Cationic Amino Acids Involved in Dicarboxylate Binding of the Flounder Renal Organic Anion Transporter

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Abstract. Three conserved cationic amino acids in predicted transmembrane domains 1, 8, and 11, respectively, of the flounder renal organic anion transporter, fROAT, were changed by site-directed mutagenesis and the resulting mutants functionally characterized in Xenopus laevis oocytes. Uptake of p-aminohippurate (PAH) in oocytes that expressed mutant H34I, K394A, or R478D was markedly reduced compared with oocytes that expressed wild-type fROAT, but was still several-fold higher than that in water-injected control oocytes. Immunocytochemically, no decrease in cell surface expression of the mutants could be detected. Only mutant R478D appeared to have a lower PAH affinity than the wild type. Similar to wild-type–dependent PAH transport, uptake induced by mutant H34I was sensitive to glutarate (GA) cis-inhibition. In contrast, mutants K394A and R478D could not be significantly affected by up to 10 mM GA, although the cRNA-dependent PAH uptake could still be almost completely suppressed by probenecid. Moreover, again in contrast to the wild type, neither PAH influx nor PAH efflux mediated by these two mutants could be trans-stimulated by GA, nor did they induce GA transport. These data suggest that amino acids K394 and R478 in fROAT are required for dicarboxylate binding and PAH/dicarboxylate exchange.

The proximal tubular basolateral organic anion exchanger, for which the simplified nomenclature is OAT1 and p-aminohippurate (PAH) is the model substrate, is critically involved in the elimination of anionic drugs and other xenobiotics via the kidney. Known early on for its broad specificity (1), cloning of this carrier from various species has meanwhile allowed the identification of a multitude of therapeutically and pharmacologically relevant substrates, including diuretics, nonsteroidal anti-inflammatory drugs, β-lactam antibiotics, anti-viral drugs, and mycotoxins (2–4). Moreover, studies in whole animals or intact tubule preparations indicated that this carrier plays a role in drug-induced nephrotoxicity of, e.g., cephaloridine (5), acetaminophen (6), or cysteine conjugates (7,8), which can be mitigated by the classical inhibitor probenecid. Recent studies that have used the cloned rat or human OAT1, respectively, have shown that this carrier does indeed confer increased cellular sensitivity to cephalosporin antibiotics (9) and antiviral drugs (10) and that their cytotoxic effects can be markedly reduced by probenecid (9) as well as by nonsteroidal anti-inflammatory drugs (11).

Effective renal elimination of organic anions first requires their basolateral uptake into the renal tubule cell against the electrochemical gradient (12), which is achieved via exchange for internal dicarboxylates (13,14). The necessary outwardly directed dicarboxylate gradient is maintained by metabolic production as well as by basolateral and luminal Na+/H+ cotransport systems (15). This tertiary active mechanism for cellular organic anion uptake is functionally conserved among all vertebrate species investigated thus far and even in a number of nonmammalian renal model systems, such as crab urinary bladder (16), flounder (17), and snake renal tubules (18), which suggests that the mechanism is critical for organic anion elimination.

Similar to PAH transport by intact renal basolateral membranes, the cloned renal PAH/dicarboxylate exchangers were cis-inhibited (19–25) and, where tested, also trans-stimulated by α-KG and/or glutarate (GA) (20–22,25). Moreover, those analyzed in this respect exhibited a similar specificity pattern for dicarboxylates of at least five carbons (22,26), as has been observed in rat renal proximal tubules (27). Only the more distantly related Caenorhabditis elegans OAT, also a PAH/dicarboxylate exchanger, differs, in that it could be trans-stimulated by the C4-dicarboxylate fumarate as well as by α-KG but not GA (28). These functional characteristics are unique to the OATs1, whereas all other related and likewise multispecific organic anion transporters cloned to date, named OAT2 to OAT4, do not appear to operate as exchangers (23,29–31). Their driving forces and thus the direction of net transport in vivo are still unclear.

To our knowledge, nothing is yet known about the structure-function relationship of any of these cloned organic anion carriers. In this study, we therefore investigated the functional importance of cationic residues for the recognition of negatively charged substrates in the flounder PAH/dicarboxylate exchanger, fROAT (22). These residues were selected on the basis of the following rationale. The OATs are related both in sequence and predicted
secondary structure to the organic cation transporters (OCTs) (4). We therefore speculated that positively charged amino acids conserved among all OATs1, which correspond to conserved, but negatively charged or neutral, amino acids in the OCTs, might be involved in the selectivity of these two groups of carriers for oppositely charged substrates. A sequence comparison of the available OATs1 and OCTs revealed only three positions fulfilling these criteria (4), corresponding to amino acids H34 in predicted transmembrane domain (TM)-1, K394 in TM-8, and R478 in TM-11 of fROAT, indicated by arrows in the model presented in Figure 1. These were exchanged individually for the corresponding amino acids present in OCTs by site-directed mutagenesis. The resulting mutants were analyzed with respect to function as organic anion transporters, as well as—in view of the importance of basolateral organic anion/dicarboxylate exchange in effective renal organic anion elimination—with respect to their interaction with dicarboxylate. Interestingly, mutations K394A and R478D resulted in a complete insensitivity of PAH transport to GA and, consistently, a loss in the ability to translocate this cation, or respectively.

Materials and Methods

Site-Directed Mutagenesis and Sequencing

Mutations were introduced in the original fROAT clone (22) by use of the QuikChange site-directed mutagenesis kit (Stratagene, Cambridge, UK) according to the manufacturer’s instructions with an extension time of 14 min and by using the following primer pairs (the position of the mutation is italicized): H34I (sense), CTGATGATGGCCAGCATCAACCTGTGAGAAGC; H34I (antisense), GTTCTGACGCAGGTTGATGCTGAGCATACTCATCAG; K394A (sense), CGACGCAGAACAATCGACGCGGAGTACAAC; K394A (antisense), GTCAGATCCCGCTGAGTTGACTGTCGTGCAG; R478D (sense), GGTTCTCCATGATGGCCACATAGAGGACCATGGT; and R478D (antisense), CACCATGCTCCTATGAGGAGCCCATGAGACC. To verify the mutated clones, both strands of the entire coding region were sequenced by dye terminator cycle sequencing (automated sequencer: ABI Prism, Applied Biosystems, Norwalk, CT). The sequence was assembled and analyzed by use of the Genetics Computer Group software package (Madison, WI).

Epitope Tagging

To be able to detect the fROAT protein, we generated a construct with a C-terminal FLAG epitope (Integra Biosciences, Fernwald, Germany). For this purpose, the fROAT 5′-untranslated region plus coding region were amplified by polymerase chain reaction (PowerScript DNA polymerase; PAN Biotech GmbH, Aidenbach, Germany) by use of a pSport1-specific primer containing the KpnI site, 5′-GCAGTACCGGTCTCGGAA-3′, and a primer specific for the 3′ end of the fROAT coding region except for the introduction of an NsiI site 5′ of the stop codon: 5′-CTCATGCAATCTGTTAAGGATTGCCCTC-3′ (the restriction sites are italicized). A pSport1 plasmid that contained a C-terminally FLAgged C-terminal fragment of the flounder NaPi-II (fragment 4-8 [34]) was sequentially restricted with KpnI and NsiI. After agarose gel electrophoresis, the larger fragment containing the FLAG sequence, the 15 3′ terminal bp of the NaPi-II coding region including the stop codon, and the vector up to the KpnI site, was extracted (Nucleotrap extraction kit; Machery and Nagel, Düren, Germany). This fragment was ligated to the KpnII/NsiI-digested fROAT polymerase chain reaction amplificate. This approach was chosen as a simple alternative to attempts to introduce the FLAG epitope by use of loop-forming primers in a site-directed mutagenesis reaction, which were hindered by unexplained base insertions. From this construct, the FLAgged mutant R478D was obtained by site-directed mutagenesis as described above. FLAgged mutants H34I and K394A were generated by combining the respective Bsu11 fragments of fROAT plasmids that contained the mutations H34I or K394A with that containing the 3′ end of fROAT with the FLAG sequence. All constructs were verified by sequencing, as described above.

Figure 1. Secondary structure model of fROAT. Positively or negatively charged amino acids and histidine residues within the predicted transmembrane domains are indicated by +, −, or ×, respectively. The positions of the amino acids mutated in this study are marked by arrows. Y denotes the potential N-glycosylation sites within the large extracellular loop between transmembrane domains 1 and 2.
To study fROAT trafficking, oocytes were defolliculated by overnight incubation at 15°C in collagenase (Type CLSII, Biochrom KG, Berlin, Germany) (0.5 mg/ml oocyte Ringer’s solution, see above) and subsequent washing for 10 min in Ca²⁺-free oocyte Ringer’s solution (10 mM NaCl, 3 KCl, 1 MgCl₂, and 5 HEPES/Tris [pH 7.6]) before cRNA was injected. On day 3 after injection, they were manually devitellinized after 5 to 10 min of incubation in 200 mM K⁺ aspartate and then fixed in Dent’s solution overnight at −20°C (35). The fixative was washed out, and oocytes were incubated with mouse anti-FLAG M2 IgG monoclonal antibody (Sigma, Deisenhofen, Germany) (dilution 1:2000) in the presence of 10% goat serum at 4°C for 12 h. After being washed with phosphate-buffered saline, incubation with secondary Alexa 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR) (dilution 1:200) was performed at room temperature for 1 h. After being washed with phosphate-buffered saline, stained oocytes were postfixed with 3.7% paraformaldehyde for 30 min. The embedding procedure in acrylamide (Technovit 7100, Heraeus Kulzer, South Bend, IN) was carried out according to the manufacturer’s instructions. Embedded oocytes were cut into 5-μm sections and analyzed with a fluorescence microscope (Nikon Optiphot, Nikon, Natick, MA).

**Results**

Mutants H34I, K394A, and R478D of fROAT were functionally characterized after expression in X. laevis oocytes. First, we determined the PAH uptake rates they induced relative to the wild type (Figure 2). All three mutations resulted in a marked decrease in PAH transport activity. However, uptake was still significantly above the levels of the respective water-injected control oocytes (8.7-fold for H34I, 6.3-fold for K394A, and 4.4-fold for R478D).

Because the lower PAH transport activity of the mutants might have been caused by impaired trafficking to the oocyte membrane because of the mutations, we generated constructs containing a C-terminal FLAG epitope to analyze cell-surface expression by immunocytochemistry. Initial experiments demonstrated that these constructs behaved similarly to their counterparts without the FLAG epitope, both with respect to absolute transport activity as well as sensitivity to GA inhibition (data not shown). No difference in membrane staining of oocytes expressing either of the mutants could be detected compared with those that expressed the wild type (Figure 3).

Alternatively, the reduced transport activity of the mutants as observed at 10 μM PAH could have been due to a decrease in PAH affinity. Thus, initial rate uptake was assayed as a function of PAH concentration over a period of 5 (wild type) or 10 min (mutants) because preliminary experiments had shown that uptake was linear at least until these time points even at the highest PAH concentration to be used (data not shown). Figure 4 depicts the PAH-dependent uptake rates, i.e., PAH uptake of cRNA-injected oocytes minus that of the respective water-injected control oocytes, normalized to uptake per min. Mutant R478D-induced transport rates reach those of the wild type at high (mM) PAH concentrations but are lower in the μM range, which suggests that mutation of R478 to D results in a lower PAH affinity of the carrier. In contrast, uptake rates mediated by mutants K394A and H34I were lower than those by the wild type over the entire PAH concentration range tested, which indicates that they are not the result of a decreased PAH affinity. An unequivocal kinetic analysis of the data turned out to be difficult, because both Eadie-Hofstee and Hanes-Woolf plots of the data were nonlinear when the entire concentration range tested (up to 10 mM) was included (not shown), revealing an additional, low-affinity component of PAH uptake at mM PAH concentrations. This could also be observed in wild-type cRNA-injected oocytes but had not been apparent in the range of PAH concentrations previously tested (22). As an approximation of the $K_m$ of the high-affinity component of uptake, we only used the cRNA-dependent uptake

![Figure 2](image-url)

**Figure 2.** Effect of site-specific mutation of H34, K394, or R478 on fROAT-mediated p-aminohippurate (PAH) uptake. Oocytes were injected with cRNA of wt fROAT (white bar), mutant H34I, mutant K394A, mutant R478D (hatched bars), or an equivalent volume of H₂O (control, black bar). 1-hr uptake of 10 μM [³H]PAH was assayed and is expressed in % of the respective controls. Data are mean ± SEM of the number of independent experiments given in parentheses, each carried out on 6 to 12 oocytes per treatment. Significance of differences in Student’s $t$ test in PAH uptake of cRNA-injected oocytes relative to control cells is indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

![Figure 3](image-url)

**Figure 3.** Immunocytochemical detection of wild-type (wt) fROAT and mutant H34I, K394A, and R478D in oocytes. Devitellinized oocytes that expressed constructs of wt fROAT or mutants H34I, K394A, or R478D containing a C-terminal FLAG epitope were immunostained with anti-FLAG M2 IgG, followed by secondary Alexa 546 goat anti-mouse IgG. They were then embedded in acrylamide, and 5-μm sections were analyzed by fluorescence microscopy at the magnifications indicated in parentheses.
rates at PAH concentrations up to 250 μM in Eadie-Hofstee analysis (Figure 4, inset), although some contribution of the low-affinity component to these uptake rates cannot be excluded. Thereby, similar apparent affinity constants for PAH were obtained for the wild type (37 ± 12 μM), mutant H34I (34 ± 8 μM), and mutant K394A (57 ± 16 μM), whereas a significantly higher value resulted for mutant R478D (277 ± 51 μM). As for maximal transport rates via the high-affinity component, the Eadie-Hofstee plot suggests values in the following order: mutant H34I > wild type > mutant H34I > mutant K394A. More accurate estimates of $V_{\text{max}}$ could only be obtained if this high-affinity transport component could be assayed separately at higher PAH concentrations.

Because cationic amino acids conserved among the OATs1 had been exchanged for amino acids conserved among the OCTs, we analyzed the three mutants for their ability to transport the model organic cation TEA, which does not significantly affect wild-type fROAT-mediated PAH uptake (22). However, similar to the wild type, none of the mutants was significantly inhibited by TEA or induced any TEA uptake above control levels (data not shown).

As a PAH/dicarboxylate exchanger, fROAT can typically be trans-stimulated and cis-inhibited by glutarate (22). Similar to the wild type, mutant H34I-induced PAH uptake was almost completely inhibited already by 1 mM GA (in pmol/h per oocyte): without GA 9.9 ± 1.2, with GA 1.9 ± 0.4; water-injected control oocytes 1.6 ± 0.6 (mean ± SEM, n = 4 independent experiments with 9 to 11 oocytes per treatment each). This mutant was therefore not included in further experiments that assessed interaction of the carrier with GA. In contrast, neither PAH uptake mediated by mutant K394A nor by mutant R478D was significantly affected by 1 mM external GA (not shown). Even at 5 and 10 mM GA, equal to a 500- or 1000-fold excess over the substrate, respectively, which reduced PAH uptake by the wild type further, these two mutants were insensitive to GA inhibition (Figure 5). In the presence of 5 or 10 mM GA, mutants K394A and R478D mediated higher PAH uptake rates than wild-type fROAT. Yet both mutants still exhibited a high sensitivity to probenecid, the classical inhibitor of renal organic anion secretion: mutant K394A- and mutant R478D-dependent 10-μM PAH uptake were inhibited by 88.3 ± 10.0% and 91.8 ± 9.0%, respectively, by 1 mM probenecid in three independent experiments (10 to 11 oocytes per experimental group each).

To assay for GA trans-stimulation of cRNA-induced PAH uptake, we first expressed either the wild-type fROAT or mutants K394A or R478D alone (17 ng/oocyte) or together with the flounder Na⁺-dicarboxylate cotransporter fNaDC-3 (23 ng/oocyte) and preloaded all oocytes for 15 min with 1 mM GA before the PAH uptake assay (10 min). Although transport by the wild type was significantly stimulated by fNaDC-3 coexpression (149 ± 15% of oocytes that expressed fROAT alone, n = 5 independent experiments on 10 to 13 oocytes per treatment each), PAH uptake by the two mutants was unaffected (with fNaDC-3: K394A 106 ± 14% and R478D 95 ± 11% of uptake in oocytes

![Figure 4](image1.png)

**Figure 4.** Concentration dependence of PAH uptake in oocytes that expressed wt fROAT or mutants H34I, K394A, or R478D. Oocytes were injected with cRNA of wt fROAT or mutants H34I, K394A, or R478D, or H₂O (control), and the initial rate of uptake (5 min for wt fROAT or 10 min for the mutants) was assayed at the given PAH concentrations. cRNA-dependent uptake rates (uprate of the respective water-injected oocytes subtracted) ($\Delta V$) were normalized to uptake per pmol/h and are expressed as mean ± SEM of n = 3 (wt fROAT [■] and mutant H34I [▲]) or n = 4 (mutant K394A [□] and R478D [●]) independent experiments, respectively, each carried out on 7 to 11 oocytes per treatment. The Eadie-Hofstee plot shown in the inset represents only those uptake data obtained at up to 250 μM PAH. When linear regression was used, apparent $K_{\text{app}}$ values for PAH of about 35 μM for wt fROAT, 35 μM for mutant H34I, 70 μM for mutant K394A, and 360 μM for mutant R478D were calculated from this analysis.

![Figure 5](image2.png)

**Figure 5.** Glutarate (GA) sensitivity of PAH uptake mediated by wt fROAT or mutants H34I, K394A, or R478D. Oocytes were injected with cRNA of wt fROAT, mutant K394A or mutant R478D, or an equivalent volume of H₂O. 1-hr uptake of 10 μM [³H]PAH was assayed in the absence or presence of 5 or 10 mM GA. Data are expressed in % of the uptake of H₂O-injected control cells in the absence of GA (dashed line) as mean ± SEM of the number of independent experiments given in parentheses, each carried out on 6 to 12 oocytes per treatment. Significance of differences in Student’s t test between uptake of mutant-cRNA–injected oocytes and that of oocytes that expressed wt fROAT under the same condition is indicated (*P < 0.05, **P < 0.01).
that expressed the respective mutant alone, \( n = 3 \) independent experiments each with 8 to 14 oocytes per group).

We also investigated PAH efflux from \(^3\text{H}\)-PAH–injected oocytes in the absence and presence of external glutarate (Figure 6A). In the absence of GA, neither \(^3\text{H}\)-PAH efflux from oocytes that expressed wild-type fROAT nor from those that expressed mutant K394A or R478D significantly exceeded that from water-injected control cells. However, when 1 mM GA was added to the extracellular buffer, efflux from oocytes that expressed the wild type increased by 271 ± 76% and was now significantly higher than that from the corresponding control group. In contrast, efflux from oocytes that expressed mutant K394A or R478D was not significantly affected by external GA, even in paired data analysis. To discriminate between a loss in the ability to interact with GA or to generally operate as an exchanger, a separate set of PAH efflux experiments was carried out without or with PAH in the extracellular buffer. Figure 6B shows that, in contrast to GA, external PAH was able to significantly stimulate PAH efflux not only from oocytes that expressed wild-type fROAT but also from those that expressed mutant K394A or R478D, albeit less strongly.

The finding that GA could no longer trans-stimulate PAH uptake when K394 in fROAT was mutated to A or R478 to D suggested that these two mutations caused a loss in the ability to mediate GA transport. As shown in Figure 7, wild-type fROAT induced a PAH-sensitive GA transport activity, whereas GA uptake in oocytes that expressed mutant K394A or R478D was not significantly different from that of water-injected control oocytes even in the absence of external PAH.

**Discussion**

All three mutants functionally analyzed in this study induced lower PAH uptake rates compared with wild-type fROAT, which were, however, still significantly higher than the control values of water-injected oocytes, which indicates that the mutants were still functional. Moreover, the uptake they induced could be almost completely suppressed by probenecid. As assessed by immunocytochemistry, none of the mutations visibly affected protein trafficking to account for the decrease in cRNA-dependent PAH uptake. Although there was some submembraneous staining, this was—if anything—more pronounced in cells that expressed the wild type than in those that expressed the mutants. This observation indicates, however, that it should have been apparent had any of the mutants been retained underneath the oocyte membrane, as has been observed for certain mutants of the rat renal NaPi-2 transporter in immunostained oocyte cryosections (36).

Only mutant R478D displayed a lower affinity for PAH, which suggests that arginine 478 is involved in PAH binding. Interestingly, mutation of the corresponding aspartate in rat OCT1 has been reported to have pronounced effects on both substrate affinity and substrate selectivity (37). In contrast, mutations H34I and K394A in fROAT appeared to only affect the maximal transport rate. Under the assumption that a comparable number of carriers

![Figure 6](image-url)

**Figure 6.** Trans-stimulation of PAH efflux from oocytes that expressed wt fROAT or mutant H34I, K394A, R478D in oocytes. Oocytes were injected with cRNA of wt fROAT, mutant K394A or mutant R478D, or an equivalent volume of \( \text{H}_2\text{O} \) (control). After 3 d of incubation, they were injected with 0.04 \( \mu \text{Ci} \) \[^3\text{H}\]PAH and placed in efflux medium with or without 1 mM GA (A) or—in a separate series of experiments—with or without 1 mM PAH (B). After an initial period of 2 min, during which oocytes showed a highly variable leak because of the injection, medium was changed and the efflux then assayed over the following 28 min. Data are mean ± SEM of three (A) or four (B) independent experiments, each carried out on four to five oocytes per treatment. Significant changes in paired data analysis in efflux after addition of organic anion to the trans side are indicated (*\( P < 0.05 \), **\( P < 0.01 \)).

![Figure 7](image-url)

**Figure 7.** GA uptake by oocytes that expressed wt fROAT or mutants H34I, K394A, or R478D. Oocytes were injected with cRNA of wt fROAT, mutant K394A or mutant R478D, or an equivalent volume of \( \text{H}_2\text{O} \) (control). 1-hr uptake of 100 \( \mu \text{M} \) \[^14\text{C}\]glutarate was assayed in the absence or presence of 5 mM PAH. Data are mean ± SEM of the number of independent experiments given in parentheses, each carried out on 9 to 12 oocytes per treatment.
as for the wild type was incorporated into the oocyte membrane, this could be due to an altered rate of conformational changes and/or to a decreased driving force as the result of a loss of affinity for the counterion, as discussed below.

Although the existence of a second, low-affinity PAH binding component, as observed for fROAT in this study, could also be detected when fROAT was analyzed electrophysiologically (38), it has not been reported for any of the other renal PAH/dicarboxylate exchangers characterized to date. Yet a similar property cannot be totally excluded for these OATs1 because—similar to fROAT in our previous study (22)—they have so far been tested only at lower ranges of PAH concentrations: up to 20 or 300 μM for hOAT1 (2,24), 500 μM or 1 mM PAH for rOAT1 (20,21), and 800 μM for mOAT1 (19) because of the high PAH affinity expected on the basis of vesicle studies.

Mutant H34I–mediated PAH uptake was similarly sensitive as the wild type to GA, in that it could be inhibited by >90% already by 1 mM of this dicarboxylate. In contrast, mutants K394A and R478D could not be significantly affected by glutarate concentrations as high as 10 mM, which inhibited wild-type–mediated PAH uptake below the corresponding values of mutants K394A and R478D. Moreover, in the absence of external glutarate, PAH efflux from oocytes that expressed wild-type fROAT or mutants K394A or R478D was similar, but only efflux from wild-type–injected cells could be stimulated by GA. In contrast, efflux from oocytes that expressed mutants K394A or R478D was unaffected by external GA, consistent with their inability to translocate GA. Taken together, these data indicate that lysine 394 and arginine 478 are directly, or indirectly by stabilizing the required conformation, involved in dicarboxylate binding, which enables this carrier to import organic anions into the proximal tubule cell against the electrochemical gradient. Lack of trans-stimulation by endogenous dicarboxylate (see below) might explain the low PAH uptake rates mediated by these mutants in the oocyte expression system. Stimulation of PAH efflux mediated by mutants K394A and R478D in the presence of external PAH indicates that they can still function as exchangers. These experiments also suggest that mutations K394A and R478D affect the carrier’s turnover rate: although similar or even somewhat higher in the absence of exchangeable anion, mutant K394A in particular appears to operate less effectively than the wild type even in the PAH/PAH exchange mode. An altered rate of conformational changes might also explain the low uptake rates mediated by mutant H34I, which could be attributed neither to a reduced surface expression nor to decreased affinity for PAH or interaction with GA.

In contrast to uptake of PAH in wild-type fROAT-expressing oocytes, PAH efflux from these cells was similar to or only slightly higher than the control values, which could suggest that this carrier functions only poorly as a uniporter. However, although a several-fold trans-stimulation of fROAT-induced PAH efflux could be achieved by glutarate, PAH uptake could be only moderately increased by 50% to 60% either by fNaDC-3–mediated glutarate preloading (this study) or by passive preloading (22), which indicates a substantial—yet unknown—content of exchangeable dicarboxylate in the oocytes under our experimental conditions. Thus, wild-type fROAT-mediated PAH efflux could possibly be cis inhibited by intracellular dicarboxylate.

Several more OATs have been cloned since the outset of this study. Among them, there are only two exceptions to the conservation pattern described above, both of which involve the positions in TM-1 and TM-8. One is OAT4, cloned from human kidney (31), which possesses a glutamine in position 34 and a conservative exchange of lysine to arginine corresponding to position 394 in fROAT. This carrier functionally deviates from the other OATs in that it transports PAH most poorly and does not interact with GA (31). The second is the OAT cloned from Caenorhabditis elegans (28), which possesses a serine corresponding to H34 of the other OATs and an asparagine corresponding to the lysine in most OATs. It is functionally most similar to the OATs1 but differs in amino acid sequence more strongly from all other OATs, including some regions that are highly conserved among all other members of this group. As was noted above, however, only CeOAT and the OATs1 appear to function as exchangers. Of the others, only rat liver OAT2 has been reported to interact with—although not exchange for—dicarboxylates (29) (to our knowledge, no information relevant to this is available for rat kidney OAT3 [30]). Yet, in a large binding pocket as such polyspecific carriers might require to accommodate chemically so diverse molecules, other charged side groups unique to the OATs2 or -3, as are present, e.g., in TM-10 and TM-11, might interfere with binding of and/or exchange for dicarboxylates.

In summary, transport induced by mutants K394A and R478D, but not mutant H34I, differed qualitatively from that of the wild type. Both showed neither cis-inhibition of PAH uptake nor trans-stimulation of PAH uptake or efflux by GA, nor did they mediate GA transport. All these results are consistent with the notion that lysine 394 and arginine 478 of fROAT are important for the binding of and exchange for dicarboxylates, required for the system to function as an efficient mechanism for renal xenobiotic elimination.

Note Added in Proofs

While this article was in press, Feng et al. (Biochemistry 40: 5511–5520, 2001) described that the amino acids K370 and R454 in rOAT3, corresponding to K394 and R478 in fROAT, differ qualitatively from that of the wild type. Both showed neither cis-inhibition of PAH uptake nor trans-stimulation of PAH uptake or efflux by GA, nor did they mediate GA transport. All these results are consistent with the notion that lysine 394 and arginine 478 of fROAT are important for the binding of and exchange for dicarboxylates, required for the system to function as an efficient mechanism for renal xenobiotic elimination.

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