Arginine 454 and Lysine 370 Are Essential for the Anion Specificity of the Organic Anion Transporter, rOAT3†

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ABSTRACT: Organic anion transporters (OATs) and organic cation transporters (OCTs) mediate the flux of xenobiotics across the plasma membranes of epithelia. Substrates of OATs generally carry negative charge(s) whereas substrates of OCTs are cations. The goal of this study was to determine the domains and amino acid residues essential for recognition and transport of organic anions by the rat organic anion transporter, rOAT3. An rOAT3/rOCT1 chimera containing transmembrane domains 1−5 of rOAT3 and 6−12 of rOCT1 retained the specificity of rOCT1, suggesting that residues involved in substrate recognition reside within the carboxyl-terminal half of these transporters. Mutagenesis of a conserved basic amino acid residue, arginine 454 to aspartic acid (R454D), revealed that this amino acid is required for organic anion transport. The uptakes of *p*-aminohippurate (PAH), estrone sulfate, and ochratoxin A were ∼10-, ∼48-, and ∼32-fold enhanced in oocytes expressing rOAT3 and were only ∼2-, ∼6-, and ∼5-fold enhanced for R454D. Similarly, mutagenesis of the conserved lysine 370 to alanine (K370A) suggested that K370 is important for organic anion transport. Interestingly, the charge specificity of the double mutant, R454DK370A, was reversed in comparison to rOAT3—R454DK370A preferentially transported the organic cation, MPP^+, in comparison to PAH (MPP^+ uptake/PAH uptake = 3.21 for the double mutant vs 0.037 for rOAT3). These data indicate that arginine 454 and lysine 370 are essential for the anion specificity of rOAT3. The studies provide the first insights into the molecular determinants that are critical for recognition and translocation of organic anions by a member of the organic anion transporter family.

Many therapeutic agents and toxic substances carry a net charge under physiological conditions, which hinders their simple diffusion across biological membranes. Organisms have evolved transporter proteins, which are involved in the absorption and disposition of charged organic compounds. Organic cation transporters (OCTs)[1] and organic anion transporters (OATs) are major protein families which appear to have evolved from a common ancestral protein (*1*) but have different substrate and, in particular, charge selectivities. In general, OCTs transport positively charged organic molecules whereas OATs transport negatively charged organic ions (*2*−*4*). During the past decade a number of mammalian OCT and OAT isoforms have been cloned and subsequently characterized in heterologous expression systems.

Three OCT isoforms (OCT1-3) (*5*−*15*) and four OAT isoforms (OAT1-4) (*2*, *16*−*23*) have been cloned from several mammalian species. Hydropathy analyses of these transporters suggest that all share a common secondary structure composed of 12 membrane spanning α-helices, and all have two large loops: an extracellular loop between TMD 1 and TMD 2 and an intracellular loop between TMD 6 and TMD 7. Although evolutionary analyses suggest that OCTs and OATs evolved from the same ancestral protein (*1*), OCTs share only about 30%−40% sequence identity with OATs, and their substrate specificities are distinct. When overexpressed in either *Xenopus laevis* oocytes or mammalian cells, OCTs mediate the electrogenic uptake of organic cations such as 1-methyl-4-phenylpyridinium (MPP^+), tetraethylammonium (TEA), and guanidine but do not interact with substrates of organic anion transporters such as *p*-aminohippurate (PAH) (*3*, *24*−*26*). Likewise, OATs have been shown to transport several organic anions but do not, in general, interact with organic cations (*2*). One notable exception to this charge specificity is rOAT3, an organic anion transporter cloned from rat brain; rOAT3 transports not only hydrophobic organic anions (e.g., estrone sulfate and ochratoxin A) and hydrophilic organic anions (e.g., PAH) but also the moderately hydrophilic organic cation cimetidine (log $P_{cimetidine} = -0.069$; $pK_a = 6.8$). The transport mechanism of rOAT3 is not known—attempts to drive substrate influx by exchange for trans organic anions were not successful (*21*). Although the transport mechanism of rOAT3 is not

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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; RT-PCR, reverse transcription polymerase chain reaction; TEA, tetraethylammonium; TMD, transmembrane domain; V_{max}, maximal uptake rate.

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known, it is possible to obtain critical information about residues responsible for substrate selectivity.

The goal of this study was to identify structural domains and amino acid residues involved in determining the substrate specificity of rOAT3. Our studies indicate that (a) the substrate recognition site resides in the carboxyl-terminal half of the transporters; (b) two conserved basic amino acid residues within this region, arginine 454 and lysine 370, contribute to the substrate specificity of rOAT3; and (c) in contrast to the wild-type rOAT3, the double mutant, R454DK370A (arginine 454 to aspartic acid and lysine 370 to alanine), preferentially transports the organic cation, MPP^+, in comparison to the organic anion, PAH. This is the first study to delineate molecular determinants involved in substrate and charge specificity of a member of the OAT family.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Transporters and Site-Directed Mutagenesis. The cDNAs of wild-type rOCT1 and rOAT3 (GenBank accession numbers X78855 and AB017446) were amplified by RT-PCR using an oligo(dT) primer (Gibcol-BRL, Gaithersburg, MD) with total RNA isolated from rat kidney and rat brain (Clontech, Palo Alto, CA), respectively. The primers for PCR were designed from the published sequences. The pair of primers for rOCT1 was 5′-aagcttc-cagccatgcccaccgtggatgatg-3′ (sense) and 5′-ggatcctcaggtact-tgaggacttgcctgtttggac-3′ (antisense); the pair of primers for rOAT3 was 5′-gaattccttgcctggtgccatgacctcttccg-3′ (sense) and 5′-ggatccgggtcctatccaccagtcttcagcggg-3′ (antisense). The cDNAs were subcloned into pOX vector (*27*) under the control of a T3 promoter.

Genetics Computer Group software (Wisconsin Package, version 10.1) was used to align the nucleotides and the deduced amino acid sequences of rOCT1 and rOAT3. Secondary structure models were generated using the TOPO program (S. J. Johns and R. C. Speth) based on output from HMMTOP, an automatic server for predicting transmembrane helices and topology of proteins (http://www.enzim.hu/hmmtop/) (*28*). A rOAT3/rOCT1 chimera consisting of TMD 1 to TMD 5 of rOAT3 and TMD 6 to TMD 12 of rOCT1 was obtained by equivalent exchange at an internal *Hae*II site (position 704 in rOAT3 and position 787 in rOCT1). That is, the fragment between *Hae*II and the C-terminus of rOAT3 was switched with the same fragment of rOCT1. The sequence of rOAT3/rOCT1 chimera was confirmed by automated DNA sequencing in the Biochemical Resource Center at the University of California at San Francisco.

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 substitutions R454D (arginine 454 to aspartic acid), R454N (arginine 454 to asparagine), K370A (lysine 370 to alanine), and double mutant R454DK370A were prepared using the cDNA of wild-type rOAT3 as the template. The sequences of R454D, R454N, K370A, and R454DK370A mutants were confirmed by directed DNA sequencing.

cRNA Transcription and Expression in X. laevis Oocytes. Oocytes were harvested from oocyte positive *X. 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 (*6*). Oocytes were maintained at 18 °C in modified Barth's medium. Healthy stage V and VI oocytes were injected with capped cRNA (1 μg/μL) 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 (*6*). The compounds were used at the following concentrations: [^{3}H]MPP^+ (1 μM) (82 Ci/mmol), [^{3}H]cimetidine (1 μM) (15 Ci/mmol), [^{3}H]PAH (10 μM) (4 Ci/mmol), [^{3}H]estrone sulfate (150 nM) (53 Ci/mmol), and [^{3}H]ochratoxin A (550 nM) (18 Ci/mmol). Uptake experiments were carried out as follows: groups of seven to nine oocytes were incubated in 100 μL of Na^+ buffer (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.2) containing a radiolabeled compound at 25 °C for 1 h. Uptake was stopped by washing the oocytes five times with 3 mL of ice-cold Na^+ buffer. Then the oocytes were lysed with 100 μL of 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.

Partition Coefficient Determinations. To study the lipophilicity of the organic anion substrates, the partition coefficient (log *P*) values were determined using the similar methods as reported before (*29*, *30*).

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. Due to 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]MPP^+ (82 Ci/mmol) (Dupont-New England Nuclear, Boston, MA), [^{3}H]cimetidine (15 Ci/mmol) (Amersham Life Sciences, Arlington Heights, IL), and [^{3}H]PAH (4 Ci/mmol), [^{3}H]estrone sulfate (53 Ci/mmol), and [^{3}H]ochratoxin A (18 Ci/mmol) (NEN Life Science Products, Boston, MA). Oligonucleotide primers were synthesized by Gibco-BRL (Gaithersburg, MD).

RESULTS

Substrate Specificity of Wild-Type rOAT3. To characterize the substrate selectivity of the wild-type organic anion transporter, rOAT3, we studied the uptake of cimetidine, MPP^+, and PAH. In oocytes expressing rOAT3, cimetidine

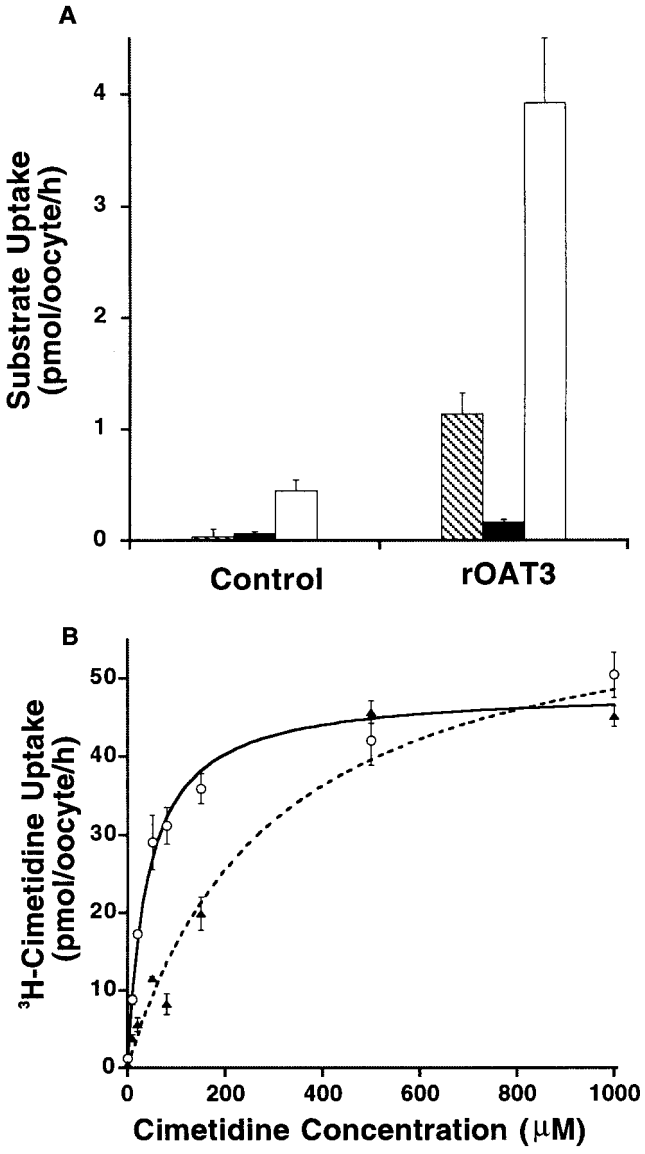

FIGURE 1: (A) Substrate selectivity of wild-type rOAT3. The uptake of [^{3}H]cimetidine (1 μM) (striped bar), MPP^+ (1 μM) (dark bar), and PAH (10 μM) (open bar) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of rOAT3 cRNA. Data are mean values ± SD for seven to nineoocytes. (B) Inhibitory effect of PAH on rOAT3-mediated cimetidine transport. The rate of [^{3}H]cimetidine uptake at various concentrations was measured without (○) or with (▲) PAH (500 μM). Oocytes were injected with 50 ng of rOAT3 cRNA. In the absence of PAH, the apparent K_m and V_{max} values for cimetidine were 40 ± 6 μM and 49 ± 2 pmol oocyte^{-1} h^{-1}. In the presence of 500 μM PAH, the apparent K_m and V_{max} values for cimetidine were 289 ± 92 μM and 63 ± 8 pmol oocyte^{-1} h^{-1}. The change of K_m was significant ($P < 0.05$). 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 ± SE for six to eight oocytes.

and PAH uptakes were ~15-fold and ~10-fold enhanced, respectively, over the uninjected oocytes (Figure 1A), similar to the previous report from Kusuhara's group (*21*). In contrast, MPP^+ uptake was only ~2-fold enhanced in oocytes expressing rOAT3. We examined the nature of the interaction between cimetidine and PAH with rOAT3. Preliminary experiments showed that PAH uptake was inhibited by 1 mM cimetidine, and cimetidine uptake was inhibited by 1 mM PAH (data not shown). Further kinetics studies (Figure 1B) showed that PAH (500 μM) significantly increased the apparent Michaelis−Menten constant (K_m) of cimetidine for rOAT3, from 40 ± 6 μM to 289 ± 92 μM ($P < 0.05$), whereas the maximum uptake rate (V_{max}) did not change significantly (49 ± 2 pmol oocyte^{-1} h^{-1} to 63 ± 8 pmol oocyte^{-1} h^{-1}). Also, cimetidine (250 μM) significantly increased the K_m of PAH for rOAT3, from 278 ± 48 μM to 1240 ± 186 μM ($P < 0.05$), whereas the V_{max} did not change significantly (72 ± 5 pmol oocyte^{-1} h^{-1} to 78 ± 7 pmol oocyte^{-1} h^{-1}). These data indicate that PAH inhibits cimetidine uptake by rOAT3 in a competitive manner and vice versa, suggesting that the two compounds share a common substrate recognition site in rOAT3.

Substrate Selectivity of a rOAT3/rOCT1 Chimera. To determine the domains of rOAT3 and rOCT1 involved in substrate recognition and permeation, chimeric transporters of rOAT3 and rOCT1 were constructed, and their substrate and charge selectivities were examined. A chimera $rOAT3_{1-5}$:$rOCT1_{6-12}$ corresponding to the TMD segments 1−5 of rOAT3 and 6−12 of rOCT1 was constructed from the wild-type transporters (Figure 2). The chimera $rOAT3_{1-5}$:$rOCT1_{6-12}$ maintained functional characteristics similar to that of wild-type rOCT1 (Figure 3). In particular, this chimera transported MPP^+, a model organic cation, but not PAH, a model organic anion. These results suggest that the domains responsible for substrate recognition and translocation largely reside in the carboxyl-terminal half of rOCT1. The data indicate that the carboxyl-terminal half of rOCT1 is sufficient for recognition and translocation of organic cations by rOCT1 and that critical amino acids required for substrate recognition reside in this half of rOCT1. However, since reverse chimeras did not function, it is difficult to interpret the data with respect to domains responsible for recognition and translocation of organic anions by rOAT3. Larger domains may be necessary for substrate recognition and translocation by rOAT3. Because OCTs and OATs are homologous, the data are consistent with the hypothesis that the domains responsible for substrate recognition may largely reside within the carboxyl-terminal half of both transporter families.

Functional Characteristics of Arginine 454 Mutants of rOAT3. Alignments of OAT and OCT sequences were performed to identify candidate amino acid residues in TMD segments 6−12 that could be responsible for the substrate selectivity of rOAT3. For anion selectivity, we looked for conserved basic amino acids in OATs in these alignments. Sequence alignments showed that there are three conserved basic amino acids in the OAT family: histidine 34, lysine 370, and arginine 454. We hypothesized that arginine 454 may be critical in substrate discrimination between anions and cations because all OCTs have a conserved negatively charged aspartic acid residue at the corresponding position (Figure 4A). According to the predicted secondary structure of OATs, arginine 454 resides in the middle of TMD 11 of rOAT3 (Figure 4B). To investigate the functional significance of this residue, the mutants R454D and R454N, in which the positively charged arginine was replaced by the negatively charged aspartic acid or the uncharged polar amino acid, asparagine, respectively, were constructed. The uptakes of [^{3}H]cimetidine, [^{3}H]MPP^+, and [^{3}H]PAH in oocytes injected with rOAT3, R454D, or R454N cRNA and unin-

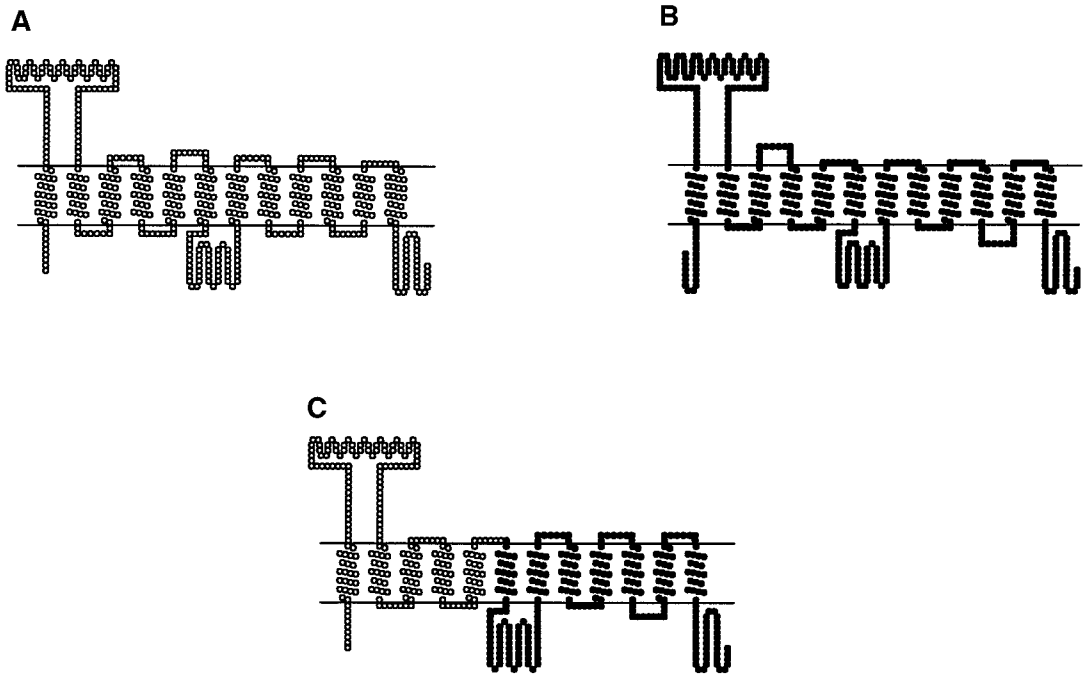

FIGURE 2: Secondary structure of wild-type and chimeric transporters. Wild-type rOAT3 (A) has 536 amino acids and wild-type rOCT1 (B) has 556 amino acids. Both wild-type transporters are predicted to have 12 transmembrane domains. The chimera (C) contains the first 235 amino acids of rOAT3, which correspond to the first five transmembrane domains, and amino acids 263−556 of rOCT1, which correspond to transmembrane domains 6−12.

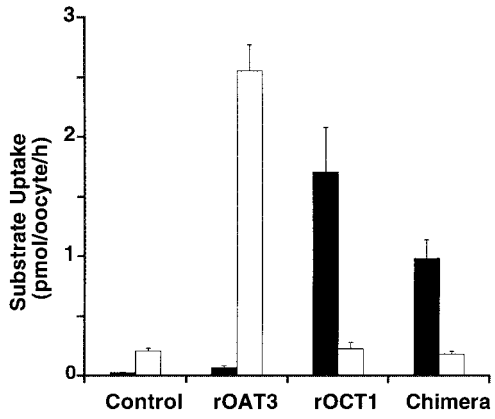

FIGURE 3: Substrate selectivity of wild-type and chimeric transporters. The uptake of [^{3}H]MPP$^+$ (1 μM) (dark bar) and [^{3}H]PAH (10 μM) (open bar) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of rOAT3, rOCT1, or chimera (rOAT3$_{1-5}$:rOCT1$_{6-12}$) cRNA. Data are mean values ± SD for seven to nine oocytes.

jected (control) oocytes are shown in Figure 5A. [^{3}H]PAH uptake was dramatically reduced in oocytes expressing the R454D and R454N mutants, which suggests that arginine 454 is essential for PAH transport by rOAT3. In contrast, both wild-type rOAT3 and the mutants R454D and R454N maintained similar cimetidine uptake activities (more than 10-fold). Further kinetic studies of cimetidine transport demonstrated that the K_m of cimetidine is slightly increased in oocytes expressing R454N and R454D (69 ± 10 μM and 103 ± 12 μM) compared to 48 ± 8 μM in oocytes expressing the wild-type transporter. Therefore, arginine 454 appears to be essential for PAH but not for cimetidine transport by rOAT3.

Estrone sulfate and ochratoxin A are also substrates of rOAT3 (*21*), and our studies showed that arginine 454 is also important in the transport of these two substrates (Figure 5B). The uptakes of estrone sulfate and ochratoxin A were ~48 and ~32-fold enhanced in oocytes expressing wild-type rOAT3 and were only ~6- (~12%) and ~5- (~16%) fold enhanced in oocytes expressing R454D, although the uptake of cimetidine was similar in both wild-type rOAT3 and the R454D mutant.

Interestingly, estrone sulfate, ochratoxin A, and the hydrophobic organic anions probenecid, indomethacin, and furosemide, as well as the hydrophobic organic cation, quinidine, potently inhibited [^{3}H]cimetidine uptake mediated by either wild-type rOAT3 or the R454D mutant (Figure 6), indicating that, for inhibition, these compounds do not require arginine 454. In contrast, the finding that PAH was not able to inhibit cimetidine transport by R454D suggests that arginine 454 is required for its ability to inhibit cimetidine translocation. Further kinetic studies of estrone sulfate transport demonstrated that the K_m of estrone sulfate is similar for the R454N mutant (32 ± 9 μM) and the wild-type rOAT3 transporter (34 ± 3 μM) (Figure 7), although the V_{max} value for wild-type rOAT3 is higher (~4-fold) than for the R454N mutant. These data indicate that the R454N mutation does not alter the binding affinity of estrone sulfate for rOAT3 but does alter its V_{max}.

Functional Characteristics of Lysine 370 Mutants of rOAT3. Lysine 370, located in TMD 8 of rOAT3, is also conserved among the OATs and may be critical to the charge selectivity of the transporter. To investigate the functional significance of this residue, the mutant K370A, in which the positively charged lysine was replaced by alanine, the amino acid found at the corresponding site in OCTs, was constructed. The uptakes of [^{3}H]cimetidine, [^{3}H]MPP$^+$, and [^{3}H]-PAH in oocytes expressing the wild-type rOAT3, R454D, or K370A and in uninjected (control) oocytes are shown in Figure 8A. [^{3}H]PAH was dramatically reduced in oocytes expressing the K370A mutant, which suggests that, like

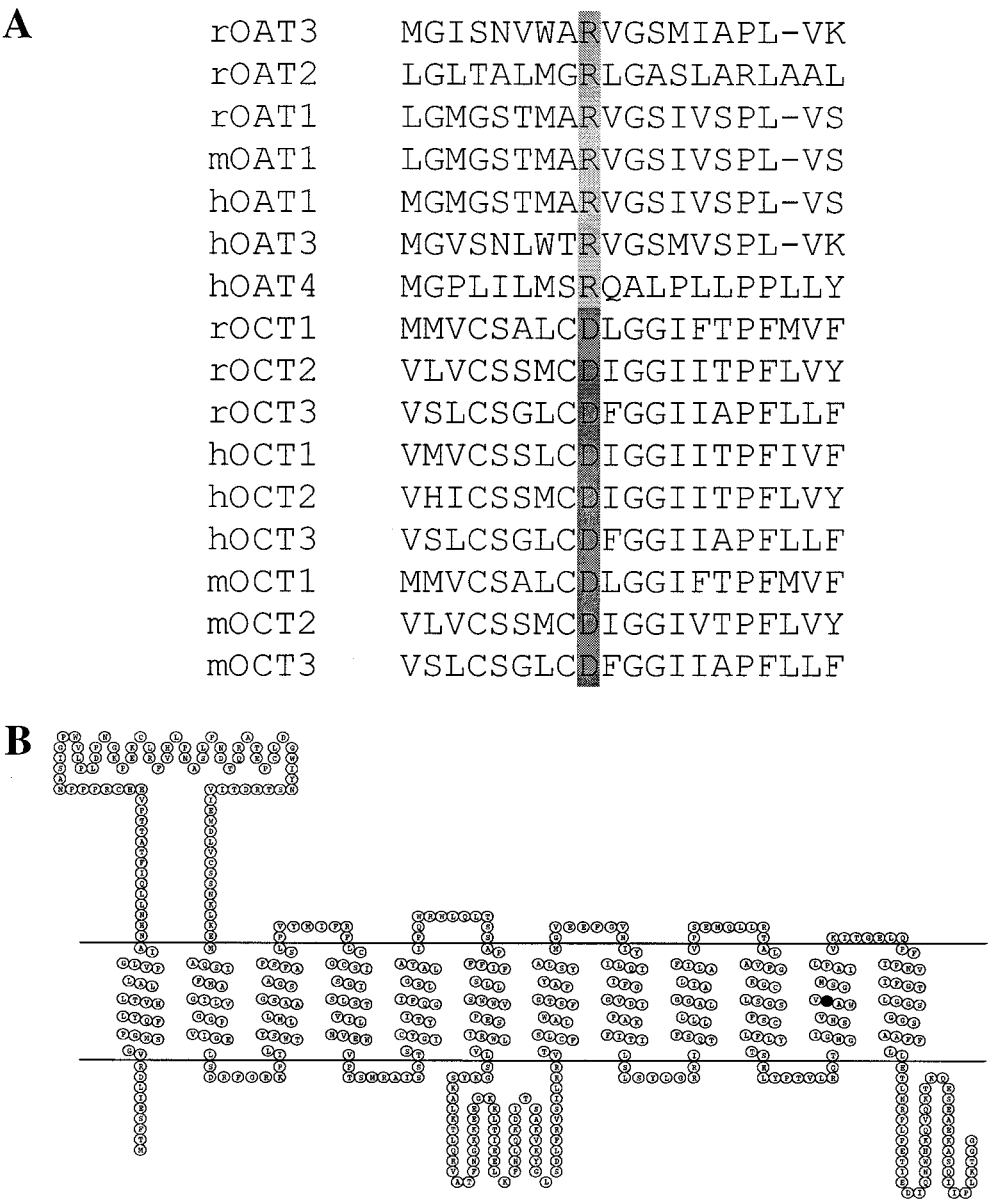

FIGURE 4: (A) Multiple alignments of transmembrane domain 11 of the OCTs and OATs. The conserved arginine in the OATs and the conserved aspartic acid in the OCTs are highlighted. (B) Model of the secondary structure of rOAT3 with arginine 454 highlighted.

arginine 454, lysine 370 is essential for [^{3}H]PAH transport by rOAT3 but not for cimetidine transport. Hence, both arginine 454 and lysine 370 appear to be important in the transport of PAH. Transport of estrone sulfate and ochratoxin A was dramatically reduced in oocytes expressing K370A, indicating that lysine 370 is also important in the transport of these two substrates (Figure 8B).

For the inhibition studies, hydrophobic organic anion substrates, estrone sulfate and ochratoxin A, as well as the hydrophobic organic cation, quinidine, potently inhibited cimetidine uptake mediated by K370A (Figure 9A), indicating that, for inhibition, these compounds do not require lysine 370. Furthermore, PAH inhibited cimetidine transport by the K370A mutant. These data suggest that, unlike arginine 454, lysine 370 is not required for PAH binding to rOAT3 to inhibit cimetidine translocation.

Functional Characteristics of Arginine 454 and Lysine 370 Double Mutants of rOAT3. The double mutant R454DK370A was constructed to study the function of rOAT3 when the two conserved basic amino acids, arginine 454 and lysine 370, were mutated. The uptakes of [^{3}H]cimetidine, [^{3}H]-MPP^+, and [^{3}H]PAH in oocytes expressing the wild-type rOAT3, R454D, K370A, or R454DK370A and in uninjected (control) oocytes are shown in Figure 8A. For the double mutant R454DK370A, PAH uptake was also decreased, whereas the uptake of cimetidine was similar to that observed in oocytes expressing K370A and R454D, which further confirms that both arginine 454 and lysine 370 are important for PAH transport by rOAT3. Transport of estrone sulfate and ochratoxin A was reduced in oocytes expressing R454DK370A, indicating that both arginine 454 and lysine 370 are important in the transport of these two substrates, too (Figure 8B). Interestingly, the uptake of the model organic cation substrate, MPP^+, was considerably enhanced in oocytes expressing the double mutant, R454DK370A, in comparison to uninjected oocytes (~8-fold). The cation/anion charge selectivity of R454DK370A was reversed in comparison to the wild-type transporter rOAT3 (ratio of MPP^+ uptake/PAH uptake = 3.21 for the double mutant vs 0.037 for the wild type) (Figure 8A inset), suggesting that replace-

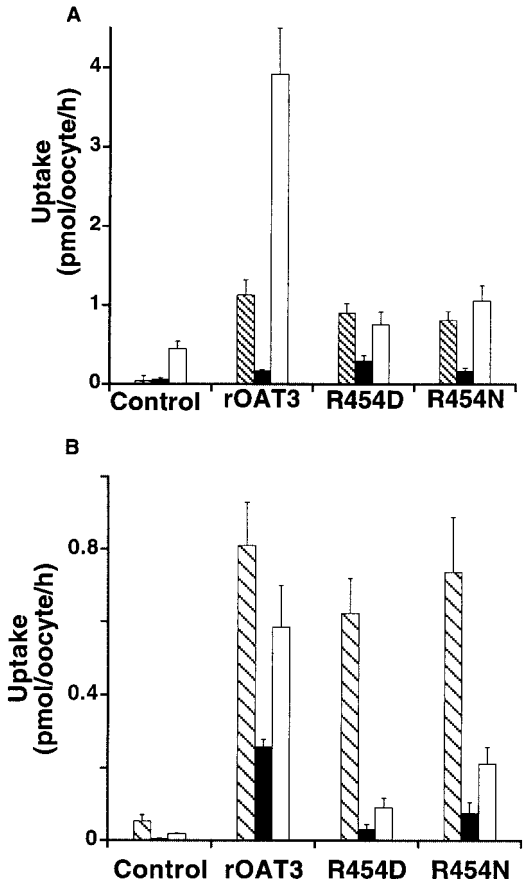

FIGURE 5: (A) Substrate selectivity of wild-type rOAT3 and the R454D and R454N mutants. The uptake of [^{3}H]cimetidine (1 μM) (striped bar), MPP$^+$ (1 μM) (dark bar), and PAH (10 μM) (open bar) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3, R454D, or R454N. (B) Substrate selectivity of wild-type rOAT3 and the R454D and R454N mutants. The uptake of [^{3}H]cimetidine (1 μM) (striped bar), estrone sulfate (150 nM) (dark bar), and ochratoxin A (550 nM) (open bar) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3 or R454D. Data are mean values ± SD for seven to nine oocytes.

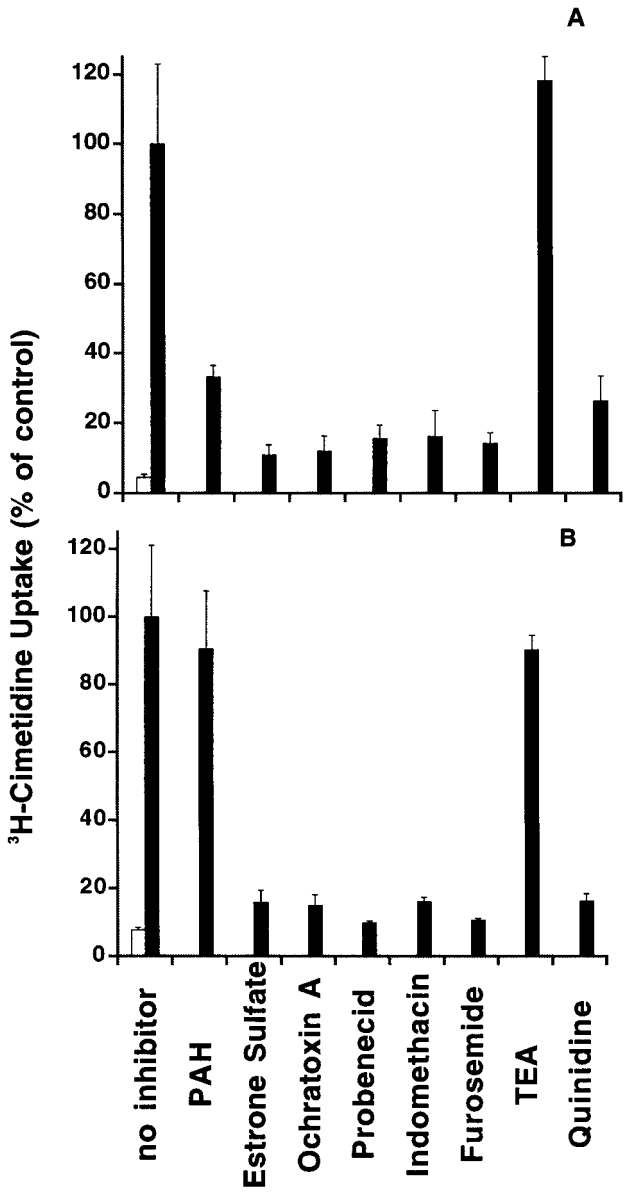

FIGURE 6: Inhibition of (A) rOAT3-mediated [^{3}H]cimetidine (1 mM) uptake by various compounds or (B) R454D-mediated [^{3}H]-cimetidine (1 mM) uptake by various compounds. Oocytes were injected with 50 ng of the cRNAs of rOAT3 or R454D. The concentration of inhibitors was 1 mM. The values are expressed as a percentage of rOAT3- or R454D-mediated ^{3}H-labeled cimetidine uptake in the absence of the inhibitor. The open bar indicates cimetidine uptake in uninjected (control) oocytes. Data are mean values ± SD for five to seven oocytes.

ment of the amino acid residues at positions 454 and 370 with the corresponding residues of the OCTs allows the transporter to accept and, furthermore, to transport the organic cationic, MPP$^+$. Further kinetic studies of MPP$^+$ transport by the R454DK370A double mutant demonstrated that the K_m of MPP$^+$ is 32 ± 7 μM and the V_{max} value is 17 ± 1 pmol oocyte^{-1} h^{-1} (Figure 10).

Hydrophobic organic anion substrates, estrone sulfate and ochratoxin A, as well as the hydrophobic organic cation, quinidine, potently inhibited cimetidine uptake in oocytes expressing the R454DK370A double mutant (Figure 9B), indicating that, for inhibition, these compounds do not require arginine 454 and lysine 370. However, unlike the K370A mutant, PAH was not able to inhibit cimetidine transport by R454DK370A.

Since R454DK370A can transport the model organic cation, MPP$^+$, we performed inhibition studies of MPP$^+$ uptake in oocytes expressing the double mutant (Figure 11). The hydrophobic organic cations, TPrA, TBA, quinidine, verapamil, and vinblastine, and anions, estrone sulfate and ochratoxin A, inhibited MPP$^+$ uptake mediated by the double mutant, whereas the hydrophilic organic cations, TMA, TEA, choline, guanidine, and NMN, did not. These data suggest that the interaction of hydrophobic organic cations or anions with rOAT3 does not require either arginine 454 or lysine 370.

DISCUSSION

The first aim of this study was to determine the structural regions involved in substrate recognition by rOAT3 and rOCT1. Since all members of the OAT/OCT gene family share a common secondary structure and appear to have evolved from the same ancestral sequence (*1*), information regarding the large structural domains responsible for substrate recognition of one isoform might provide insights into the binding domains of other members of this gene family as well. To this end a number of rOAT3/rOCT1

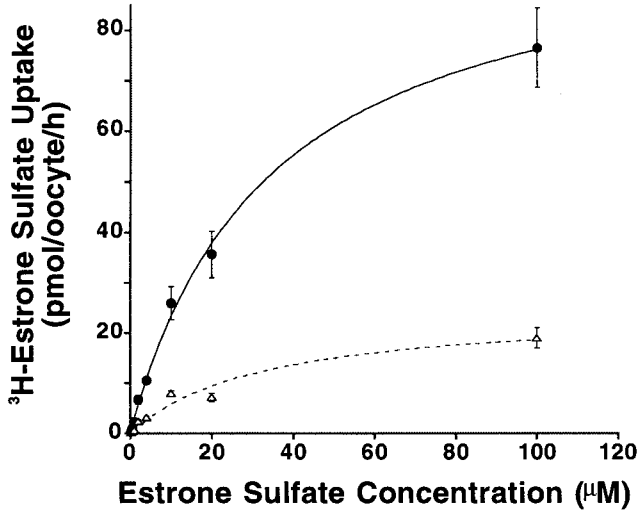

FIGURE 7: Kinetic studies of estrone sulfate transport mediated by wild-type rOAT3 and the R454N mutant. The rate of [^{3}H]estrone sulfate uptake by wild-type rOAT3 and the R454N mutant at various concentrations was measured. The apparent K_m and V_{max} values for estrone sulfate by wild-type rOAT3 were 33 ± 3 μM and 102 ± 4 pmol oocyte^{-1} h^{-1}. The apparent K_m and V_{max} values for estrone sulfate by the R454N mutant were 32 ± 9 μM and 24 ± 3 pmol oocyte^{-1} h^{-1}. Kinetic parameters were determined by fitting the data to the Michaelis−Menten equation using a nonlinear least-squares regression-fitting program. rOAT3- and R454N-mediated transport was obtained by subtracting the transport velocity in uninjected oocytes from that in rOAT3 expressing oocytes. Data are mean values ± SE for six to eight oocytes.

chimeras were constructed and their transport properties assessed. Our results demonstrate that the substrate recognition site for these transporters resides primarily in the TMD 6−12 region (Figure 3). Our attempts to construct a functional reciprocal chimera with regions containing TM 6−12 of rOAT3 transplanted into rOCT1 and to construct functional OAT/OCT chimeras with smaller OCT domains to identify more specific regions important in substrate recognition were unsuccessful. These chimeras were nonfunctional, possibly because they were not expressed in the plasma membrane due to trafficking or stability problems (*31*, *32*). Since further chimera studies were unsuccessful, site-directed mutagenesis studies of rOAT3 were performed to determine if conserved, basic amino acids are involved in rOAT3 transport function.

We hypothesized that basic amino acids, such as arginine and lysine, which carry a positive charge, in the TMD 6−12 region are important in rOAT3 transport function. In particular, basic residues may be important for interactions between rOAT3 and charged anionic compounds. Of the three conserved basic residues in the OATs (histidine 34, lysine 370, and arginine 454), only arginine 454 aligned with a conserved negatively charged residue (aspartic acid) at the corresponding position in the OCTs (Figure 4A). Recently, aspartic acid 475 in rOCT1 has been suggested to play a role in the transport function of rOCT1 (*33*).

In this study, we observed that the mutants, R454D and R454N, did not interact with or transport the organic anion, PAH, and only weakly transported the organic anions, estrone sulfate and ochratoxin A (Figure 5, Table 1). In contrast, the mutants retained the ability to transport the weak base, cimetidine (Figure 5A). These data suggest that arginine 454 represents an important site for binding and translocation

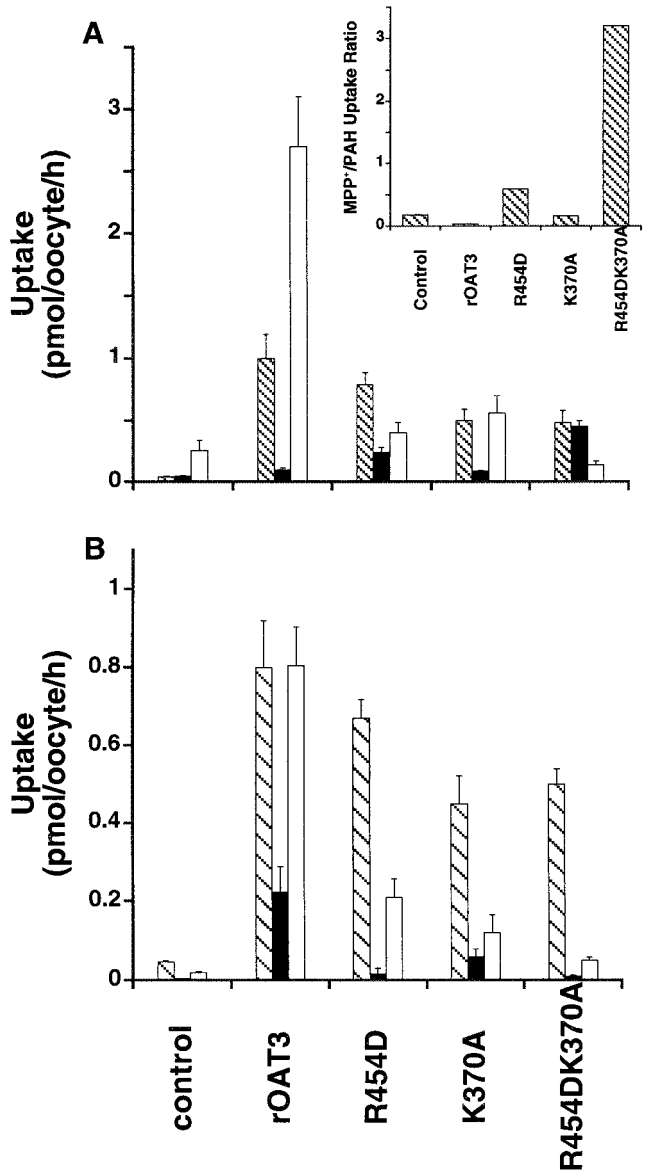

FIGURE 8: (A) Substrate selectivity of wild-type rOAT3 and the R454D, K370A, and R454DK370A mutants. The uptake of [^{3}H]-cimetidine (1 μM) (striped bar), MPP$^+$ (1 μM) (dark bar), and PAH (10 μM) (open bar) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3, R454D, K370A, or R454DK370A. The inset shows the ratio of MPP$^+$/PAH uptake by rOAT3, R454D, K370A, or R454DK370A. (B) Substrate selectivity of wild-type rOAT3 and the R454D, K370A, and R454DK370A mutants. The uptake of [^{3}H]cimetidine (1 μM) (striped bar), estrone sulfate (150 nM) (dark bar), and ochratoxin A (550 nM) (open bar) was measured in uninjected (control) oocytes and in oocytes injected with 50 ng of the cRNA of rOAT3, K370A, and R454DK370A. Data are mean values ± SD for seven to nine oocytes.

of organic anions and that the basic compound, cimetidine, does not require arginine 454 for the interaction with the transporter.

Hydrophobic organic anions and cations such as probenecid, indomethacin, furosemide, and quinidine as well as organic anion substrates, estrone sulfate and ochratoxin A, still potently inhibited cimetidine transport by the R454D mutant, suggesting that these compounds interact with a shared site which is distinct from arginine 454. In contrast, PAH, which is considerably more hydrophilic than estrone sulfate or ochratoxin A (log $P_{PAH} = -2.18$; log $P_{estrone\ sulfate} = 0.04$;

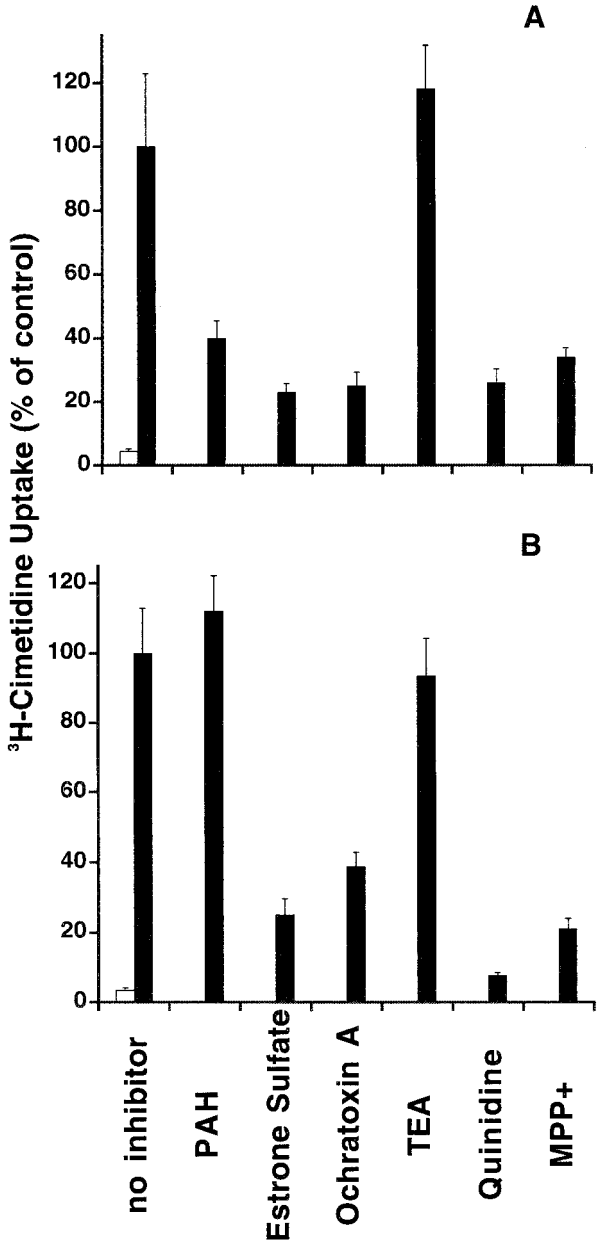

FIGURE 9: Inhibition of (A) K370A mutant-mediated [^{3}H]cimetidine (1 μM) uptake by various compounds or (B) R454DK370A double mutant-mediated [^{3}H]cimetidine (1 μM) uptake by various compounds. Oocytes were injected with 50 ng of the cRNAs of K370A or R454DK370A. The concentration of inhibitors was 1 mM. The values are expressed as a percentage of K370A- or R454DK370A-mediated ^{3}H-labeled cimetidine uptake in the absence of the inhibitor. The open bar indicates cimetidine uptake in uninjected (control) oocytes. Data are mean values ± SD for five to seven oocytes.

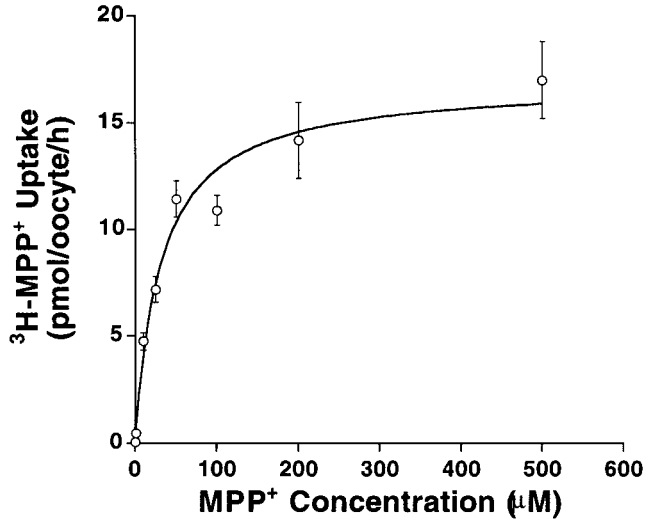

FIGURE 10: Kinetic studies of MPP$^+$ transport mediated by the R454DK370A double mutant of rOAT3. The rate of [^{3}H]MPP$^+$ uptake by the R454DK370A double mutant at various concentrations was measured. The apparent K_m and V_{max} values for MPP$^+$ by R454DK370A were 32 ± 7 μM and 17 ± 1 pmol oocyte^{-1} h^{-1}. Kinetic parameters were determined by fitting the data to the Michaelis−Menten equation using a nonlinear least-squares regression-fitting program. R454DK370A-mediated transport was obtained by subtracting the transport velocity in uninjected oocytes from that in R454DK370A expressing oocytes. Data are mean ± SE for six to eight oocytes.

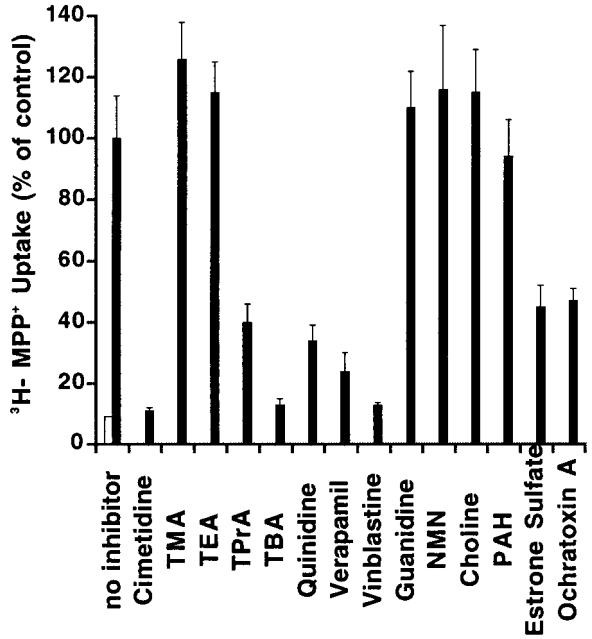

FIGURE 11: Inhibition of the R454DK370A double mutant-mediated [^{3}H]MPP$^+$ (1 μM) uptake by various compounds. Oocytes were injected with 50 ng of the cRNA of R454DK370A. The concentration of inhibitors was 1 mM, except for vinblastine (10 μM). The values are expressed as a percentage of R454DK370A-mediated ^{3}H-labeled MPP$^+$ uptake in the absence of the inhibitor. The open bar indicates MPP$^+$ uptake in uninjected (control) oocytes. Data are mean values ± SD for five to seven oocytes.

log $P_{\text{ochratoxin A}} = 0.06$), did not inhibit cimetidine transport by R454D. Clearly, arginine 454 is essential for the interaction of organic anion substrates with rOAT3. However, it appears that other domains or residues are involved in the transport of cimetidine and interaction of hydrophobic compounds with the transporter.

Our data also indicate that lysine 370 is important for the transport of the organic anion substrates, PAH, estrone sulfate, and ochratoxin A, but not for the weak base cimetidine (Figure 8, Table 1). In addition, similar to arginine 454, lysine 370 is not essential for the inhibitory effects of hydrophobic organic anions and cations on cimetidine transport. However, in contrast to arginine 454, lysine 370 is not important for inhibition of cimetidine uptake by PAH. Hence, arginine 454 and lysine 370 play important but not identical roles in determining the specificity of rOAT3. These results suggest that K370 is involved in the transport and recognition of PAH, however, not in its ability to inhibit transport. One

Table 1: Summary of the Substrate and Inhibitor Specificity Data for Wild-Type, Chimeric, and Mutant Organic Anion Transporters

	substrate uptake (*S*)[a] and inhibition (*I*)[b] data													
	rOAT3		rOCT1		chimera		R454D		R454N		K370A		R454DK370A	
	S	I	S	I	S	I	S	I	S	I	S	I	S	I
organic cations														
cimetidine[c]	+++	ND	ND	ND	ND	ND	+++	ND	+++	ND	+++	ND	+++	ND
MPP^+ [c]	−	−[e]	+++	ND	+++	ND	+	+++[e]	−	ND	−	+++	++	+++
organic anions														
estrone sulfate[d]	+++	+++	ND	ND	ND	ND	+	+++	++	ND	++	+++	−	+++
ochratoxin A[d]	+++	+++	ND	ND	ND	ND	+	+++	++	ND	+	+++	−	++
PAH[c]	+++	+++	−	ND	−	ND	−	−	−	ND	−	++	−	−

[a] Substrate uptake mediated by wild-type, chimeric, and mutant transporters. [b] Inhibition of [^{3}H]cimetidine mediated by wild-type and mutant transporters by various compounds (1 mM). The maximal inhibition (100%) was defined as complete inhibition of uptake on the model compound in the presence of an inhibitor. Key: −, 0%−15% inhibition; +, 15%−35% inhibition; ++, 35%−65% inhibition; +++, 65%−100% inhibition. [c] Key: +++, ≥10-fold over uninjected oocytes (controls); ++, 5−9-fold over controls; +, 3−5-fold over controls; −, ≤2-fold over controls. [d] Key: +++, ≥30-fold over controls; ++, 11−29-fold over controls; +, 3−10-fold over controls; −, ≤2-fold over controls. [e] Data not shown; ND, not determined.

possibility is that distinct sites are involved in transport and inhibition.

The R454DK370A double mutant had characteristics similar to those of R454D. However, in contrast to the single mutant, the double mutant could transport MPP^+ (Figure 8A). Thus, replacement of the amino acid residues at positions 370 and 454 with the corresponding residues of the OCTs changes the charge selectivity of rOAT3; the double mutant prefers MPP^+ to PAH as a substrate. The demonstration that substitutions of a few amino acids can switch the charge specificity of OAT/OCT transporters suggests that this class of transporters may have evolved from a common intermediate that lacked charge specificity but that recognized different organic molecules. However, the R454DK370A double mutant did not accept other hydrophilic organic cations, such as TEA, guanidine, and carnitine as substrates (data not shown), which suggests that, in addition to charge recognition domains, other domains are involved in the transport of organic compounds by the transporter. The demonstrations (Figure 11) that hydrophobic organic cations and also hydrophobic organic anions can inhibit MPP^+ uptake mediated by R454DK370A suggest that replacement of arginine 454 and lysine 370 does not alter the interaction of hydrophobic organic ion inhibitors with the transporter.

It is possible that the mutation of an amino acid residue will result in a transporter which is not functional due to alterations in the sites required for driving the uptake of the substrate. Since the mechanism of transport by rOAT3 is not known, i.e., attempts to drive the transporter with countergradients of organic anions failed, it is difficult to directly test this possibility. Thus, it is possible that mutation of lysine 370 and arginine 454 resulted in changing a region required for translocation rather than substrate recognition. Our data suggest, however, that this is not the case since both mutant transporters as well as the double mutant still transported cimetidine.

Our studies support the following major conclusions. First, the substrate recognition site of the organic cation and anion transporters resides in the carboxyl-terminal half of the transporters. Second, multiple domains differently contribute to the binding and translocation of hydrophilic and hydrophobic organic anions. Third, two conserved basic amino acid residues within this region, arginine 454 and lysine 370, contribute to the substrate specificity of rOAT3. Fourth, changing these two amino acid residues switches the specificity of the transporter from preferential transport of the organic anion, PAH, to the organic cation, MPP^+. These studies suggest how charge selectivity of transporters can evolve by mutations of charged amino acid residues.

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