Cysteine Accessibility in the Hydrophilic Cleft of Human Organic Cation Transporter 2

Organic cation transporters (OCTs) are involved in the renal elimination of many cationic drugs and toxins. A hypothetical three-dimensional structure of OCT2 based on a homology model that used the *Escherichia coli* glycerol 3-phosphate transporter as a template has been described (Zhang, X., Shirahatti, N. V., Mahadevan, D., and Wright, S. H. (2005) *J. Biol. Chem.* 280, 34813–34822). To further define OCT structure, the accessibility to hydrophilic thiol-reactive reagents of the 13 cysteine residues contained in the human ortholog of OCT2 was examined. Maleimide-PEO2-biotin precipitated (surface biotinylated) and reduced tetraethylammonium transport by OCT2 expressed in Chinese hamster ovary cells, effects that were largely reversed by co-exposure to substrates and transport inhibitors, suggesting interaction with cysteines that are near to or part of a substrate-binding surface. Cysteines at amino acid position 437, 451, 470, and 474 were identified from the model as being located in transmembrane helices that participate in forming the hydrophilic cleft, the proposed region of substrate-protein interaction. To determine which residues are exposed to the solvent, a mutant with all four of these cysteines converted to alanine, along with four variants of this mutant each with an individual cysteine restored, were created. Maleimide-PEO2-biotin was only effective at precipitating and reducing transport by wild-type OCT2 and the mutant with cysteine 474 restored. Additionally, the smaller thiol-reactive reagent, methanethiosulfonate ethylsulfonate, reduced transport by wild-type OCT2 and the mutant with cysteine 474 restored. These data demonstrate that cysteine 474 of OCT2 is exposed to the aqueous milieu of the cleft and contributes to forming a pathway for organic cation transport.

Renal excretion, accomplished by active proximal tubular secretion, is the principal pathway for elimination of a diverse array of potentially toxic organic compounds, including clinically important therapeutics, environmental toxins, and endogenous metabolites (1). Many of these compounds fall into the chemical class commonly referred to as “organic cations” (OCs), which includes a diverse array of primary, secondary, tertiary, or quaternary amines that have a net positive charge on the amine nitrogen at physiological pH. Transport proteins of the renal proximal tubule epithelium mediate OC secretion, thus performing a critical role in detoxification (see reviews in Refs. 2–7). Three homologous OC transporters (OCT1, OCT2, and OCT3) have been cloned and shown to be expressed in the peritubular (i.e. basolateral) membrane of proximal tubule cells where they mediate OC uptake (8–10). The OCTs are a potential site of harmful drug-drug interactions because they are multispecific, handling OCs of diverse structure and chemistry. Consequently, understanding the structure of the OCT substrate-binding surface is a critical tool for predicting the interaction of OCs with these transport proteins.

The OCTs are members of a larger family of solute carriers (SLC22A), which includes the OCTNs (OCTN1–3) and OATs (OAT1–5). SLC22A family members have common structural features, including 12 putative transmembrane-spanning helices (TMHs), intracellular C and N termini, a large extracellular loop between TMHs 1 and 2, and a large intracellular loop between TMHs 6 and 7. These and other structural features further place SLC22A transport proteins into the major facilitator superfamily (MFS). The elucidation of high-resolution crystal structures of two MFS transporters, the *Escherichia coli* lactose permease (LacY (11)) and glycerol 3-phosphate transporter (GlpT (12)), led to the contention that all MFS transporters share a common structural fold with similar topological organization of α-helices (13). This realization has permitted the application of homology modeling to develop hypothetical three-dimensional structures of several MFS transport proteins, including the rat ortholog of OCT1 (rOCT1 (14)) and rabbit ortholog of OCT2 (rbOCT2 (15)). Using these models others have focused on the organization and alignment of residues within the 12 TMHs, resulting in the identification of a large hydrophilic cleft centrally located within the protein that is proposed to contain the substrate-binding surface, as is the

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1 Supported by Ruth L. Kirchstein National Research Service Award Training Grant HL07249 and an individual fellowship (DK075242) from the National Institutes of Health. To whom correspondence should be addressed: Dept. of Physiology, University of Arizona, College of Medicine, P.O. Box 245051, Tucson, AZ 85743. Tel.: 520-626-4307; Fax: 520-626-2383; E-mail: rpelis@email.arizona.edu.

2 The abbreviations used are: OC, organic cation; OCT, organic cation transporter; rOCT2, human OCT2; rOCT1, rat OCT1; rbOCT2, rabbit OCT2; TMH, transmembrane helix; MFS, major facilitator superfamily; TFA, tetraethylammonium; TMA, tetramethylammonium; TpropA, tetrapropyrammonium; TBA, tetrabutylammonium; TPA, tetrapentylammonium; CHO, Chinese hamster ovary; maleimide-PEO2-biotin, (†)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; GlpT, glycerol 3-phosphate transporter; PBS, phosphate-buffered saline.

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case for both LacY (11) and GlpT (12). These OCT homology models were suggested to be valid because of the alignment of adjacent TMHs (15). Vardy et al. (15) casted substantial doubt on the precise alignment between “target” and “template” in the homology modeling process. For example, the long extracellular loop (a structure unique to SLC22A family members) and long cytoplasmic loop (a structure shared by all MFS transporters) were omitted from the rOCT1 and rbOCT2 sequences (14, 15) to facilitate the modeling process, and the authors acknowledge that this approach introduced ambiguity with respect to the alignment of adjacent TMHs (15). Vardy et al. (13) present data suggesting that the accuracy of homology modeling of MFS transporters is significantly enhanced when based on a “manually optimized alignment,” i.e., when the sequence alignment is based on several criteria including experimental data. Thus, although the general organization of α-helices within the postulated three-dimensional structures of rOCT1 and rbOCT2 is probably reasonably accurate, experimental validation of the relative location of amino acid residues within these hypothetical structures is required if the models are to serve as the basis for predicting substrate-transporter interactions.

In the present study, the three-dimensional model of the human ortholog of OCT2 (hOCT2) was used to make inferences about the relative positions of the thirteen cysteine residues contained within the transport protein, i.e., are they exposed to the external solvent compartment or embedded in the membrane. Based on their relative position in the model, three populations of cysteine residues were identified in hOCT2, i.e., six residues in the long extracellular loop, three residues in TMHs peripheral to the hydrophilic cleft, and four residues in TMHs that form the cleft. Of the 13 residues, only the cleft cysteine at position 474 of TMH 11 was accessible to the cleft cysteine residues in TMHs that form the cleft. Of the 13 residues, only the cleft cysteine at position 474 of TMH 11 was accessible to the thiol-reactive reagents used. These findings are discussed in relation to the proposed three-dimensional structure of hOCT2.

EXPERIMENTAL PROCEDURES

Chemicals—[3H]Tetraethylammonium (54 Ci/mmol) was synthesized by Amersham Biosciences. (+)-Biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine (maleimide-PEO₂-biotin) was obtained from Pierce Biotechnology. (2-Trimethylammonium)ethyl[methanethiosulfonate bromide (MTSES) were obtained from Toronto Research Chemical. (3-Hydroxypropyl)aminomethyl-3,6-dioxaoctanediamine (maleimide-PEO₂-biotin) was obtained from Pierce Biotechnology.

Cell Culture and Stable Expression of hOCT2—Wild-type hOCT2 contained in the pcDNA3.1 expression vector was stably transfected into CHO-K1 cells and maintained as described previously (18). CHO cells containing a single integrated Flp recombination target (FRT) site were acquired from Invitrogen (CHO Flp-In) and were used for stable expression of the hOCT2 mutant constructs. Prior to transfection, CHO Flp-In cells were grown in Ham’s F12 Kaighn’s modification medium supplemented with 10% fetal calf serum and zeocin (100 μg/ml). Cultures were split every 3 days. 5 × 10⁶ cells were transfected by electroporation (BTX ECM 630, San Diego, 260 volts and time constant of ~25 ms) with 10 μg of salmon sperm, 18 μg of pOG44, and 2 μg of pcDNA5/FRT/V5-His TOPO containing the mutant constructs of hOCT2. Cells were seeded in a T-75 flask following transfection and maintained under selection pressure with hygromycin (100 μg/ml). Cells were used for experiments ~21 days after electroporation.

Cell Surface Biotinylation with Maleimide-PEO₂-biotin—The method described here is a minor modification of that described by Pelis et al. (19). All solutions were kept ice-cold throughout the procedure, and long incubations were conducted on ice with gentle shaking. Cells plated to confluence in a 12-well plate were initially washed three times with 2 ml of phosphate-buffered saline (PBS) solution containing calcium and magnesium (PBS/CM) (in mM): 137 NaCl, 2.7 KCl, 8 Na₂HPO₄, 1.5 KH₂PO₄, 0.1 CaCl₂, and 1 MgCl₂, pH 7.0 with HCl) followed by a single incubation in maleimide-PEO₂-biotin diluted in PBS/CM. The concentration and time of exposure to maleimide-PEO₂-biotin is described under “Results.” In some cases, the cells were pre-exposed to the quaternary ammonium compounds (TMA (10.5 mM), TEA (1 mM), TpropA (400 μM), TBA (390 μM), or TPA (210 μM) for 2 min followed by inclusion of these compounds in the biotinylation reaction. The quaternary ammonium compounds were used at concentrations 20-fold higher than their reported IC₅₀ values for inhibition of TEA transport by hOCT2 (18). After biotinylation, the cells were rinsed twice briefly with 3 ml of PBS/CM followed by a 20-min incubation in the same solution. The cells were lysed in 1 ml of lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100).
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X-100, 1% sodium deoxycholate, and 0.1% SDS, pH 7.4) containing protease inhibitors (in μM: 200 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.16 aprotinin, 4 leupeptin, 8 bestatin, 3 pepstatin A, 2.8 E-64; Sigma) for 1 h and centrifuged at 15,800 × g (4 °C) for 30 min to remove insoluble material. 50 μl of streptavidin-agarose beads (Pierce) were added to the lysates and incubated overnight at 4 °C with constant mixing. After extensive washing with the above lysis buffer, 50 μl of Laemmli sample buffer was added, and the proteins were eluted from the beads at 100 °C for 5 min. Proteins were separated on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes, and immunoreactivity corresponding to the V5-tagged hOCT2 constructs was detected as described previously (19).

Immunocytochemistry—CHO cells grown on coverslips in 12-well plates were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4). All subsequent washes were performed in triplicate at room temperature in PBS. Cells were fixed in ice-cold 100% methanol for 20 min, washed, and incubated for 1 h with mouse anti-V5 antibody (Invitrogen) diluted in PBS (final concentration of 2 μg/ml). The cells were washed and incubated for 1 h in the dark with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Invitrogen) diluted to 2 μg/ml in PBS. The cells were washed before staining the nuclei with propidium iodide (5 μg/ml in PBS; Sigma) for 10 min. Cells were washed again and the coverslips mounted onto microscope slides. A confocal microscope (Nikon PCM 2000 scan head fitted to a Nikon E800 microscope) was used for detection of immunoreactivity in CHO cells.

Transport Experiments—CHO cells grown to confluence in 12-well plates were rinsed twice with Waymouth’s buffer (WB) and incubated overnight at 4 °C with constant mixing. After extensive washing with the above lysis buffer, 50 μl of Laemmli sample buffer was added, and the proteins were eluted from the beads at 100 °C for 5 min. Proteins were separated on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes, and immunoreactivity corresponding to the V5-tagged hOCT2 constructs was detected as described previously (19).

Interaction of Maleimide-PEO2-biotin with Wild-type hOCT2—Fig. 1 shows the secondary structure model of hOCT2 based upon the homology model of hOCT2 (15), emphasizing the placement within the sequence of 13 cysteine residues contained within this transport protein. There are six residues in the long extracellular loop and one or two cysteines in TMHs 3, 6, 9, 10, and 11. There are no cysteine residues present in any of the predicted short extracellular loops or intracellular loops. The presence of cysteines in the hOCT2 sequence led to the initial hypothesis that the membrane-impermeable, thiol-reactive reagent maleimide-PEO2-biotin would interact with one or more of these cysteine residues.

With a fixed concentration of maleimide-PEO2-biotin (0.5 mg/ml) in the biotinylation reaction, immunoreactivity on Western blots corresponding to biotinylated hOCT2 expressed at the plasma membrane of CHO cells increased with increasing time of exposure to the thiol-reactive reagent, with only a slight increase in immunoreactivity with an exposure time longer than 20 min (supplemental Fig. 1A). The relative molecular mass of hOCT2 was ~85 kDa, a profile similar to that of

FIGURE 1. Secondary structure model of hOCT2 based upon the high-resolution crystal structure of GlpT as a template (15). The 13 cysteine residues contained within the transport protein are highlighted (black circles).

RESULTS

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FIGURE 2. A, effect of the quaternary ammonium compounds TMA, TEA, TpropA, TBA, and TPA on precipitation of wild-type hOCT2 with maleimide-PEO$_2$-biotin (0.5 mg/ml for 20 min). The quaternary ammonium compounds were included in the biotinylation reaction at concentrations 20-fold higher than their reported IC$_{50}$ values for inhibition of TEA transport by hOCT2 (18). B, [H]TEA (14 nM) uptake (at 30 s) by wild-type hOCT2 following treatment for 20 min with TPA (210 µM), maleimide-PEO$_2$-biotin (MB; 0.5 mg/ml), or TPA and maleimide-PEO$_2$-biotin in combination. Following treatment, cells were rinsed extensively over a 20-min period to remove residual maleimide-PEO$_2$-biotin and/or TPA. The different letters above the columns (A versus B) indicate significant differences among treatments (n = 5; p < 0.05, Student-Newman-Keuls test).

rbOCT2 (19). The precipitation of hOCT2 also increased in a concentration-dependent manner, with only a modest increase in precipitated immunoreactivity produced by exposure to maleimide-PEO$_2$-biotin above 0.1 mg/ml (supplemental Fig. 1B). The effectiveness of maleimide-PEO$_2$-biotin to precipitate hOCT2 shows that the cysteine-modifying reagent interacts with this transport protein.

To determine the effect on hOCT2-mediated transport activity of covalent modification of cysteine residues, TEA uptake was measured after exposure of CHO cells expressing hOCT2 to maleimide-PEO$_2$-biotin. Uptake of [H]TEA by cells expressing hOCT2 increased with time in a near linear manner for 30 s, with more than 95% of transport blocked by the addition of 2 mM unlabeled TEA to the transport reaction (supplemental Fig. 2, inset). At a concentration of 0.5 mg/ml, maleimide-PEO$_2$-biotin reduced TEA transport in a time-dependent manner with a 70% reduction in transport at 20 min (supplemental Fig. 2A). Using a fixed exposure time of 20 min, there was a concentration-dependent decrease in TEA transport with a maximum effect at 0.5 mg/ml (80% reduction in TEA uptake) (supplemental Fig. 2B). Subsequent biotinylation and transport experiments were conducted by exposing the cells for 20 min to 0.5 mg/ml maleimide-PEO$_2$-biotin.

Protection of Biotinylation and Transport by Quaternary Ammonium Compounds—The quaternary ammonium compounds, TMA, TEA, TpropA, TBA, and TPA all interact with OCT2, as shown by the ability of each of these compounds to inhibit OCT2-mediated transport of radiolabeled TEA (and other organic cations) (18). To determine whether the presence of a substrate/inhibitor within the binding surface/region influences the ability of maleimide-PEO$_2$-biotin to access one or more of the reactive thiols within the hOCT2 sequence, we tested the effect of exposing cells to these quaternary ammonium compounds on precipitation of transport protein. The addition of TMA, TEA, TpropA, TBA, or TPA to the biotinylation reaction almost completely prevented the precipitation of hOCT2 (Fig. 2A). Furthermore, exposure to TPA ameliorated the reduced level of TEA transport elicited by exposure of hOCT2-expressing cells to maleimide-PEO$_2$-biotin (Fig. 2B). These data suggest that maleimide-PEO$_2$-biotin interacts with one or more cysteine residues that are near to or part of the binding surface for the quaternary ammonium compounds.

Cysteine Accessibility in the Hydrophilic Cleft of OCT2—Five mutants were created: a quadruple mutant in which all four of the cleft cysteines were converted to alanines and four variants of the quadruple mutant in which one of the cleft cysteines was restored at each individual position (Cys-437, Cys-451, Cys-470, and Cys-474). All of the mutant transporters were expressed in the plasma membrane and displayed considerable transport activity, albeit at a reduced level compared with the wild-type transport protein (TEA uptake 3–6-fold lower than wild type) (Fig. 4). Of the mutant transporters, only the mutant containing Cys-474 exhibited TEA transport activity that was sensitive to maleimide-PEO$_2$-biotin (Fig. 5A). Indeed, biotinylation experiments showed that only wild-type hOCT2 and the Cys-474 add-back mutant could be precipitated with maleimide-PEO$_2$-biotin. As noted previously for wild-type hOCT2, the reduction caused by maleimide-PEO$_2$-biotin of TEA transport by the Cys-474 add-back mutant was prevented by co-treatment with TPA (Fig. 5B). Precipitation of the quadruple mutant and the Cys-437, Cys-451, and Cys-470 add-back mutants was rescued following permeabilization of the plasma membrane with 0.1% saponin (supplemental Fig. 3). This observation confirms that maleimide-PEO$_2$-biotin does not readily permeate the plasma membrane under the experimental procedures used. The precipitation of the transport protein following permeabilization probably reflects the interaction of maleimide-
FIGURE 3. Hypothetical two- and three-dimensional structures of hOCT2 based on a homology model that used the high-resolution crystal structure of GlpT as a template (15). A, secondary structure of hOCT2 emphasizing the TMHs peripheral to the hydrophilic cleft (green) and TMHs that participate in forming the hydrophilic cleft (blue). Three populations of cysteine residues were identified from the hOCT2 sequence, i.e. six cysteines in the long extracellular loop (loop cysteines (yellow triangles)), three cysteines in peripheral TMHs (peripheral cysteines; TMHs 3, 6, and 9 (orange circles)), and four cysteines in cleft TMHs (cleft cysteines; TMHs 10 and 11 (yellow circles)). B, side view of the OCT2 homology model, with the extracellular aspect of the transporter oriented upward. Peripheral cysteines (orange) and cleft cysteines (at amino acid position 437, 451, 470, and 474 (yellow)) are shown, along with Glu-448 and Asp-475 (red). C, view of the model from the cytoplasmic aspect of the protein. TMHs 10 and 11, which contain Cys-437, Cys-451, Cys-470, Cys-474, Glu-448, and Asp-475, are numbered. D, the relative orientation of TMHs 10 and 11, emphasizing the juxtaposition of Cys-437, Cys-451, Cys-470, Cys-474, Glu-448, and Asp-475. Overlaying these two helices is the postulated substrate docking surface determined for rBOCT2, depicted as the van der Waals surface of the included residues (15).
PEO₂-biotin with one or more of the cleft and/or peripheral cysteines that are not typically accessible to the reagent.

Maleimide-PEO₂-biotin is a hydrophilic reagent (Mr 525) that contains a thiol-reactive maleimide moiety and biotin moiety that are separated by a 29.Å polyethylene oxide (PEO₂) group. To determine whether steric hindrance precludes access of maleimide-PEO₂-biotin to reactive thiols on cysteines 437, 451, and 470, two smaller hydrophilic thiol-reactive reagents, MTSET (Mr 278) and MTSES (Mr 242), were used. MTSET is a quaternary ammonium compound like the OCT2 substrate TEA, whereas MTSES is an organic anion. Both reagents have been used extensively to probe the structures of numerous transmembrane proteins, including LacY (21) and the GABAA receptor (22).

Although MTSET reduced TEA transport, as expected, by wild-type hOCT2 (94%), it also inhibited transport by the quadruple mutant (70%) (Fig. 6A). However, the likelihood that MTSET is transported by hOCT2, thereby allowing the reagent access to free thiols in the intracellular compartment (including those associated with proteins other than OCT2), cannot be dismissed. The anionic MTSES, which exhibits none of the structural properties of an OCT substrate, significantly reduced TEA transport by wild-type hOCT2 (40%) and the mutant with the cleft cysteine restored at position 474 (25%; Fig. 6B). Contrarily, TEA transport mediated by cells expressing the

![FIGURE 4](image-url)

**FIGURE 4.** A, immunocytochemical localization in CHO cells of expressed wild-type hOCT2 and mutant constructs lacking cysteine residues in TMHS 10 and 11 (i.e. cleft cysteines) (green). In the quadruple mutant, all four cleft cysteines were mutated to alanine. The quadruple mutant DNA served as a template for constructing the other mutants, with each having one of the four cleft cysteine residues (Cys-437, Cys-451, Cys-470, or Cys-474) restored. Nuclei were stained with propidium iodide (red). B, 30 s uptakes of 14 nM [³H]TEA by CHO cells expressing wild-type and mutant constructs of hOCT2. Uptake of [³H]TEA was conducted in the absence and presence of 2 mM unlabeled TEA (n = 3–6).

![FIGURE 5](image-url)

**FIGURE 5.** A, mediated [³H]TEA (14 nM) uptake (at 30 s) and precipitation (Western blot) of wild-type hOCT2 and mutant constructs expressed in CHO cells following treatment with maleimide-PEO₂-biotin (0.5 mg/ml for 20 min). In the quadruple mutant, all four cleft cysteines were mutated to alanine. The quadruple mutant DNA served as a template for constructing the other mutants, with each having one of the four cleft cysteine residues (Cys-437, Cys-451, Cys-470, or Cys-474) restored. Transport data are expressed as a percentage of mediated [³H]TEA uptake in the absence of maleimide-PEO₂-biotin (Not treated), *, significantly different from control (not treated) (n = 3–5; Student’s t test, p < 0.05). B, [³H]TEA (14 nM) uptake (at 30 s) by the mutant construct of hOCT2 containing Cys-474 (but not Cys-437, Cys-451, or Cys-470) following treatment for 20 min with TPA (210 μM), maleimide-PEO₂-biotin (MB; 0.5 mg/ml), or TPA and maleimide-PEO₂-biotin in combination. Following treatment, cells were rinsed extensively over a 20-min period to remove residual maleimide-PEO₂-biotin and/or TPA. The different letters above the columns (A versus B) indicate significant differences among treatments (n = 4; p < 0.05, Student-Newman-Keuls test).
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FIGURE 6. Effect of MTSET (A) or MTSES (B) on mediated \[^{3}H\]TEA (14 nm) uptake (at 30 s) by wild-type and mutant constructs of hOCT2. In the quadruple mutant, all four cleft cysteines were mutated to alanine. The quadruple mutant DNA served as a template for constructing the other mutants, with each having one of the four cleft cysteines (Cys-437, Cys-451, Cys-470, or Cys-474) restored. Transport data are expressed as a percentage of mediated \[^{3}H\]TEA uptake in the absence of MTSET or MTSES (Not treated). *, significantly different from control (not treated) (n = 3–5; Student’s t test, p < 0.05).

mutants with Cys-437, Cys-451, or Cys-470 restored appeared to be insensitive to MTSES.

DISCUSSION

The extremely broad structural selectivity of the OCTs (18, 23–27) make them potential sites of harmful drug-drug interactions, and an understanding of the structural basis of the binding of substrate with OCTs holds the promise of anticipating the interaction of drugs with these transport proteins. Recently, hypothetical three-dimensional models of rOCT1 (14) and rOCT2 (15) were developed; these models should prove useful for generating hypotheses concerning OCT structure and function. To further elucidate OCT structure, the present study examined the accessibility to hydrophilic thiol-reactive reagents (maleimide-PEO2-biotin, MTSET, and MTSES) of cysteine residues contained within hOCT2. The hOCT2 sequence contains 13 cysteine residues, and three populations were identified from the model: six cysteines in the long extracellular loop (loop cysteines), three in peripheral TMHs (peripheral cysteines; TMHs 3, 6, and 9), and four in TMHs 10 and 11 that are within the cleft (cleft cysteines). The probable importance of these cysteines in OCT structure/function is implied from the observation that each of the 13 residues is conserved as a cysteine at a homologous position in the pig, rabbit, mouse, and rat orthologs of OCT1 and the human ortholog of OCT1. Our initial hypothesis was that the membrane-impermeable maleimide-PEO2-biotin would interact with one or more of the cysteine residues contained within hOCT2, and indeed, maleimide-PEO2-biotin precipitated hOCT2 and reduced its transport activity in a time- and dose-dependent manner. The presence of quaternary ammonium compounds largely prevented precipitation of hOCT2 and eliminated the reduction of its transport activity that followed exposure of hOCT2-expressing cells to maleimide-PEO2-biotin (Fig. 2), suggesting that the thiol-reactive reagent interacts with cysteines that are near to or part of the substrate-binding surface.

There is substantial evidence that a common feature of MFS proteins is a fluid-filled pore or cleft composed of TMHs 1, 2, 4, 5, 7, 8, 10, and 11. Mutagenesis studies (14–17) suggest that in the SLC22A family, including the OCTs, the cleft contains several regions that interact with substrate. For example, mutation of Asp-475 in TMH 11 to glutamate significantly increases the affinity of rOCT1 for some substrates (e.g. TEA) but not others (e.g. N-methylphenylpyridinium), and this has been interpreted as demonstrating that the cleft has multiple sites of interaction and that Asp-475 is part of or near the binding surface for several substrates, including TEA (17). In TMH 10, Ala-443, Leu-447, and Gln-448 of the rat ortholog of OCT2 (rOCT2) together confer a higher affinity for corticosterone compared with rOCT1 (16); and in the rabbit, replacement of Glu-447 with glutamine shifts selectivity toward a more OCT1-like phenotype (15). Intriguingly, each of these residues is directed toward the fluid-filled cleft within the current homology models for structure of OCT1 and OCT2 (14, 15). In addition to these key residues important for substrate selectivity and binding, nearby are four cysteine residues occurring within TMHs 10 and 11 at amino acid positions 437, 451, 470, and 474 of hOCT2 (Fig. 3). To determine whether maleimide-PEO2-biotin interacts with cleft cysteines, a quadruple mutant was created in which all four of the cleft cysteines were mutated to alanine. The mutant transporter was expressed at the plasma membrane and retained function (Fig. 4), but unlike wild-type hOCT2, the quadruple mutant could not be precipitated with maleimide-PEO2-biotin, and its transport activity was insensitive to the reagent (Fig. 5).

The refractoriness of the peripheral cysteines to maleimide-PEO2-biotin supports the model of OCT2 structure that shows the cysteines residing in TMHs 3, 6 and 9 as being embedded in and exposed to the lipid bilayer. However, the possibility that the peripheral cysteines are accessible from the hydrophilic cleft to smaller thiol-reactive reagents cannot be dismissed. Somewhat surprisingly, maleimide-PEO2-biotin also failed to interact with any of the six cysteines in the long extracellular loop. The loop cysteines are clearly important for OCT structure, as they are conserved in all orthologs of OCT1, OCT2, and OCT3 currently identified (and four of the six loop cysteines are conserved at homologous positions in all OAT homologues). Although virtually nothing is known about the structure of the long extracellular loop, the inaccessibility of the six loop cysteines is not likely due to their being obscured by the lipid bilayer, as they are in proximity to several known sites of N-glycosylation (19) (Fig. 1). Potential explanations for the inaccessibility of the loop cysteines include: (i) their involvement in disulfide bridges; and (ii) the possibility that that steric hindrance, perhaps caused by the presence of N-glycosylation and/or the topology of the extracellular loop, may preclude access of maleimide-PEO2-biotin to otherwise reactive thiols. Regardless, the loop cysteines may be involved in trafficking and/or stabilization of the transport protein in the plasma membrane, because mutation of any of the cysteine residues in the long extracellular loop of hOCT2 causes retention of the transport protein in an intracellular compartment (28).3

To identify which of the cleft cysteines are accessible, we created four variants of the quadruple mutant, each of which

3 John B. Pritchard, personal communication.
had one of the cleft cysteines (Cys-437, Cys-451, Cys-470, or Cys-474) restored. Of these four mutant transporters, only hOCT2 with Cys-474 restored could be precipitated and had TEA transport activity that was sensitive to maleimide-PEO₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻‥-

MTSES further supports the contention that Cys-474 is adjacent to or comprises a substrate binding surface.

In conclusion, three populations of cysteine residues have been identified in hOCT2 based on their relative location in a postulated homology model of the three-dimensional structure of OCT2, i.e. six cysteines in the long extracellular loop (loop cysteines), three in peripheral TMHs (peripheral cysteines; TMHs 3, 6, and 9), and four in TMHs 10 and 11 that provide the framework for the hydrophilic cleft (cleft cysteines) comprising the proposed region of substrate-transporter interaction. The loop and peripheral cysteines were inaccessible to maleimide-PEO₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻=-=-=-=-=-

Acknowledgment—We thank the Faculty of Medicine Siriraj Hospital, Mahidol University, for its support of Y. Dangprapai during the course of this work.

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