The Chloride Dependence of the Human Organic Anion Transporter 1 (hOAT1) Is Blunted by Mutation of a Single Amino Acid

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Organic anion transporter 1 (OAT1) is key for the secretion of organic anions in renal proximal tubules. These organic anions comprise endogenous as well as exogenous compounds including frequently used drugs of various chemical structures. The molecular basis for the polyspecificity of OAT1 is not known. Here we mutated a conserved positively charged arginine residue (Arg466) in the 11th transmembrane helix of human OAT1. The replacement by the positively charged lysine (R466K) did not impair expression of hOAT1 at the plasma membrane of Xenopus laevis oocytes but decreased the transport of p-aminohippurate (PAH) considerably. Extracellular glutarate inhibited and intracellular glutarate trans-stimulated wild type and mutated OAT1, suggesting for the mutant R466K an unpaired interaction with dicarboxylates. However, when Arg466 was replaced by the negatively charged aspartate (R466D), glutarate no longer interacted with the mutant. PAH uptake by wild type hOAT1 was stimulated in the presence of chloride, whereas the R466K mutant was chloride-insensitive. Likewise, the uptake of labeled glutarate or ochratoxin A was chloride-dependent in the wild type but not in R466K. Kinetic experiments revealed that chloride did not alter the apparent Km for PAH but influenced Vmax in wild type OAT1-expressing oocytes. In R466K mutants the apparent Km for PAH was similar to that of the wild type, but Vmax was not changed by chloride removal. We conclude that Arg466 influences the binding of glutarate, but not interaction with PAH, and interacts with chloride, which is a major determinant in substrate translocation.

Since the organic anion transporters were first cloned in 1997, much work has been done to understand their structure, functioning, and regulation (1–3). It has been established that OAT1s2 are exchangers that are functionally coupled to Na+/K+-ATPase and sodium dicarboxylate cotransporter 3 that generate the necessary driving forces for organic anion/α-keto-glutarate exchange via OATs (4). Located at the basolateral membrane of proximal tubules in the kidneys, OAT1 has been shown to interact with a wide variety of structurally diverse therapeutically important compounds like antivirals, diuretics, and nonsteroidal anti-inflammatory drugs (5).

As regards structure-function studies in OAT1, phosphorylation and glycosylation states of the transporter have been shown to be important. Phosphorylation through protein kinase C down-regulated hOAT1 through carrier internalization, but so far no amino acid residue was implicated (6), whereas glycosylation of asparagines in the first large extracellular loop of mouse and human OAT1 is necessary for proper trafficking to the plasma membrane. One of these, Asn109, also plays a role in substrate binding/recognition (7). Apart from this an alanine scanning mutagenesis was made in the first transmembrane domain (TMD) of hOAT1 that demonstrated the importance of leucine 30 and threonine 36 for transport (8). Cysteine scanning mutagenesis of mOAT1 revealed that although individual cysteines are not required for transport function, they collectively influence targeting to the plasma membrane (9). Recently it was shown that the C terminus of hOAT1 has two critically important amino acids: the anionic aspartate 506 and Leu512 (10). Asp506 was reported to be important because it may maintain structural integrity through the formation of salt bridges with cationic amino acids elsewhere in the transporter. Because the mutant L512V showed similar Km but reduced Vmax compared with the wild type (wt), it was said to critically affect the turnover of hOAT1.

Further amino acid residues that are involved in substrate binding or translocation have been investigated with the flounder OAT1 and mouse OAT3. In the flounder OAT1, it was shown that two nonconservative amino acid mutations K394A and R478D resulted in a loss of interaction with dicarboxylates but not PAH, suggesting that these cationic residues are important for dicarboxylate but not PAH binding (11). In the study by Feng et al. (12), the same corresponding amino acids in mouse OAT3 were mutated. Neutral and opposite charge replacements were made as K370A, R454D, and R454N. All of the mutants showed considerably reduced transport of PAH, estrone sulfate, and ochratoxin A, no transport of the organic cation 1-methyl-4-phenylpyridinium, but uptake of cimetidine similar to that of wild type. Interaction with the counter ion α-ketoglutarate was not tested. Interestingly, although the R454D mutant could not transport 1-methyl-4-phenylpyridinium, the double mutant R454D/K370A did so in preference to the anion PAH. In this case too, charge-conserving muta-

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2 The abbreviations used are: OAT1, organic anion transporter 1; PAH, p-aminohippurate; TMD, transmembrane domain; wt, wild type; OCT, organic cation transporter; ORI, oocyte Ringer’s solution.
TABLE 1
Mutations introduced into hOAT1
Shown are the primer pairs used for conservative (R466K) and nonconservative (R466D) amino acid substitutions. The bases that were changed in the codon are underlined. The codon itself is in parentheses.

|       | Primer Pair A               | Primer Pair B               | Primer Pair C               |
|-------|-----------------------------|-----------------------------|-----------------------------|
| R466K | 5’-GGGACACCAATGCCC (AAA)   | GTGGAGCAGCTGCTGTTG          | 3’-AACCACTTGAGCCCAAC (TTT) |
|       | (sense)                     | (antisense)                 | (antisense)                 |
| R466D | 5’-GCGGACATGCCC (GAC)       | GTGGAGCAGCTGCTGTTG          | 3’-AACCACTTGAGCCCAAC (TTT) |
|       | (sense)                     | (antisense)                 | (antisense)                 |

MATERIALS AND METHODS

Chemicals—All of the chemicals were of reagent grade. p-[glycyl-2-3H]Aminohippurate ([3H]PAH), 1–5 Ci/mmoll, was obtained from PerkinElmer Life Sciences. [1,5,14C]Glutaric acid (55 mCi/mmoll), was obtained from MP Biomedicals (Heidelberg, Germany). [3H(G)]Ochratoxin A (7.2 Ci/mmoll) was obtained from Moravek Biochemicals Inc. (Brea, CA). Unlabeled PAH, glutaric acid, adipic acid, and malonic acid and all other chemicals were obtained from Sigma.

Site-directed Mutagenesis and Plasmid Constructs—Mutations were created in the hOAT1 clone described previously that had a FLAG epitope in the large first extracellular loop between amino acid residues 107 and 108 (6). Mutations were introduced by use of the QuickChange site-directed mutagenesis kit (Stratagene, Cambridge, UK) according to the manufacturer’s instructions using two synthetic oligonucleotide primer pairs (sense and antisense) containing the desired mutation (Table 1). The presence of the mutation was confirmed on both strands in each clone by automated DNA sequencing (ABI 377; Applied Biosystems, Weiterstadt, Germany) followed by alignments using online tools (e.g. CusTaLW).

cRNA Transcription, Oocyte Injection, and Transport Experiments—wt and mutant hOAT1 plasmid DNA from the constructs described above were used as templates for respective cRNA synthesis. Plasmids were linearized with NotI, and in vitro cRNA transcription was performed using a T7 mMessage mMACHINE Kit (Ambion, Austin, TX). The resulting cRNAs were resuspended in purified, RNase-free water to a final concentration of 1 μg/μl.

Stage V–VI oocytes were defolliculated by overnight incubation at 18 °C with collagenase (0.5 mg/ml, Type CLSII; Biochrom, Berlin, Germany) in oocyte Ringer’s solution (ORI) (90 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES-Tris, pH 7.6) followed by washing for 10 min in Ca²⁺-free ORI. Oocytes were then injected with 23 nl of water or 23 ng of cRNA in an equivalent volume. After injection, the oocytes were incubated for 3 days at 18 °C in modified Barth’s solution (88 mM NaCl, 2 mM KCl, 0.82 mM MgSO₄, 0.66 mM NaNO₃, 0.77 mM CaCl₂, 5 mM HEPES/NaOH, pH 7.6) containing 12 μg/ml gentamycin.

Uptake of [3H]PAH, [14C]glutarate, or [3H]ochratoxin A was assayed at room temperature (experimental parameters are described in the figure legends) in ORI. FLAG-tagged OAT1 and mutants were used in all of the experiments. The oocytes were then rinsed with ice-cold ORI (3 × 4 ml) and dissolved in 1 N NaOH, and their 3H or 14C content was determined by liquid scintillation counting.

Cl⁻-free ORI was prepared by substitution with gluconate (90 mM Na⁺-gluconate, 3 mM K⁺-gluconate, 2 mM Ca²⁺-gluconate, 1 mM Mg²⁺-gluconate, 5 mM HEPES-Tris, pH 7.6). For transport assays in the absence of Cl⁻, the oocytes were initially washed three times with Cl⁻-free ORI over 15 min and then incubated at room temperature for the appropriate time periods, in the Cl⁻-free ORI containing the radiolabel.

For cis-inhibition experiments 1 mM malonic, glutaric, or adipic acid solutions were prepared in ORI, and pH was adjusted to 7.6. Uptake of [3H]PAH was assayed in the presence of each dicarboxyrate at room temperature for 1 h using uptake of [3H]PAH in ORI without any dicarboxylate as control.

Trans-stimulation experiments were performed by injecting oocytes with 46 nl of 5 mM unlabeled glutaric acid solution in water (pH adjusted to 7.6). They were then washed and kept on ice for 15 min, and then the uptake of [3H]PAH was assayed over 1 h in ORI. Oocytes not preloaded with glutarate served as controls.

Immunocytochemistry—To study hOAT1 trafficking, oocytes were injected with the cRNA of FLAG-tagged wt or mutant hOAT1 or an equivalent volume of water (mocks). On day 3 after injection, they were manually devitellinized after 5–10 min of incubation in 200 mM K⁺ aspartate and then fixed in Dent’s solution (80% methanol, 20% Me₂SO) overnight at −20 °C. The fixative was washed out, and oocytes were incubated with mouse anti-FLAG M2 IgG monoclonal antibody (Sigma) (dilution 1:1000) in the presence of 10% goat serum at 4 °C for 12 h. After being washed with phosphate-buffered saline, incubation with secondary Alexa 488 goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) (dilution 1:200) was performed at room temperature for 3 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 (Zeiss Axioshot, Germany).
Quantification of Surface Expression—Photographs were taken under exactly the same conditions with 1-s exposures, and no changes were made in the acquired image files for adjusting brightness, contrast, sharpness, etc. All of the images were 8-bit gray scale. Following this, the program Image J was used to assess the intensity of fluorescence as represented by their gray values. Rectangular selections of equal area were made on individual oocytes and the function analyze/measure was used to estimate minimum and maximum vertically averaged pixel intensity (gray values) along the section. We took three such measurements from each oocyte, and 10 oocytes were used for each group, i.e. mock, wild type hOAT1, and mutants R466K and R466D (see Fig. 2C).

Km Determinations—An indirect approach was applied for determining changes in affinity between mutant and wild type transporters and between normal and chloride free conditions. Oocytes expressing the respective transporter were assayed for 1 μM [3H]PAH uptake over 30 min in ORI with or without chloride and in the presence of increasing concentrations of unlabeled PAH (0–500 μM).

The addition of unlabeled PAH inhibited uptake of [3H]PAH by a process adequately described by the Michaelis-Menten equation for competitive interaction of the labeled and unlabeled substrate (15).

\[ V = \frac{V_{\text{max}} \cdot [S]}{K_m + [S] + C} \]

(Eq. 1)

where \( V \) is the rate of [3H]PAH transport from a concentration of labeled substrate equal to \([S] \); \( V_{\text{max}} \) is the maximum rate of mediated PAH transport; \( K_m \) is the PAH concentration that resulted in half-maximal transport; \([S]\) is the concentration of unlabeled PAH in the transport reaction; and \( C \) is a constant representing the component of total PAH uptake that was not saturated (over the range of substrate concentrations tested) and presumably reflected the combined influence of diffusive flux, nonspecific binding, and/or incomplete washing.

Kinetics of glutarate transport were determined using 1.9 μM [14C]glutarate and increasing concentrations of unlabeled glutarate, and \( K_m \) values were calculated as described for PAH above.

Statistical Analysis—All of the data presented are the means ± S.E. of the number of observations indicated in the text or figure legends. \( n \) is given as the number of experiments on oocytes from different donor animals, with the number of oocytes used per treatment in each individual experiment being 9–12. Statistical analysis was performed using SigmaPlot version 10 (Systat Software GmbH, Erkrath, Germany). Significant \( p \) values are indicated and were calculated by Student’s \( t \) test and one-way analysis of variance.

RESULTS

In Fig. 1A we show the currently accepted secondary structure model of the human organic anion transporter 1 that contains a positively charged arginine at position 466 in the middle of the putative 11th transmembrane helix (16, 17). This arginine residue is conserved in OAT1 homologues from human, monkey, pig, rabbit, mouse, rat, flounder, and Caenorhabditis elegans, as well as in other organic anion transporters of the SLC22 family: OAT2, OAT3, and OAT4 (Fig. 1B), and is also oppositely charged in the OCTs, which suggested to us an important role for this residue for function.
When expressed in Xenopus laevis oocytes, the FLAG-tagged wild type human OAT1 transports the model anion, p-aminohippurate (PAH; Fig. 2A). 1 μM [3H]PAH uptake into wt hOAT1 expressing oocytes was 2.7 ± 0.4 pmol/oocyte·30 min. By site-directed mutagenesis, we then replaced the arginine 466 of FLAG-tagged hOAT1 by lysine (mutant R466K), keeping the positive charge at this position untouched. This subtle mutation resulted in a considerably decreased uptake of PAH (Fig. 2A), although PAH uptake by R466K was still significantly higher than in mock oocytes. 1 μM [3H]PAH uptake by mutant R466K was 0.30 ± 0.05 pmol/oocyte·30 min. These values represent ~31-fold and 3.5-fold increases in PAH uptake over mocks by wt and mutant R466K-hOAT1 expressing oocytes, respectively. Charge reversal by substitution with aspartic acid (mutant R466D) led to a further loss of function by the transporter to about half that of R466K (see Fig. 4).

Because the lower transport rate of R466K could be the result of a decreased expression of this mutant, we used anti-FLAG antibodies to detect the protein at the cell membrane. As shown in Fig. 2B, mock oocytes did not show any immunoreactivity, excluding a nonspecific labeling of endogenous proteins by anti-FLAG antibodies. Oocytes expressing the FLAG-tagged wt OAT1, R466K, or R466D containing a C-terminal FLAG epitope were immunostained with anti-FLAG mouse IgG, followed by secondary Alexa 488 goat anti-mouse IgG. Thereafter, the oocytes were embedded in acrylamide, and 5 μM sections were analyzed by fluorescence microscopy. All of the images are 8-bit gray scale taken under the same conditions with a 20× fluorescence objective lens. For densitometry analysis, the plasma membrane fluorescence was quantified by determining the average maximum pixel intensities from a sample of 10 oocytes using Image J software. The gray values corresponding to fluorescence intensities are shown for the respective oocytes. ns, no significant difference from wt hOAT1.

Physiologically, OAT1 exchanges extracellular organic anions with intracellular α-ketoglutarate. We therefore preloaded oocytes with glutarate, the nonmetabolizable analogue of α-ketoglutarate. As shown in Fig. 4, this preloading significantly stimulated PAH uptake into wt-expressing oocytes. This increase in PAH uptake is termed trans-stimulation, because
glutarate was offered from the trans-side, and labeled PAH from the cis-side, of the oocyte cell membrane. A trans-stimulation of PAH uptake by intracellular glutarate was also observed with the mutant R466K, suggesting that the replacement of arginine 466 by lysine did not impair the interaction of OAT1 with dicarboxylates. A replacement of arginine by the negatively charged aspartate (R466D), however, not only decreased PAH uptake below that observed with R466K, but in the absence of chloride and by R466K in the presence or absence of chloride was of the same magnitude, suggesting that arginine 466 is required for the chloride sensitivity of OAT1.

The mutation of Arg466 may influence the chloride sensitivity of only the PAH uptake or of organic anion transport in general. Therefore we investigated the effect of chloride on the uptake of radiolabeled glutarate (Fig. 6, left panel) and ochratoxin A (right panel), two compounds with unrelated chemical structures. Chloride removal clearly inhibited glutarate uptake as well as ochratoxin A transport in wt-expressing oocytes. Uptake of both glutarate and ochratoxin A was found to be reduced in the mutant R466K and no longer to be dependent on the presence or absence of chloride. These results suggest that optimal transport of PAH, glutarate, and ochratoxin A requires chloride in wt OAT1 but not in the mutant R466K.

The strongly decreased uptake of PAH by the wt in the absence of chloride and by R466K in the presence and absence of chloride may be due to either a decrease in affinity or in maximal velocity of OAT1. To discriminate between these possibilities, we measured uptake at various concentrations of PAH and used the method of Malo and Berteloot (15) to determine $K_m$ and $V_{max}$ of PAH uptake (see “Materials and Methods” for details). The results are shown in Table 2. With the wt OAT1, the $K_m$ is $3.1 \pm 0.8 \mu M$ in the presence of chloride and $4.0 \pm 1.5 \mu M$ in its absence. The mutant R466K exhibited an apparent $K_m$ for PAH of $6.4 \pm 0.2 \mu M$ in the presence of chloride and of $4.5 \pm 1.1 \mu M$ in its absence. Therefore, chloride removal did...
Effect of Mutating Arg^{466} in hOAT1

It is recognized that the OATs have a truly multispecific substrate selectivity. In recent years the number of characterized pharmaceutically and physiologically important compounds interacting with the OATs has increased. Among pharmaceuticals, nonsteroidal anti-inflammatory drugs, antibiotics, antivirals, and antineoplastics (5, 20) and, among endogenous compounds, neurotransmitter metabolites and prostaglandins (1, 21, 22) have been shown to be handled by OAT1s. The next step, i.e. investigating interindividual variations or the pharmacogenomics has also been taken. Single nucleotide polymorphisms have been identified for OAT1, and pharmacologically relevant effects on drug handling have been demonstrated, for example, for antivirals (23–25).

This calls for greater understanding of the physiology of organic anion transport through OAT1s. Until the exact structure of OAT1s is known through crystallography, mutational analyses may provide insight. The existing crystal structures of the SLC family, i.e. that of H⁺lactose symporter (LacY) and the phosphate/glycerol-3-phosphate antiporter (GlpT), already provide some clues toward the structure of OAT1s and serve as templates for putative in silico molecular models (26). Indeed, positively charged residues embedded in the middle of TMDs have been found to be involved in substrate binding/translocation in both LacY and GlpT. Many researchers have used these models as templates to aid functional studies in transporters related to OAT1. In particular, the functional importance of anionic amino acids in the 11th TMD of rOCT1 (27) and in the 10th TMD of rOCT2 (28) have been demonstrated using these models as templates. Recently, Perry et al. (29) provided a theoretical three-dimensional model for hOAT1 based on the crystal structure of GlpT where they showed Arg^{466} (the residue investigated in this study) to be one of the residues that surround the putative active cavity of OAT1. In the present study we report that the positively charged arginine 466 in the helix 11 of hOAT1 is critically important for the transporter.

**Arg^{466} Is Important for Both Binding of Dicarboxylates and Conformational Changes Required for Substrate Translocation**

Mutation of this positively charged residue Arg^{466} to a negative aspartate D led to severely reduced transport of PAH. No trans-stimulation of radiolabeled PAH influx could be seen upon pre-loading with the exchange partner glutarate, suggesting that interaction with dicarboxylates was abolished. In our earlier studies with the flounder OAT1 (11), it was shown that this mutation led to a loss of interaction with dicarboxylates, and no trans-stimulation or cis-inhibition of PAH transport could be demonstrated along with a reduction of PAH affinity. Because of the extremely low uptake of R466D mutant in hOAT1, we were unable to measure affinity.

Because in flounder OAT1 only a nonconservative Arg to Asp substitution was made, in the present study, we made a conservative mutation wherein the cationic arginine 466 in hOAT1 was mutated to Lys, another cationic amino acid. We report that this conservative mutation could rescue the Arg to Asp mutant in many ways. First, the transport rate was increased; second, the affinity remained similar to the wild type transporter; third, cis-inhibition profile of the mutant by a...
series of dicarboxylates of increasing chain lengths remained qualitatively similar to the wild type; and fourth, the mutant transported glutarate and could also be trans-stimulated by it.

The counterparts of Arg⁴⁶⁶ in hOAT1 have been investigated in other transporters of the SLC22 family, namely, in hOAT1 (11), rOCT1 (27, 30), rOCT2 (28), rOAT3 (12), and rOCT2 (31). In the above cases, both a conservative mutation and $K_m$ determination was only made for hOAT1 (present study) and for rOCT1. In both cases it was found that the $K_m$ values remained the same (PAH for hOAT1 and 1-methyl-4-phenylpyridinium for OCT1) or were decreased (choline, tetraethylammonium, $N^\text{2}$-methylnicotinamide for OCT1), whereas the maximal transport rate ($V_{\text{max}}$) went down severely. Because the low $V_{\text{max}}$ could have been due to reduced surface expression, we checked for membrane trafficking and found similar expression of the mutant and wild type transporters.

Because arginine is ~1.5 Å longer than lysine and has a different $pK_a$ and hydrogen bonding profile, one possible explanation why lysine does not substitute for arginine is that Arg⁴⁶⁶ plays a role in conformational change via short range interactions and shortening the residue even by this little is not able to rescue the reduced turnover. The low turnover number indicates that this residue also contributes structurally to the transporter apart from its interaction with dicarboxylates. This may be through stabilizing the binding site through the formation of salt bridges with adjacent/nearby amino acids.

Because of the large substrate specificity of hOAT1, it is likely that a number of residues will contribute to substrate binding. The fact that in the R466K mutant affinity for PAH has remained unchanged and interaction with glutarate could be rescued by simply manipulating the charge implies that this residue is important for binding of dicarboxylates and may or may not be a player in PAH binding.

Arg⁴⁶⁶ Interacts with Chloride as a Major Determinant in Substrate Movements through the Transporter—Our second set of observations was concerned with determining what role chloride plays in OATs. Stimulation by chloride has been reported for a number of the cloned OATs (6, 19, 32), and an earlier study using basolateral membrane vesicles from rat kidney (33). Although OAT1 and OAT3 have been reported to be dependent on chloride such that the rate of transport decreases in the absence of chloride, OAT4 was stimulated under chloride-free conditions; however, it is not stimulated by chloride.

There arise two ways of interpreting these findings: 1) chloride is co-transported with organic anions via a binding site different from that of PAH, and 2) chloride binds to OAT1 but is not transported and serves other purposes important for turnover. The possibility of chloride transport has been tested in the earlier study by J. B. Pritchard (33) using basolateral membrane vesicles, and it was found that $^{36}\text{Cl}^-$ uptake by basolateral membrane vesicles was not altered by PAH transport, nor could a chloride gradient (in > out) stimulate PAH entry. Therefore chloride is not co-transported. We favor the other possibility that chloride binding stimulates the transporter through conformational changes resulting in increased turnover of the transporter. That is why chloride depletion leads to lowered $V_{\text{max}}$ at unaltered $K_m$. Based on our results, it is likely that chloride exerts these effects through the amino acid arginine 466, because the mutant R466K essentially behaves like hOAT1 under chloride free conditions; it has similar affinity for PAH, and it can be cis-inhibited and trans-stimulated by dicarboxylates; however, it is not stimulated by chloride.

In summary, we report that hOAT1 is stimulated by chloride, and in the absence of chloride its turnover number decreases, whereas PAH affinity does not change. The chloride effect is observed for other substrates as well. A charge-conserving mutation of the arginine in helix 11 of hOAT1, R466K, results in a functional mutant with low transport rates but similar affinity to PAH and similar interaction with dicarboxylates. The mutation of this single amino acid has two effects: 1) $V_{\text{max}}$ is reduced although surface expression remained the same, suggesting a structural role for this amino acid, and 2) chloride dependence is abolished, suggesting that this residue may be the one through which chloride exerts its stimulatory effects.

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