A Conserved Glutamate Residue in Transmembrane Helix 10 Influences Substrate Specificity of Rabbit OCT2 (SLC22A2)*§

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OCT1 and OCT2 are involved in renal secretion of cationic drugs. Although they have similar selectivity for some substrates (e.g. tetraethylammonium (TEA)), they have distinct selectivities for others (e.g. cimetidine). We postulated that "homolog-specific residues," i.e. the 24 residues that are conserved in OCT1 orthologs as one amino acid and in OCT2 as a different one, influence homolog-specific selectivity and examined the influence on substrate binding of three of these conserved residues that are found in the C-terminal half of the rabbit orthologs of OCT1/2. The N353L and R403I substitutions (OCT2 to OCT1) did not significantly change the properties of OCT2. However, the E447Q replacement shifted substrate selectivity toward an OCT1-like phenotype. Substitution of glutamate with cationic amino acids (E447K and E447R) abolished transport activity, and the E447L mutant displayed markedly reduced transport of TEA and cimetidine while retaining transport of 1-methyl-4-phenylpyridinium. In a novel homology model of the three-dimensional structure of OCT2, Glu447 was found in a putative docking region within a hydrophilic cleft of the protein. In addition, six residues identified in separate studies as exerting significant effects on OCT binding were also found within the putative cleft region. There was a significant correlation (r² = 0.82) between the IC₅₀ values for inhibition of TEA transport by 14 different compounds and their calculated Kᵣ values for binding to the model of rabbit OCT2. The results suggest that homology modeling offers an opportunity to direct future site-directed studies of OCT/substrate interaction.

Renal excretion is the principal pathway for elimination of many clinically used drugs and is the exclusive pathway for eliminating many end products of drug-metabolizing enzymes (1–3). Transporters in the renal tubule epithelium mediate secretion and thus play a critical role in detoxification (4). In addition, transporters control the exposure of renal cells to nephrotoxic drugs and environmental toxins and thereby influence xenobiotic-induced nephrotoxicity. A large fraction of these agents fall into the chemical class commonly referred to as "organic cations" (OCs), i.e. a diverse array of primary, secondary, tertiary, or quaternary amines that have a net positive charge on the amine nitrogen atom. At physiological pH, the proximal tubule is the primary site of renal OC secretion (3, 5), and the first step in the process is OC entry from the blood into proximal cells across the peritubular (i.e. basolateral) membrane. Three homologous transporters (OCT1, OCT2, and OCT3) have been cloned and subsequently shown to be expressed in the basolateral membrane of proximal cells (1, 6). In all species examined, including the human (7), the kinetic and selectivity profile of OCT3 and the comparatively low levels of mRNA and protein expression of this homolog in the kidney suggest that renal secretion is dominated by some combination of OCT1 and OCT2 activity. Perhaps the most convincing evidence of the significance of OCT1 and OCT2 in renal OC secretion is the observation that secretion of the prototypic OC, tetraethylammonium (TEA), is completely eliminated in OCT1/2−/− mice (8). In human kidney, OCT2 appears to be the predominant OC transporter. Expression of mRNA for OCT2 far exceeds that for OCT1, and immunocytochemistry clearly shows a basolateral expression for OCT2 and little or no presence of OCT1 (9). There are, however, clear species differences in this profile, with substantial expression of both OCT1 and OCT2 evident in both rat and rabbit kidney (10, 11). Notably, the relative functional expression of OCT1 and OCT2 has only been established in the mouse (8) and rabbit (12), with clear evidence indicating that both OCT1 and OCT2 can play a quantitatively significant role in OC secretion. Nevertheless, OCT2, which interacts with a variety of OCs, including many clinically used drugs (e.g. procainamide and cimetidine), hormones (e.g. norepinephrine and dopamine), and toxic substances (e.g. 1-methyl-4-phenylpyridinium (MPP⁺)), evidently plays a key role in all species in governing the entry of OCs from the blood into the renal tubule, thereby controlling the first step in renal secretion of OCs.

The orthologs of OCT1 and OCT2 have been cloned from human (13, 14), rat (15, 16), mouse (17, 18), and rabbit (19, 20), and in each species, these homologs share ~70% amino acid identity and >90% sequence similarity. This high degree of shared structure is consistent with the shared functional characteristics of these homologous transporters. Indeed, there is strong evidence supporting the contention that these transporters arose by gene duplication (21). However, despite the similar affinities for some substrates generally observed between OCT1 and OCT2 homologs in any of these species (e.g. for TEA) (11), these transporters are typically distinguished from one another by markedly different affinities for selected substrates (11, 22). These differences in selectivity presumably reflect differences in the identity of a subset of the amino acid residues that distinguish OCT1 from OCT2 homologs. Here, we have tested the hypothesis that shifts in selectivity of OCT2 toward a more OCT1-like phenotype will result from mutation of one or more of the residues that distinguish these two homologous proteins. We identified one such residue in OCT2 (Glu447 in the rabbit ortholog) that exerted a marked influence on substrate selectivity. This observation has been interpreted in light of a proposed homology model of the structure of rabbit OCT2 and a recently described model of the structure of rat OCT1 (23), both of which are based upon recent develop-
ments in the understanding of the structure of proteins in the major facilitator superfamily (MFS) of transporters.

EXPERIMENTAL PROCEDURES

Preparation of Site-directed Mutants—Wild-type rabbit (rb) OCT2 was subcloned into the pcDNA3.1 expression vector as described previously (20). To facilitate the immunocytological localization of expressed mutants within transiently transfected cells, the V5 epitope tag (amino acid sequence GKP IPN PLLG LDST, nucleotide sequence GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG) was added to the N terminus of rbOCT2 using PCR amplification. Mutations of the V5-tagged rbOCT2 sequence were introduced by site-directed mutagenesis using the QuikChange system (Stratagene) following the manufacturer’s instructions. Plasmid DNA was prepared using standard methods (Geneseek Scientific, San Diego, CA). The final plasmids were sequenced to confirm the presence of the mutation and the absence of PCR artifacts.

Cell Culture and Transfection—Chinese hamster ovary (CHO) cells were grown at 37 °C in a humidified atmosphere (5% CO2) in plastic culture flasks. The medium was Kaighn’s modified Ham’s F-12 medium supplemented with 10% fetal calf serum. Cultures were split every 3 days. Cells were transfected via electroporation (ECM 630, BTX, San Diego, CA) with a plasmid containing the eDNA sequence for the desired construct. Briefly, cells were transfected with 10 μg of DNA at 260 V (time constant of ~25 ms) and seeded onto 12-well plates at 320,000 cells/well. Uptake was typically measured 48 h after transfection, after the cells had reached confluence.

Measurement of Transport—CHO cells were incubated at room temperature (~25 °C) in Waymouth buffer (135 mM NaCl, 13 mM HEPES-NaOH, pH 7.4, 28 mM d-glucose, 5 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, and 0.8 mM MgSO4) to which labeled substrate and appropriate test agents were added. Uptake was stopped by rinsing the cells three times with 2 ml of ice-cold Waymouth buffer. The cells were then solubilized in 400 μl of 0.5 N NaOH with 1% (v/v) SDS. (The extract was neutralized with 200 μl of 1 N HCl.) Accumulated radioactivity was determined by liquid scintillation spectrometry. Rates of uptake are expressed as moles/cm2 of nominal cell surface of the confluent monolayer.

Immunocytochemistry—CHO cells were electroporated with plasmid DNA containing the various epitope-tagged sequences and seeded onto coverslips. Immunocytochemistry was generally performed on a confocal microscope. Briefly, cells were fixed and permeabilized with methanol for 20 min, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na2HPO4, and 1.5 mM KH2PO4, pH 8; all washes were done in triplicate), and incubated for 1 h with anti-V5 antibody (Invitrogen) diluted 1:500 in PBS. The cells were then washed with PBS and incubated for 1 h in the dark with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Molecular Probes, Inc.) diluted 1:1000 in PBS.

Western Blotting—Crude CHO membranes were prepared as described (24), and protein concentration was determined by the Bradford protein assay (Bio-Rad). Thirty micrograms of total membrane protein was separated on a 10% Tris/glycine/SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (25). The membrane was blocked for 1 h with 6% milk in PBS at room temperature, followed by incubation overnight with anti-V5 antibody (1:5000 dilution) at 4 °C. After extensive washing with 0.05% PBS/Tween, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Promega Corp.). The secondary antibody was detected with the SuperSignal West Femto maximum sensitivity substrate kit (Pierce).

Homology Modeling of the Three-dimensional Structure of rbOCT2—
The rbOCT2 sequence was used as a “probe” to search homologous sequences (PSI-BLAST, NCBI Database) and sequence-based structural relatives (3D-PSSM) (26). GlpT (Protein Data Bank code 1PW4) and LacY (code 1PV6) were returned as the top hits in both search algorithms. Notably, although the sequence identity (between rbOCT2 and GlpT or LacY) was low (<15%), the sequence similarity was ~50%. The transmembrane α-helices of rbOCT2 were predicted using TMHMM (27). The putative transmembrane-spanning helices (TMHs) of rbOCT2 were then aligned with the respective α-helices of LacY and GlpT using ClustalW. In parallel, we also computed the hydropathy plots and helical wheel plots. These suggested that TMH1 and TMH12 were not aligned correctly with respect to the crystal structures. Significantly, the highly conserved motifs that fall between TMH2 and TMH3 and between TMH7 and TMH8 of MFS proteins (28) were well aligned between the rbOCT2 and GlpTs sequences. Based on these factors, we performed some deletions and modifications to fine-tune the sequence alignment. The final sequence alignment was used for target-template homology modeling, which was performed using ICM 3.0 (MolSoft L.L.C., La Jolla, CA). The model was refined in SYBYL 6.9 (Tripos, Inc., St. Louis, MO) using the Powell method (29), and the minimization routine was run for 7000 cycles. The model was validated using PROCHECK (30), WHATIF (31), and PROVE (32).

RESULTS

Multiple alignment of the human, rat, mouse, and rabbit orthologs of OCT1 and OCT2 revealed 24 residues that are conserved in all orthologs of OCT2 as one amino acid, but are conserved as a different amino acid in all orthologs of OCT1. Most of these conserved differences involve either strongly or weakly similar amino acids, e.g. F23L (OCT2 to OCT1) and L249V. However, seven involve residues with comparatively different physicochemical characteristics, e.g. a charged residue replaced with a neutral residue or a polar residue replaced with a lipophilic residue. We further narrowed our focus to residues found in the C-terminal half of OCT2 in light of several studies that implicated the C-terminal half as playing a major role in influencing selectivity of SLC22A family members (33, 34). Consequently, our initial work, described here, focused on three residues in the C-terminal half of OCT2 (N353L, R403I, and E447Q) and the influence on OCT2 selectivity of mutating these residues from their conserved OCT2 identity to that expressed in OCT1.

Effect of Mutations on the Kinetics of Substrate Interaction—We elected to use the rabbit ortholog of OCT2 to assess the influence of the selected mutations on transport activity because rbOCT2 generally displays a greater degree of homolog-specific selectivity over rbOCT1 than noted for the human orthologs of these transporters (35). In particular, the >20-fold difference in affinity for cimetidine that distinguishes rbOCT2 from rbOCT1 (11, 20) was anticipated as being a valuable tool for determining the bases of such differences in selectivity. We also reasoned that structural information gained from the study of rbOCT orthologs is likely to be relevant to understanding the structure of OCTs in other species as well. Fig. 1 compares the uptake of [3H]TEA by wild-type rbOCT2 with that supported by the N353L, R403I, and
E447Q single, double, and triple mutants of rbOCT2. Each mutant displayed significant transport of TEA. Uptake into transiently transfected CHO cells was nearly linear for at least 5 min (data not shown), and 5-min uptakes were generally used to generate initial rates of transport for kinetic analyses.

OCT1 and OCT2 have a similar apparent affinity for TEA ($K_v = 141$ and 165 $\mu M$, respectively; $p > 0.05$) (TABLE ONE; see also Ref. 22), and we hypothesized that replacing OCT2-specific residues with those found in OCT1 would have comparatively little effect on the affinity of the mutant transporter for TEA. As shown in Fig. 2 and TABLE ONE, this proved to be the case: although there was a general trend for a slight increase in apparent affinity of some of the mutant transporters for TEA (i.e. a decrease in $IC_{50}$), it was not significant. We did expect, however, that one or more of the residue replacements would result in a decrease in affinity for cimetidine and for the fluorescent cation $N,N,N$-trimethyl-2-(methyl-7-nitro-2,1,3-benzoxadiazol-4-yl)amine ethanaminium iodide (NBD-MTMA) (36), i.e. a shift toward a more “OCT1-like” selectivity. This proved to be the case: although neither the N353L nor R403I mutation by itself resulted in a significant change in the $IC_{50}$ for inhibition of TEA uptake by cimetidine and NBD-MTMA, every protein that included the E447Q mutation displayed a significant decrease in apparent affinity for cimetidine and NBD-MTMA (Fig. 2). The $IC_{50}$ for cimetidine inhibition of TEA uptake by the E447Q mutant was increased >15-fold (from 2.1 to 33.4 $\mu M$; $p < 0.05$), and that for NBD-MTMA was increased 2.5-fold (from 11.6 to 28.3 $\mu M$; $p < 0.05$). The inclusion of both the E447Q and N353L conversions produced a further decrease in apparent affinity for both cimetidine ($IC_{50}$ increase from 33.4 to 104 $\mu M$) and NBD-MTMA (from 28.0 to 78.9 $\mu M$; $p < 0.05$). Interestingly, the affinity of the N353L/R403I/E447Q triple mutant for both cimetidine and NBD-MTMA did not differ significantly from that displayed by OCT1.

These results implicated E447Q in the homolog-specific binding characteristics of OCT2. Consequently, we examined in more detail the influence of replacing Glu$^{447}$ with other amino acids. Not surprisingly,

![Graph showing the influence of single, double, and triple residue replacements on the rate of uptake TEA into CHO cells transiently transfected with the cDNA for rbOCT2 containing the indicated mutations.](image)

**FIGURE 1.** Influence of single, double, and triple residue replacements on the rate of uptake TEA into CHO cells transiently transfected with the cDNA for rbOCT2 containing the indicated mutations. The height of each bar represents the mean uptake $\pm$ S.E. of three to five determinations of uptake based upon 5-min incubations in buffer containing 55 nm [3H]TEA. Uptake into cells containing wild-type (WT) rbOCT2 averaged 10-fold over non-transfected CHO cells. *, $p < 0.05$ compared with the wild-type protein.

| Mutant          | $K_v$ (μM) | Cimetidine $IC_{50}$ (μM) | NBD-MTMA $IC_{50}$ (μM) |
|-----------------|------------|---------------------------|-------------------------|
| rbOCT2          | 165±50 (3.6±1.11) | 2.1±0.2                  | 11.6±0.6                |
| rbOCT1          | 141±44 (5.0±1.65)  | 80.6±6.0$^*$              | 70.9±21.0$^*$           |
| N353L           | 94.4±19.3 (2.9±1.76) | 3.4±0.3                  | 11.1±1.5                |
| R403I           | 60.2±4.7 (0.90±0.192$^*$ | 2.5±0.6                  | 13.4±2.4                |
| E447Q           | 136±45 (4.9±0.37)  | 33.4±3.9$^*$              | 28.0±3.1$^*$            |
| N353L/E447Q     | 68.0±21.4 (5.0±0.07$^*$ | 104±29$^*$               | 78.9±6.5$^*$            |
| R403I/E447Q     | 60.9 (4.0)       | 27.0±4.0$^*$              | 28.3±3.4$^*$            |
| N353L/R403I/E447Q | 92.1±18.4 (5.0±2.06) | 54.4±5.9$^*$             | 62.5±10.2$^*$           |

$^*$ The measured value differed significantly from the value determined for wild-type rbOCT2.

![Graph showing the effect of selected residue replacements on the kinetics of TEA transport or inhibition of TEA transport by cimetidine or NBD-MTMA mediated by rbOCT2.](image)

**FIGURE 2.** Effect of selected residue replacements on the kinetics of TEA transport or inhibition of TEA transport by cimetidine or NBD-MTMA mediated by rbOCT2. The initial rates of uptake of 55 nm [3H]TEA were based upon 5-min uptakes measured in the presence of increasing concentration of unlabeled TEA (A), unlabeled cimetidine (CIM; B), or unlabeled NBD-MTMA (NBD; C) into CHO cells transiently transfected with the cDNA for the indicated transporter: rbOCT2 (C), rbOCT1 (A), N353L (●), R403I (■), E447Q (▲), N353L/E447Q (★), and N353L/R403I/E447Q (◆). Uptake is expressed as the percentage of uptake observed in the absence of added unlabeled compound. Each point represents the mean uptake $\pm$ S.E. measured in three to nine separate experiments.
replacement of the anionic glutamate with cationic residues, i.e. arginine and lysine, exerted a profound effect on activity of the resulting mutant. In fact, we could detect no transport of either TEA or the anionic substrate \( p \text{-aminohippurate} \) (PAH) by these constructs (data not shown). The failure to support transport did not appear to reflect a failure of the constructs to be synthesized following transfection; as shown in Fig. 3 (left panel), a Western blot of crude membranes isolated from CHO cells transiently transfected with a plasmid containing recombinant rbOCT2 constructs expressed in CHO cells. Nuclei and rbOCT2 protein were stained in transiently transfected CHO cells using propidium iodide (red) and the antibody against the C-terminal V5 tag of the indicated OCT2 constructs (green).

Although we cannot reject the idea that the mutant protein was restricted to an intracellular compartment just below the plasma membrane, it was clearly not confined to the perinuclear endoplasmic reticulum, as might be expected if the mutant protein was misfolded.

Replacement of Glu\textsuperscript{447} with either leucine or alanine was also associated with markedly decreased TEA transport. However, despite a profound reduction in TEA transport, we noted that both E447L and E447A retained the ability to accumulate the fluorescent cation NBD-MTMA (data not shown). Consequently, we examined the characteristics of one of these mutants, i.e. E447L, in more detail. Fig. 4 compares the rates of TEA, MPP\textsuperscript{+}/H\textsubscript{11001} (55 nM) (A), \([\text{H}]\text{MPP}^+\) (16 nM) (B), and \([\text{H}]\text{cimetidine} \) (54 nM) (C). Each point is the mean ± S.E. of the 30-s uptake into CHO cells that stably expressed rbOCT2 or the E447L mutant measured in three separate experiments. CIM, cimetidine.
the apparent affinity of OCT2 and its E447L mutant for TEA was estimated by determining the IC\textsubscript{50} for TEA inhibition of MPP\textsuperscript{+} transport mediated by both transporters. As shown in Fig. 5A, the E447L mutation decreased the apparent affinity for TEA as shown by the significant increase in the IC\textsubscript{50} for inhibition of MPP\textsuperscript{+} transport, from a control value of 83 ± 16.0 \mu M to 750 ± 127.3 \mu M. (Note that the IC\textsubscript{50} for TEA inhibition of OCTC-mediated MPP\textsuperscript{+} transport did not differ significantly from the K\texttextsubscript{m} for TEA transport, as expected.) Given the very large decrease in TEA uptake measured in the E447L mutant in the face of maintained rates of MPP\textsuperscript{+} uptake (Fig. 4), we think it likely that the mutation also produced a substantial decrease in turnover number of the transporter when bound to TEA. This contention is further supported by the interesting observation that, although the rate of cimetidine transport was markedly reduced in the E447L mutant (Fig. 4), its apparent affinity for cimetidine (9.0 ± 0.73 \mu M), as determined in lines of CHO cells that stably expressed either the wild-type transporter or the E447L mutant, remained comparable with that of wild-type rbOCT2 (16.9 ± 2.48 \mu M) (Fig. 5C). Thus, although retaining a structure consistent with comparatively high affinity binding to cimetidine, the presence of leucine at position 447 may have caused a change in protein structure that resulted in reduced turnover of the transporter-substrate complex.

Homology Model of the Three-dimensional Structure of rbOCT2—The three-dimensional structures of three MFS transporters have been determined and found to share a common tertiary fold (37–39). Two of these, the sugar/H\textsuperscript{+} cotransporter LacY (37) and the glycerol phosphate/phosphate exchanger GlpT (38), have been resolved at 3.5 \AA. Furthermore, the LacY structure was solved with (Protein Data Bank code 1PV7) and without (code 1PV6) a ligand in the substrate-binding site. There is now strong evidence to support the contention that all members of the MFS share a common structural fold with similar topological organization of their \alpha-helices (40). With that in mind, we developed a homology model of the three-dimensional structure of rbOCT2 using the GlpT structure as the template. Because of the comparatively low degree of similarity between the full-length sequences of GlpT and rbOCT2 (13.8\% amino acid identity and a total of 41.5\% similarity), aspects of the alignment were, of necessity, based on structural insight (see supplemental Fig. A). An 11-residue motif between TMM2 and TMH3 and between TMH8 and TMH9 that is highly conserved in MFS members (28) was evident in the alignment of GlpT and rbOCT2, and this enhances the confidence in the alignment of the adjacent helices (i.e. helices 2/3 and 7/9). However, there is less confidence associated with the alignment adjacent to the "long loops,” i.e. the long cytoplasmic loops found in both GlpT and the OCTs between TMH6 and TMH7 and the long external loop found between TMH1 and TMH2 in the OCTs (which is not found in other MFS transporters). To facilitate modeling, these loops were edited out of the sequences, but the rationale for selection of precise regions for elimination introduced a degree of arbitrariness to the resulting alignment of the adjacent helices. The use of TMH prediction algorithms provided a degree of independent assessment of the general accuracy of the model. It should be noted that the computationally derived position of TMH1 of both OCT1 and OCT2 routinely placed the valine at position 40 within helix 1. However, the homologous position in the closely related protein OAT1 (Asn\textsuperscript{39}) has been shown to be a site of glycosylation (41). Consequently, we adjusted the alignment to ensure that Val\textsuperscript{40} was outside of the membrane (supplemental Fig. A).

Fig. 6 shows the resulting three-dimensional homology model of rbOCT2 based on the GlpT template. The root mean square deviation for the superposition of the refined and minimized rbOCT2 model
Homolog-specific Residues and OCT2 Selectivity

**TABLE TWO**

Predicted docking characteristics of the OCT2 homology model

The measured IC$_{50}$ values are compared with the calculated $K_d$ values generated from three postulated docking surfaces within the central cleft of the homology model of the OCT2 structure or homologous docking surfaces found within the central clefts of the GlpT and LacY structures (see ‘Discussion’). The $K_d$ values were based upon G scores generated by FlexX. IC$_{50}$ values were taken from Ref. 35.

| Compound | IC$_{50}$ (µM) | 5 Å Specific residues | 10 Å Specific residues | 5 Å + specific residues | GlpT | LacY |
|----------|----------------|-----------------------|------------------------|-------------------------|------|------|
| TEA      | 86.5           | 9.3 (−115)            | 6.2 (−119)             | 5.6 (−120)              | 115 (−90) | 11,900 (−44) | 957 (−69) |
| MPP      | 1.43           | 0.8 (−139)            | 3.1 (−126)             | 3.4 (−125)              | 9.3 (−115) | 34.4 (−102) | 94.3 (−92) |
| Cimetidine | 3.3           | 10.3 (−114)           | 3.8 (−124)             | 3.1 (−126)              | 17.0 (−109) | 523 (−75) | 640,000 (−4) |
| Phenformin | 7.33          | 12.6 (−112)           | 38.1 (−101)            | 63.0 (−96)              | 0.3 (−148) | 89.100 (−24) | 190 (−90) |
| Serotonin | 664.00         | 156.0 (−87)           | 8.4 (−116)             | 13.9 (−111)             | 17.0 (−109) | 2370 (−60) | 316 (−80) |
| Tyramine | 426            | 127.5 (−89)           | 31 (−80)               | 85.2 (−93)              | 2370 (−60) | 316 (−80) | 957 (−69) |
| Pindolol | 67.5           | 7.6 (−117)            | 10.3 (−114)            | 0.7 (−140)              | 3.4 (−125) | 63.0 (−96) | 12.6 (−112) |
| Propranolol | 30.0         | 1.9 (−131)            | 15.4 (−110)            | 34.4 (−102)             | 5.1 (−121) | 0.01 (−183) | 58.2 (−82) |
| NMN      | 180            | 285.5 (−81)           | 28.1 (−104)            | 316 (−80)               | 1750 (−63) | 48,700 (−30) | 233 (−83) |
| Clonidine | 0.19           | 0.6 (−142)            | 5.6 (−120)             | 4.1 (−123)              | 0.6 (−142) | 25.4 (−105) | 2370 (−60) |
| PAH      | 5000           | 472.5 (−76)           | 473 (−76)              | 427 (−77)               | 639 (−73) | 20.8 (−107) | 4330 (−54) |
| Probenecid | 1800          | 248.0 (−82.4)         | 1020 (−68.4)           | 2370 (−60)              | 865 (−70) | 85. (−93) | 115 (−90) |
| NBD-MTMA | 2.3            | 2.0 (−130)            | 7.6 (−117)             | 20.8 (−107)             | 6.9 (−118) | 34. (−102) | 31.1 (−103) |
| Choline  | 1390           | 315.8 (−80)           | 427 (−77)              | 12.6 (−112)             | 1430 (−65) | 38 (−78) | 1940 (−62) |

Based on the C-α backbone with the crystal structure of GlpT was 2.3 Å. Clearly evident is the pseudo 2-fold symmetry that arises from the helical organization of the N- and C-terminal halves of the protein and that appears to be a common structural feature of MFS proteins (40). The water-filled “pocket” that exists between these two domains is suspected to contain the sites involved in substrate binding (as found for both GlpT (38) and LacY (37)). This pocket is formed by the relative positions of TMH1, TMH2, TMH4, and TMH5 (in the N-terminal half of the protein) and TMH7, TMH8, TMH10, and TMH11 (in the C-terminal half of the protein). Consequently, it is reasonable to hypothesize that selected residues found in these helices may reside in or adjacent to the water-filled cavity that composes the pocket and could strongly influence the binding of substrates to rbOCT2. Lending support to this hypothesis was the observation that Glu447 proved to reside within TMH10 and was directed toward the putative binding pocket. Additional support for the structure presented in Fig. 6 was derived from the observation that residues shown in other studies to influence binding were also found to be directed toward the putative binding region of OCT2, including residues in TMH4 (Trp217, Tyr221, and Thr225) (23), TMH11 (Asp475) (42), and two additional residues in TMH10 (Leu446 and Leu449) (34). Interestingly, Asp475, the first residue implicated in the binding of substrates to OCTs (i.e. Asp575 in rat (r) OCT1) (42), was found at a position within the putative binding pocket that was virtually identical to the positions of the residues associated with the binding of sugar to LacY (37) and the postulated binding of glycerol phosphate to GlpT (38).

Docking Analysis—One of the principal reasons for developing a three-dimensional structure of OCT2 is development of a predictive model of transporter/substrate interactions. Consequently, it was of particular interest to compare the predicted docking energies of substrates within the putative binding region of the rbOCT2 homology model with experimentally determined kinetic parameters for substrate/inhibitor interactions with rbOCT2. We generated three distinct docking surfaces, each derived from appropriate “receptor description files,” for use with the FlexX docking program (BioSolvIT GmbH). The first two were based upon the location of ligand within the LacY binding site. The rbOCT2 model was superimposed over the LacY ligand crystal structure (Protein Data Bank code 1PV7), and the rbOCT2 residues that were within either 5 or 10 Å (first and second models, respectively) of the ligand (β-D-galactopyranosyl 1-thio-β-D-galactopyranoside) were identified and used to prepare receptor description files. The third model was based upon a docking surface that contained only those residues identified in this and previous studies (23, 34, 42) to study substrate binding to OCTs (i.e. the residues shown in Fig. 6, A and B). We then used FlexX to generate G scores for the binding interaction of each of 14 different compounds selected from a set of compounds examined in a previous study on the molecular determinants of substrate interaction with OCT2 (35). The selected compounds (TABLE TWO) provided a range of three-dimensional structures and apparent affinities for OCT2. FlexX generates four parameters related to ligand docking: PMF score, G score, D score, and F score. Scatter diagrams of these scores versus the experimentally determined IC$_{50}$ values for the test compounds showed that the measured IC$_{50}$ values most closely correlated with the G score (values listed in TABLE TWO). The G score is related to the binding free energy ($G_b$) by the empirically determined relationship $\Delta G_b = (G \text{ score})/4$ (reflecting the uniform weighting of the four FlexX parameters in our analysis) (43, 44). Apparent dissociation constants ($K_{d}$) for ligand transport interactions were then derived from the relationship $K_{d} = \exp(\Delta G_b/RT)$.

Fig. 7 shows the relationship between the measured IC$_{50}$ values for the battery of test compounds and the corresponding $K_d$ values predicted employing each of the three model docking surfaces. Although all three models displayed a significant correlation ($p < 0.05$) between measured and predicted binding interactions, the docking surface that was restricted to only those residues currently known to exert influence on OCT/substrate interactions showed the weakest correlation ($r^2 = 0.32$); it is likely that this docking surface did not include one or more key residues. The 5 Å model showed the strongest correlation ($r^2 = 0.82$) and distinguished with surprising precision between ligands that interacted poorly with rbOCT2 (e.g. PAH, probenecid, and choline) and those that interacted quite well (e.g. clonidine, NBD-MTMA, and MPP ). Interestingly, the 5 Å docking surface did not include several of the specific residues shown to influence binding of substrates to rOCT1 (23, 34), i.e. three within TMH4 (Trp217, Tyr221, and Thr225) and one...
within TMH10 (Ile<sup>442</sup>), indicating that each of these residues resides >5 Å from the volume defined by the coordinates of the LacY β-D-galactopyranosyl 1-thio-β-D-galactopyranoside ligand. To determine whether the inclusion of these residues in the 5 Å docking surface of rbOCT2 would improve its calculated interaction with the test battery of OC ligands, we constructed a new docking surface consisting of the "5 Å residues" plus the additional "specific" residues noted above. Although the predicted $K_D$ values obtained using this docking surface (TABLE TWO) correlated with the measured $IC_{50}$ values for the test OCs, the correlation ($r^2 = 0.544$) was not as good as that obtained using the 5 Å surface (data not shown). Fig. 6C shows a structural model of the 5 Å docking surface and the predicted lowest energy configuration of TEA within the associated cleft.

It was relevant to consider the extent to which the calculated interaction of the test compounds with the rbOCT2 docking surface was dependent upon the residues that composed that surface. To that end, we determined the binding energies for the interaction of the test compounds with 5 Å docking surfaces created from the structures of GlpT and LacY, again based upon the position of the LacY β-D-galactopyranosyl 1-thio-β-D-galactopyranoside ligand within the putative binding pocket. As shown in Fig. 7D, there was no correlation between the measured $IC_{50}$ values and $K_D$ values predicted for docking to either GlpT or LacY.

DISCUSSION

Although a number of cationic substrates, including TEA, quinidine, and histamine, typically interact nearly equally with both OCT1 and OCT2 (11, 22), these two transporters show very different affinities for other compounds. The markedly higher affinity of rbOCT2 for cimetidine compared with rbOCT1 (11) is a consequence of the influence of a comparatively small number of amino acid residues within the sequences of these two homologous proteins that are conserved across all orthologs of OCT1 as one amino acid, but are conserved as a different amino acid in all OCT2 orthologs. There are 24 such residues in OCT1/OCT2 (supplemental Table A). To test this hypothesis, we focused on the effect of rbOCT2 selectivity of these residues, i.e. N353L, R403I, and E447Q. These sites were targeted because the replacement residue for each (i.e. the residue expressed in OCT1) has markedly different physicochemical properties compared with that in OCT2. In addition, preliminary studies on the activity of OCT1/OCT2 chimeric constructs suggested that the structural factors that influence homolog-specific selectivity reside predominantly in the C-terminal half of these transporters. Consistent with our hypothesis, none of the mutants (single, double, or triple) displayed a significant change in apparent affinity for TEA, a substrate for which OCT1 and OCT2 have a similar affinity (Fig. 2). Selected residue replacements did, however, exert a significant effect on the apparent affinity of rbOCT2 for cimetidine and NBD-MTMA. All the replacement combinations that included E447Q had a decreased affinity (i.e. increased $IC_{50}$) for these selective substrates (TABLE ONE). Interestingly, although neither N353L nor R403I by itself had an effect on the interaction with either compound, the inclusion of N353L with E447Q exerted a synergistic effect on the decrease in affinity for cimetidine and NBD-MTMA (Fig. 2). These data suggest that Glu<sup>447</sup> and Asn<sup>353</sup> play significant roles in defining a binding surface within rbOCT2 that is responsible for its characteristic homolog-specific selectivity.

Replacing Glu<sup>447</sup> with residues other than glutamine also caused altered binding profiles in the resulting rbOCT2 mutants. Replacing the anionic glutamate residue with either lysine or arginine completely eliminated transport activity; the resulting mutants did not support uptake of TEA, cimetidine, MPP<sup>+</sup>, or PAH despite the fact that the mutant proteins appeared to be expressed in the plasma membrane of transfected CHO cells (Fig. 3). The E447A mutant displayed greatly reduced uptake of both TEA and MPP<sup>+</sup>, although it showed accumulation of the fluorescent cation NBD-MTMA. The E447L mutant sup-

We hypothesized that the homolog-specific selectivity of OCT2, as exemplified by its high affinity for cimetidine and NBD-MTMA compared with rbOCT1 (11), is a consequence of the influence of a comparatively small number of amino acid residues within the sequences of these two homologous proteins that are conserved across all orthologs of OCT1 as one amino acid, but are conserved as a different amino acid in all OCT2 orthologs. There are 24 such residues in OCT1/OCT2 (supplemental Table A). To test this hypothesis, we focused on the effect of rbOCT2 selectivity of these residues, i.e. N353L, R403I, and E447Q. These sites were targeted because the replacement residue for each (i.e. the residue expressed in OCT1) has markedly different physicochemical properties compared with that in OCT2. In addition, preliminary studies on the activity of OCT1/OCT2 chimeric constructs suggested that the structural factors that influence homolog-specific selectivity reside predominantly in the C-terminal half of these transporters. Consistent with our hypothesis, none of the mutants (single, double, or triple) displayed a significant change in apparent affinity for TEA, a substrate for which OCT1 and OCT2 have a similar affinity (Fig. 2). Selected residue replacements did, however, exert a significant effect on the apparent affinity of rbOCT2 for cimetidine and NBD-MTMA. All the replacement combinations that included E447Q had a decreased affinity (i.e. increased $IC_{50}$) for these selective substrates (TABLE ONE). Interestingly, although neither N353L nor R403I by itself had an effect on the interaction with either compound, the inclusion of N353L with E447Q exerted a synergistic effect on the decrease in affinity for cimetidine and NBD-MTMA (Fig. 2). These data suggest that Glu<sup>447</sup> and Asn<sup>353</sup> play significant roles in defining a binding surface within rbOCT2 that is responsible for its characteristic homolog-specific selectivity.

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FIGURE 7. Relationship between $IC_{50}$ values measured for the inhibition of rbOCT2-mediated TEA transport by a set of 14 compounds (TABLE TWO) and the calculated $K_D$ values for their binding on postulated docking surfaces of the rbOCT2 homology model. See text for details of the assumptions associated with each surface. A, 5 Å docking surface of the rbOCT2 model. B, 10 Å docking surface of the rbOCT2 model. C, docking surface restricted to the specific (Spec) residues noted above to influence binding to OCTs. The dashed lines show the linear regression between measured and predicted values (with the indicated $r^2$ values). All correlations were significant ($p < 0.05$). D, relationship between $IC_{50}$ values for the battery of test compounds and their calculated $K_D$ values for binding to the homologous 5 Å docking surface derived from the structures of GlpT and LacY. The solid line is the regression against calculated LacY $K_D$ values. Neither correlation was significant.
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reported nearly normal uptake of MPP\(^+\) (and of NBD-MTMA), but only very low rates of TEA and cimetidine uptake. Notably, although the low rate of TEA transport was associated with a marked decrease in apparent affinity of the E447L mutant for TEA, cimetidine continued to be a high affinity inhibitor of E447L transport activity (of MPP\(^+\)) (Fig. 5) despite the fact that cimetidine was itself transported only poorly (Fig. 4).

The evidence presented here implicating Glu\(^{447}\) as a key contributor to the binding properties of rOCT2 is consistent with the recent observation by Gorboulev et al. (34) that the homologous residue in rOCT1, i.e. Gln\(^{448}\), plays a key role in the low affinity of rOCT1 for corticosterone, in contrast with rOCT2. Two additional residues in the putative TMH10 in rOCT1, i.e. Ala\(^{443}\) and Leu\(^{447}\), were also shown to influence the affinity of that transporter for corticosterone, with replacement of these residues with those found in OCT2 resulting in a marked increase in affinity of the OCT1 mutant for corticosterone. In addition, mutation of Leu\(^{447}\) and Gln\(^{448}\) was associated with differential changes in affinity for rOCT1 substrates, e.g. although the L447Y/Q448E double mutant showed no change in affinity for TEA (consistent with our observations (Fig. 2)), its affinity for MPP\(^+\) was substantially increased. Significantly, Gorboulev et al. (34) reported that corticosterone binding to one of their mutants exerted an allosteric effect on the binding of other cationic substrates, leading to the conclusion that the binding surface of OCTs is large enough to support the simultaneous binding of multiple ligands, which can then exert short-range allosteric interactions with the binding region.

The differential influence of selected residues on substrate interactions has been observed in several additional studies. In their assessment of the influence on transport of amino acid residues in TMH4 of OCT1, Popp et al. (23) reported that replacement of Trp\(^{218}\) with tyrosine and Tyr\(^{222}\) with leucine resulted in significant decreases in \(K_d\) values for both TEA and MPP\(^+\), whereas the Y222F and T226A mutants decreased the \(K_d\) values for only TEA or MPP\(^+\), respectively. In addition, in their earlier study on the influence of acidic residues on the transport activity of rOCT1, Gorboulev et al. (42) found that the D475E mutant displayed a marked increase in apparent affinity for TEA, with no change in interaction with MPP\(^+\). Lending further support to the view that Asp\(^{225}\) plays a key role in defining a binding surface in OCT1 is the observation by Wolff et al. (45) that the homologous position in the sequence of all members of the OAT family (which are also members of the OCT transport family (21)) is filled with a cationic residue. Although the R478D mutant of the flounder ortholog of OAT1 still supported transport of the monovalent anion PAH (albeit at a reduced rate compared with the wild-type protein), this mutation eliminated interaction with the dicarboxylate glutarate. In the rat ortholog of OAT1, similar differential effects on interaction with different substrates have also been reported in studies that used site-directed methods to probe the influence of selected residues on the transport activity of rOAT3. The homologous replacement in rOAT3 (i.e. R454D) showed a profound change in interaction with PAH (33), and mutations of several hydrophobic residues in TMH7 (W334A, F335A, Y341A, and Y342Q) and one in TMH8 (F362S) resulted in a marked decrease in transport of the hydrophilic substrates PAH and cimetidine, with comparatively little effect on transport of the more hydrophobic substrate estrone sulfate (46), leading to the conclusion that the structure of OAT3 contains a general binding domain with no single binding site.

The homology model of the three-dimensional structure of rOCT2 provides a means to interpret, in the context of the potential mechanism of substrate binding and translocation, the effects of specific residues on transport activity as determined in site-directed studies. The development of the rOCT2 model (Fig. 6) was made possible by the recent observation that the high resolution crystal structures of two other MFS transporters, LacY (37) and GlpT (38), revealed a common organization of TMHs. Despite the low degree of sequence identity of these two transporters (~13%), the observed structural homology supported the contention that the structure of other MFS transporters may be deduced using the methods of homology modeling (40). This approach has been subsequently used to develop structural models of human GLUT1 (47, 48) and rOCT1 (23) based upon the template structures of GlpT and LacY, respectively. Not surprisingly, the GlpT-based model of the rOCT2 structure reported here displays a number of key similarities to the LacY-based model of the rOCT1 structure developed by Popp et al. (23). Most significantly, all the residues that were identified in rOCT1 as exerting influence on ligand binding (on both TMH4 and TMH10) and that were observed to be directed toward the putative pore (or "cleft") region of the rOCT1 model structure were also observed to face the pore region of the rOCT2 model described here (Fig. 6).

There are, however, differences in the proposed models as well. First, the rOCT2 and rOCT1 models were based upon different template structures (i.e. GlpT and LacY, respectively). Although these structures share a remarkably similar fold, they nevertheless show distinct differences in the absolute positions of residues that are otherwise homologous in their positions within the two sequences (the C-α root mean square deviation for superimposition of the two sequences is on the order of 3.5 Å), and these differences in position of homologous residues clearly carry over to the homology models of rOCT2 and rOCT1. Second, the alignment of rOCT2 with GlpT used to generate the OCT2 structural model differs slightly from the alignment of rOCT1 with LacY used by Popp et al. (23). Interestingly, these differences are particularly evident in TMH4 and TMH10, where the alignments are shifted by four residues or approximately a full helical turn. Consequently, both of the resulting homology models direct the same set of residues toward the putative binding cleft, but these residues reside at different levels relative to the "center point" of the membrane. The differences in alignments reflect different decisions concerning the length of selected loops found between the TMHs. Given the ambiguities associated with constructing alignments of sequences that share comparatively little amino acid identity, it is premature to conclude whether either alignment is correct.

The similarity of the protein fold of LacY and GlpT and the identification in both structures of similar ligand-binding regions (i.e. within the cleft between the N- and C-terminal halves of the proteins) suggested that the homologous region in rOCT2 might be involved in binding interactions associated with transporter/substrate interaction. This hypothesis was supported by the strong correlation between the binding energies calculated for a battery of test compounds and their measured IC\(_{50}\) values for inhibition of OCT2 transport activity (Fig. 7). Notably, no correlation was noted between the measured IC\(_{50}\) values and the binding energies calculated from the structures of GlpT and LacY, suggesting that the rank order of substrate/inhibitor docking calculated to occur within the modeled OCT2 reflects the specific amino acid composition of the docking surface within the putative binding region of the protein.

The relationship between predicted and measured binding interactions of substrate with the homology model of the rOCT2 structure must be interpreted cautiously. Despite the evident correlation between these parameters for the test set of substrates (Fig. 7), not all predictions based upon the postulated docking surface of the OCT2 homology model proved to be as accurate. For example, although the calculated \(K_D\) for cimetidine of the 5 Å docking surface that included the E447Q muta-
tion (following energy minimization of the E447Q-containing mutant protein) was increased slightly (i.e. from the wild-type value of 10 μM to the “mutant” value of 34 μM), the $K_d$ for TEA was increased much more (from 9 μM to 20 μM). Similarly, the $K_d$ profile of the E447L mutant showed some consistencies with the observed behavior of the mutant protein, i.e. maintenance of reasonably high affinity for MPP$^+$ and a marked decrease in affinity for TEA; the profile also included a profound decrease in affinity for cimetidine, which contrasted sharply with the observed behavior of the mutant protein (Fig. 5). These deviations from predicted behaviors serve to underscore the current limitations of homology modeling with respect to providing a quantitative predictive model of substrate interactions. Even comparatively slight differences in the spatial distribution of amino acid residues within a docking region can, as we have seen, result in marked differences in predicted binding interactions. As noted earlier, the alignment of target and template sequences offers many opportunities. As noted earlier, the alignment of target and template sequences offers many opportunities. As noted earlier, the alignment of target and template sequences offers many opportunities. As noted earlier, the alignment of target and template sequences offers many opportunities.
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A Conserved Glutamate Residue in Transmembrane Helix 10 Influences Substrate Specificity of Rabbit OCT2 (SLC22A2)
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