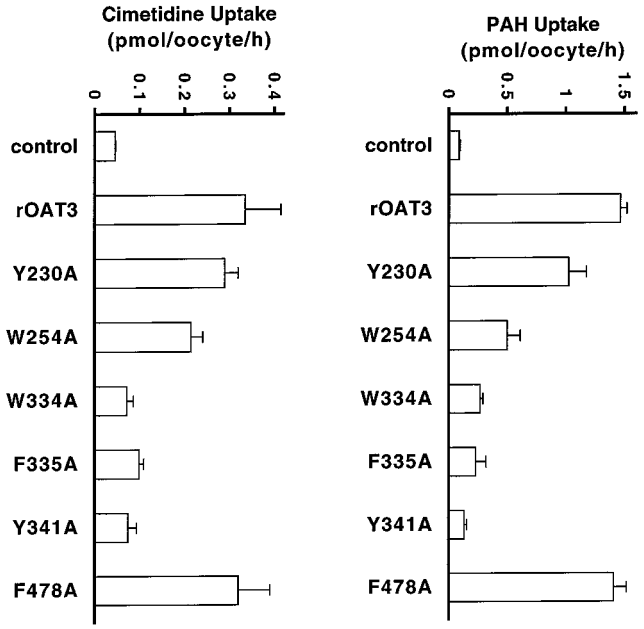


FIGURE 2: Alanine scanning of the conserved aromatic residues in rOAT3. The aromatic residues which are conserved in all OATs and OCTs were mutated to alanine. The uptake of $^3$H-PAH (10 $\mu$M) and $^3$H-cimetidine (1 $\mu$M) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3 or mutants. Data are mean values ± SD for seven to nine oocytes.

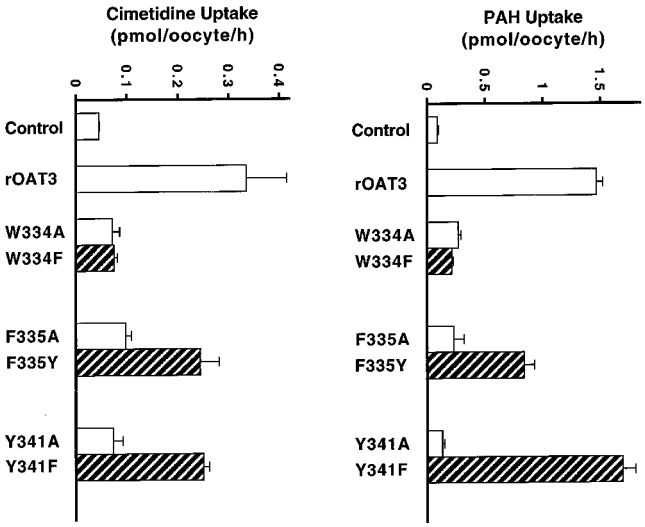


FIGURE 3: Substrate selectivity comparison of wild-type rOAT3, rOAT3 mutants with alanine substitution of the aromatic residues (open bar), and rOAT3 mutants with conservative mutation of the aromatic residues (striped bar). The uptake of $^3$H-PAH (10 $\mu$M) and $^3$H-cimetidine (1 $\mu$M) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3 and mutants. Data are mean values ± SD for seven to nine oocytes.

cimetidine transport. In the indole ring of tryptophan, the indole −NH groups could interact with substrates by hydrogen bonds. However, unlike the F335A and Y341A mutants, the F335Y and Y341F mutants recovered or partially recovered the ability to transport PAH and cimetidine, suggesting that the aromatic rings of F335 and Y341 contribute to PAH and cimetidine transport by rOAT3.

*Site-Directed Mutagenesis Studies of the Conserved Aromatic Amino Acids in OATs.* To elucidate the role of the three aromatic amino acid residues only conserved in the OATs [F339 (TMD 7), Y342 (TMD 7), and F362 (TMD 8)], each residue was mutated to the corresponding conserved amino acid in OCTs independently [F339V, Y342Q and F362S (Figure 1)]. The F339V mutation barely affected the transporter's ability to take up substrates, suggesting that the conserved F339 of rOAT3 does not contribute to substrate recognition and transport (Table 1). However, in comparison to the wild-type rOAT3, the Y342Q and F362S mutants exhibited markedly decreased PAH and cimetidine uptake (Figure 4) but still mediated estrone sulfate uptake (data not shown). The data suggest that Y342 in TMD 7 and F362 in TMD 8 are important for PAH and cimetidine transport.

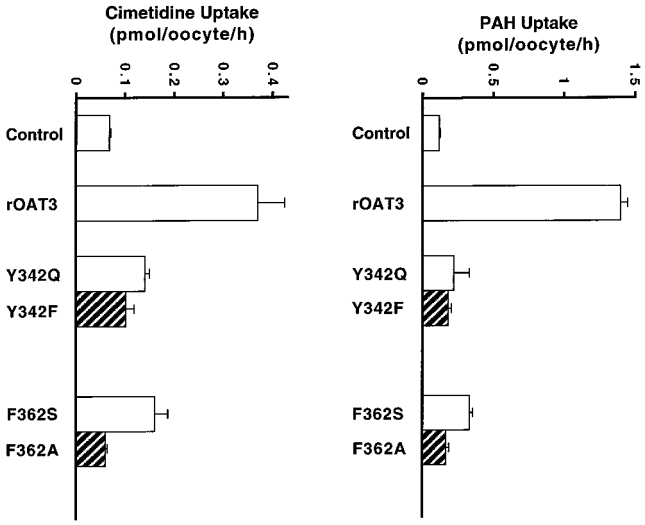


FIGURE 4: Substrate selectivity comparison of wild-type rOAT3, rOAT3 mutants with aromatic residues mutated to the corresponding amino acids in OCTs (open bar), and rOAT3 mutants with conservative mutation of the aromatic residues (striped bar). The uptake of $^3$H-PAH (10 $\mu$M) and cimetidine (1 $\mu$M) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3 and mutants. Data are mean values ± SD for seven to nine oocytes.

To further evaluate the possible contribution of the −OH group of Y342 and the aromatic ring of F362 in PAH and cimetidine transport, single amino acid replacements (Y342F and F362A) were constructed (Figure 1). Y342F and F362A lost the ability to take up PAH and cimetidine (Figure 4) but retained the ability to take up estrone sulfate (data not shown). The Y342F mutant functioned like the Y342Q mutant, indicating that the −OH of Y342 may be directly involved in PAH and cimetidine transport. On the other hand, the F362A mutant functioned like F362S, suggesting that the aromatic ring of F362 may be directly involved in PAH and cimetidine uptake.

*Inhibition Studies of W334A, F335A, Y341A, Y342Q, and F362S.* The mutagenesis studies suggested that W334, F335, Y341, Y342, and F362 are important for PAH and cimetidine recognition and transport but not for estrone sulfate. For the inhibition studies, cimetidine and PAH potently inhibited $^3$H-estrone sulfate uptake mediated by wild-type rOAT3, W334A, F335A, Y341A, Y342Q, or F362S mutant (Figure 5), indicating that, for inhibition of estrone sulfate uptake, these compounds do not require W334, F335, Y341, Y342, or F362 to bind to rOAT3. These data suggest that W334, F335, Y341, Y342, or F362 is not part of a shared binding site for PAH, cimetidine, and estrone sulfate.

*Interactions of PAH and Estrone Sulfate in rOAT3.* To determine whether estrone sulfate and PAH share a common

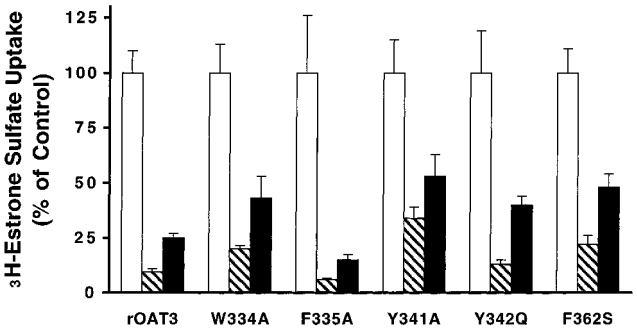


FIGURE 5: Inhibition of $^3$H-estrone sulfate (150 nM) uptake by cimetidine and PAH in oocytes expressing rOAT3 or rOAT3 mutants. Oocytes were injected with 50 ng of the cRNA of rOAT3 or mutants. The concentration of inhibitors was 1 mM. The values are expressed as a percentage of rOAT3- or mutant-mediated $^3$H-estrone sulfate uptake in the absence of the inhibitor. The transport rates for 100% values for rOAT3 or rOAT3 mutants are as follows (pmol/oocyte/h): water control, 0.006 ± 0.001; rOAT3, 0.22 ± 0.02; W334A, 0.08 ± 0.01; F335A, 0.07 ± 0.02; Y341A, 0.26 ± 0.04; Y342Q, 0.19 ± 0.04; F362S, 0.14 ± 0.02. Open bar indicates $^3$H-estrone sulfate uptake in rOAT3 and mutants. Striped bar and dark bar indicate $^3$H-estrone sulfate uptake in the presence of cimetidine and PAH, respectively. Data are mean values ± SD for five to seven oocytes.

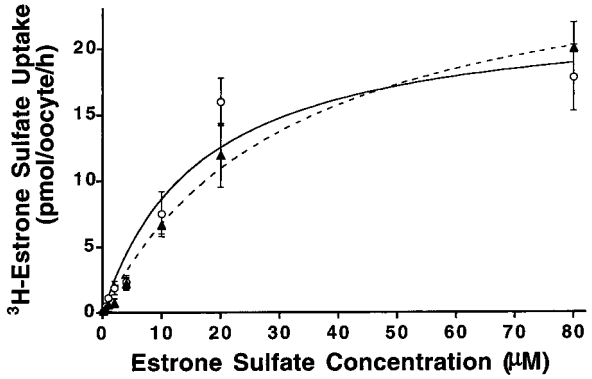


FIGURE 6: Inhibitory effect of PAH on rOAT3-mediated estrone sulfate transport. The rate of $^3$H-estrone sulfate uptake at various concentrations was measured without (○) or with (▲) PAH (800 $\mu$M). Oocytes were injected with 50 ng of rOAT3 cRNA. In the absence of PAH, the apparent $K_m$ and $V_{max}$ values for estrone sulfate were 16 ± 6 $\mu$M and 23 ± 3 pmol/oocyte/h. In the presence of 800 $\mu$M PAH, the apparent $K_m$ and $V_{max}$ values for estrone sulfate were 31 ± 5 $\mu$M and 28 ± 2 pmol/oocyte/h. Kinetic parameters were determined by fitting the data to the Michaelis−Menten equation using a nonlinear least-squares regression-fitting program. rOAT3-mediated transport was obtained by subtracting the transport velocity in uninjected oocytes from that in rOAT3 expressing oocytes. Data are mean ± SD for six to eight oocytes.

recognition site, we examined the nature of the interaction between PAH and estrone sulfate with rOAT3. Kinetics studies (Figure 6) showed that PAH (800 $\mu$M) significantly increased the apparent Michaelis−Menten constant ($K_m$) of estrone sulfate for rOAT3 (16 ± 6 $\mu$M vs 31 ± 5 $\mu$M), whereas the maximum uptake rate ($V_{max}$) did not change significantly (23 ± 3 pmol/oocyte/h vs 28 ± 2 pmol/oocyte/h), indicating that PAH inhibits estrone sulfate transport by rOAT3 in a competitive manner. The studies suggest that PAH and estrone sulfate share a common substrate recognition site in rOAT3.

*Distribution of Conserved Aromatic Residues in TMD 7.* Among the 12 TMDs of rOAT3, TMD 7 has the greatest number of conserved aromatic residues (Figure 7). When

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rOAT3   VTFCLSLAWFSTGFAYYSLAM
rOAT1   LFLCLSMLWFATSFAYYGLVM
rOAT2   ISLCCMMVWFGVNFSYYGLTL
hOAT1   LFLCLSMLWFATSFAYYGLVM
hOAT3   HDLLLSLAWFATGFAYYSLAM
mOAT1   LFLCLSMLWFATSFAYYGLVM
rOCT1   HTVILMYLWFSCAVLYQGLIM
rOCT2   HTLILMYNWFTSSVLYQGLIM
rOCT3   CTLILMFAWFTSAVVYQGLVM
hOCT1   RTFILMYLWFTDSVLYQGLIL
hOCT2   HTMILMYNWFTSSVLYQGLIM
mOCT1   HTLILMYLWFSCAVLYQGLIM
mOCT2   HTLILMYNWFTSSVLYQGLIM
mOCT3   CTLILMFAWFTSAVVYQGLVM
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FIGURE 7: Multiple alignments of transmembrane domain 7 of the OATs and OCTs. The conserved aromatic amino acid residues in the OATs and OCTs are highlighted in gray; the aromatic residues conserved in the OATs only are highlighted in gray, and the corresponding conserved residues in the OCTs are highlighted in black.

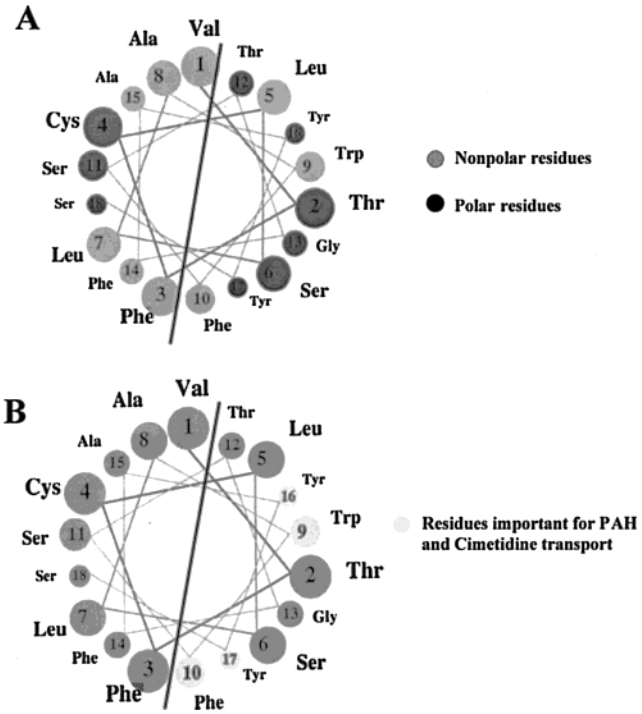



FIGURE 8: Helical wheel analysis of the TMD 7 of rOAT3. The transmembrane domain is assumed to be a standard α helix (3.6 residues/helical turn). Each residue in TMD 7 is plotted every 100° (360/3.6) around the center of a circle. The transmembrane residues are numbered from the top to the bottom in the helical wheel. Residues 1−18 are equivalent to residues 326−343 in TMD 7 of rOAT3. The straight line indicates an arbitrary boundary between the hydrophilic and the hydrophobic region. (A) Nonpolar hydrophobic residues are gray, whereas polar and hydrophilic residues are black. (B) The four aromatic residues important for PAH and cimetidine transport are gray.

TMD 7 of rOAT3 was observed in a helical wheel projection (Figure 8A), a nonrandom distribution of aromatic residues and hydrophobic residues was readily apparent. On a helical wheel projection, TMD 7 is amphipathic, with both hydro-

phobic and hydrophilic faces. The conserved aromatic amino acids, which showed the greatest sensitivity to amino acid substitution, were located close to the middle of the hydrophilic face of the helix (Figure 8B).

## DISCUSSION

The organic anion transporter, rOAT3, transports structurally diverse organic anions and cations in the kidney. Its location on the basolateral membrane suggests that it plays a role in the uptake of its substrates from the blood into the proximal tubule cell, although its transport mechanism has not been defined (*3*, *4*). rOAT3 is approximately 40% identical to rOAT1 and rOAT2 in sequence, exhibits high activity when expressed in *Xenopus laevis* oocytes, and does not appear to be an exchanger (*4*). Therefore, rOAT3 is ideally suited to begin to explore the molecular determinants of substrate recognition and translocation of a member of the organic anion transporter family, SLC21.

In this study, we tested the hypothesis that conserved aromatic amino acid residues are required for the substrates transport of rOAT3. Substrates of rOAT3 are composed of a charged group (e.g., either an anion or a cation) and an organic moiety which may be hydrophilic (e.g., PAH and cimetidine) or hydrophobic (e.g., estrone sulfate). In this study, we hypothesized that aromatic residues would be important in the interaction of the organic moieties of rOAT3 substrates with the transporter but not in the interaction of the charged moiety of the substrates. Our previous studies indicated that the basic amino acids, Arg 454 and Lys 370, are critical in the charge specificity of rOAT3. Arg 454 and Lys 370 probably interact electrostatically with the anionic charged group of substrates of rOAT3 (*5*).

There were three major findings of this study: (a) four conserved aromatic amino acid residues in TMD 7 of rOAT3 (W334, F335, Y341, and Y342) and one additional aromatic residue (F362) in TMD 8 are essential for the transport of PAH and cimetidine (Table 1); (b) inhibition of estrone sulfate transport by PAH and cimetidine does not require W334, F335, Y341, Y342, and F362 of rOAT3 (Figure 5); (c) the indole ring of W334, the −OH group of Y342, and the aromatic rings of F335, Y341, and F362 are required for transport of cimetidine and PAH by rOAT3 (Table 1). These findings are discussed next in the context of amphipathic analysis of TMD 7 and a simplified model for the interaction of diverse substrates of rOAT3 with the transporter.

Systematic alanine scanning followed by site-directed mutagenesis was used to identify the role of aromatic amino acids, conserved in OCTs and OATs, in substrate recognition of rOAT3. The residues that were conserved only in the OATs were also replaced by the corresponding conserved residues in the OCTs to obtain further information on structural requirements for substrate interactions. The data showed that the conserved residues W334, F335, Y341, and Y342 in TMD 7 are important for PAH and cimetidine transport (Figures 2 and 4; Table 1). In addition, F362 in TMD 8 is important for PAH and cimetidine but not for estrone sulfate transport (Figure 4; Table 1). These data suggest that aromatic residues in TMD 7 and 8 are important in the recognition and translocation of cimetidine and PAH.

We found that W334, F335, Y341, Y342, and F362 in rOAT3 were required for transport of PAH and cimetidine. However, these residues were not required for the inhibitory effects of PAH and cimetidine on estrone sulfate transport (Figure 5). These results suggest that these aromatic residues are involved in the translocation of PAH and cimetidine but not, however, in their binding to a common site shared by estrone sulfate. The residues may contribute to another step in the transport process.

The finding that estrone sulfate and PAH interacted competitively (Figure 6) suggests that the two compounds share a common binding site in rOAT3. However, because estrone sulfate transport was inhibited by PAH in the absence of W334, F335, Y341, Y342, or F362, the data are consistent with the notion that the shared binding site does not involve these aromatic residues. It is possible that the common binding site for PAH and estrone sulfate involves arginine 454 or lysine 370 because these amino acid residues are essential for the transport of both compounds.

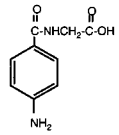
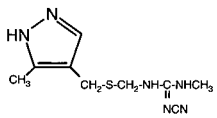
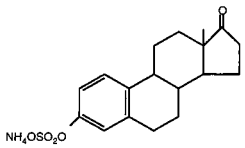
Our data suggest that the polar side chains, the indole ring of W334 and the −OH of Y342, are essential for the interaction of PAH and cimetidine with rOAT3 (Table 1). An aromatic ring alone will not support transport. Because both indole −NH and −OH can form hydrogen bonds, the data suggest that hydrogen bonding of these residues may be required for transport of cimetidine and PAH either directly in the interaction of the substrates with the transporter or indirectly, for example, through helical packing. In contrast, the findings that the conservative mutants, F335Y and Y341F, can still transport PAH and cimetidine suggests that the aromatic rings of F335 and Y341 are important for PAH and cimetidine transport (Figure 3; Table 1). Loss of PAH and cimetidine transport function by F362S and F362A suggests that the aromatic ring of F362 may be important for PAH and cimetidine transport (Figure 4; Table 1). Aromatic rings could contribute to substrate recognition through aromatic interactions that could include $\pi-\pi$ interactions with a variety of ring-containing substrates.

*Amphipathic Analysis of TMD 7.* When TMD 7 was projected in an α-helical configuration, the distribution of amino acids was not random but segregated with a more hydrophobic face and a more hydrophilic face (Figure 8A). Of all of the putative transmembrane domains of rOAT3, TMD 7 and TMD 11 show the strongest amphipathic character. The grouping of polar residues along one plane of the α helix suggests that this face may be important in the interaction and translocation of hydrophilic substrates. The four aromatic residues, sensitive to mutation (W334, F335, Y341, and Y342), are very close to the middle of the hydrophilic face of the helix (Figure 8B).

Studies of a number of membrane transporters suggest that the substrate permeation pathway in a transporter is a channel-like structure formed by several transmembrane helices, where residues facing the channel acquire a more hydrophilic character (*8*−*10*). Therefore, charged and polar residues, often found on one side of these helices, are likely to play critical roles in interacting with the substrates (*8*, *11*). It is very possible that the hydrophilic face of TMD 7 consisting of the four conserved aromatic residues, sensitive to mutation, are involved in substrate recognition and may interact directly with the small organic anion substrate, PAH, and organic cation substrate, cimetidine.

*Simplified Model for Interaction of PAH and Cimetidine with rOAT3.* The finding that the conserved aromatic residues

Table 2: Chemical Structure and log *P* Values of the Three Substrates of rOAT3

| Substrate | Structure | Log P |
|---|---|---|
| PAH |  | -2.18 |
| Cimetidine |  | -0.069 |
| Estrone Sulfate |  | 0.04 |

of TMD 7 and 8 are important for cimetidine and PAH, but not for estrone sulfate transport, suggests that those compounds interact with distinct sites in the transporter. PAH and cimetidine are smaller, more polar compounds than estrone sulfate, as indicated by their log *P* values, octanol−water (pH 7.4) partition coefficients, and their chemical structures (Table 2). PAH and cimetidine may form stronger hydrogen bonds with these residues (e.g., Y342 or W334) in comparison to estrone sulfate. It is also possible that the large side chains of the aromatic residues form a smaller aromatic pocket that precludes the interaction of rOAT3 with larger substrates such as estrone sulfate.

On the basis of these data and data from our previous study, we can propose a simplified substrate interaction model of rOAT3 (Figure 9). In the model, rOAT3 contains a large substrate binding pocket with several interaction domains responsible for the high affinity binding of structurally diverse substrates. One site contains the essential membrane-embedded positively charge residues arginine 454 and lysine 370. These residues act like a magnet to attract negatively charged substrates through charge−charge interactions and are important in the interaction of PAH as well as estrone sulfate but not cimetidine. The aromatic residues W334 and Y342 may form strong hydrogen bonds with some small, hydrophilic substrates (e.g., PAH and cimetidine) through indole −NH of W334 and −OH of Y342 groups, and the aromatic residues F335, Y341, and F362 could act through $\pi-\pi$ stacking by aromatic rings with the ring-containing substrates. The large size of the aromatic residues also restricts the size and shape of the substrates that are allowed. For example, estrone sulfate is excluded because of its large size. Therefore, aromatic residues are not important for the transport of this compound. The aromatic domains modulate the substrate specificity of rOAT3, suggesting that this domain may participate in the formation of the substrate recognition site.

In conclusion, the overall picture of substrate binding within rOAT3 supports the idea of a general transmembrane

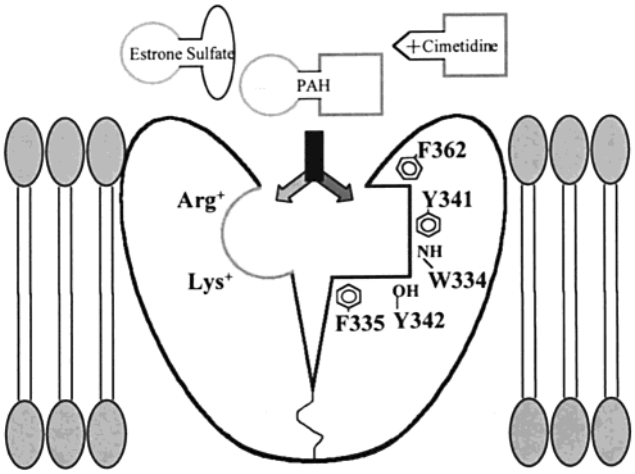



FIGURE 9: Simplified model of the substrate recognition site of rOAT3. The domain containing Arg454 and Lys370 could interact with the negative charge of PAH and estrone sulfate. The domain containing W334, F335, Y341, Y342, and F362 could interact with the organic moiety of PAH and cimetidine. The distinct contributions of the side chains of the five aromatic residues are shown in the cartoon.

binding domain with no single binding site. Multiple domains and residues participate in binding and translocation of various substrates depending upon their charge and the nature of their organic moieties. Arginine 454 and lysine 370 are required for interaction with anionic substrates; aromatic amino acid residues identified in this study (W334, F335, Y341, Y342, and F362) are essential for the interaction of PAH and cimetidine. It is possible that there are other domains in the general substrate binding site which may be important for the large, hydrophobic organic anion substrates (e.g., estrone sulfate and ochratoxin A).

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