Essential Molecular Determinants for Thyroid Hormone Transport and First Structural Implications for Monocarboxylate Transporter 8\(^*\)\(^{[8]}\)

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Monocarboxylate transporter 8 (MCT8, SLC16A2) is a thyroid hormone (TH) transmembrane transport protein mutated in Allan-Herndon-Dudley syndrome, a severe X-linked psychomotor retardation. The neurological and endocrine phenotypes of patients deficient in MCT8 function underscore the physiological significance of carrier-mediated TH transmembrane transport. MCT8 belongs to the major facilitator superfamily of 12 transmembrane-spanning proteins and mediates energy-independent bidirectional transport of iodothyronines across the plasma membrane. Structural information is lacking for all TH transmembrane transporters. To gain insight into structure-function relations in TH transport, we chose human MCT8 as a paradigm. We systematically performed conventional and liquid chromatography-tandem mass spectrometry-based uptake measurements into MCT8-transfected cells using a large number of compounds structurally related to iodothyronines. We found that human MCT8 is specific for L-iodothyronines and requires at least one iodine atom per aromatic ring. Neither thyronamines, decarboxylated metabolites of iodothyronines, nor triiodothyroacetic acid and tetraiodothyroacetic acid, TH derivatives lacking both chiral center and amino group, are substrates for MCT8. The polyphenolic flavonoids narigenin and F21388, potent competitors for TH binding at transferrin, did not inhibit \(T_3\) transport, suggesting that MCT8 can discriminate its ligand better than transferrin. Bioinformatic studies and a first molecular homology model of MCT8 suggested amino acids potentially involved in substrate interaction. Indeed, alanine mutation of either Arg\(^{445}\) (helix 8) or Asp\(^{498}\) (helix 10) abrogated \(T_3\) transport activity of MCT8, supporting their predicted role in substrate recognition. The MCT8 model allows us to rationalize potential interactions of amino acids including those mutated in patients with Allan-Herndon-Dudley syndrome.

Thyroid hormones (TH)\(^3\) are amino acid derivatives. Because of their zwitterionic nature, TH and their derivatives require transmembrane transporters to mediate their translocation across the plasma membrane (1). Several classes of transmembrane transporter proteins of the major facilitator superfamily (MFS) are capable of TH transport; organic anion transporters two and three (Oatp2 and Oatp3) were the first TH transporters cloned (2). Later, L-type amino acid transporters (Lat1, Lat2) (3) and monocarboxylate transporters eight and ten (MCT8 and MCT10) were shown to transport TH (4, 5). Among these transporters, only MCT8 is specific for TH. Mutations in MCT8/SLC16A2 lead to severe psychomotor retardation and TH abnormalities in humans (6, 7), including the Allan-Herndon-Dudley Syndrome (8). Patients suffering from mutations in MCT8 are characterized by high serum \(T_3\) values associated with low \(T_4\) and inappropriately normal thyroid-stimulating hormone. This clinical phenotype suggested that MCT8 is involved in pituitary feedback control of the TH axis. Sensitivity to TH thus depends on the expression of TH transporter molecules. Expression of MCT8 in the mouse brain (9) supported the idea that TH import into neurons may be affected in patients afflicted with the Allan-Herndon-Dudley syndrome. Mice deficient in MCT8 recapitulate the disturbed serum thyroid hormone parameters (10, 11) but exhibit only mild neurological abnormalities (12). Murine neurons are apparently protected from the lack of MCT8 by expression of alternative \(T_3\) transporters such as Lat2, which is not present in human developing neurons (12).

More than two dozen mutations in MCT8 have been identified in human patients (13). Genotype-phenotype correlations suggested that most missense mutations cause a complete loss-of-function phenotype in patients. A comparison of 12 missense mutants revealed that pathogenic MCT8 mutations may affect expression, surface translocation, or specifically the substrate transport mechanism (14). Possibly, homodimerization may also play a role that we currently do not understand (15, 16). To our knowledge, no data are yet available relating structural features and transport mechanism of MCT8.

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\(^3\) The abbreviations used are: TH, thyroid hormone; GlpT, glycerol-3-phosphate transporter; ICL, intracellular loop; MCT8, monocarboxylate transporter 8; MDCK, Madin-Darby canine kidney; MFS, major facilitator superfamily; PDB, protein data bank; \(T_{9-iodo-L-thyronine} \); \(T_{9-3',5'-triiodothyronine} \); \(T_{9-3',3',5'}-tetraiodothyronine, \(T_{9-3',5'-triiodothyroacetic acid} \); 3-T, 3-T, 3-iodothyronamine.
Our goal in this study was to obtain new functional and structural insights in the complex system of substrate binding and transport. Systematic analysis of substrate molecules for MCT8-mediated cellular uptake revealed several structural determinants for substrate recognition by MCT8: an L-amino acid side chain and at least an iodine substitution in the three positions of both aromatic rings of the thyronine moiety. We also designed a homology model of human MCT8 to identify amino acids potentially involved in substrate recognition. Strikingly, mutations of charged amino acids predicted to interact with substrate disrupted T₃ import into cells. Thus, functional data support the first predictions from our structural MCT8 model.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Stable Cell Lines—**Mutations were introduced into human MCT8 by overlap extension PCR with the following primers (mutated bases in boldface): R445A, forward, 5'–CAGGCTTGGGCTTGTGCAAGCCAC-3’ and R445A, reverse, 5’–GTGGCCTGACACAAAGAGCCCAAAGGCTG-3’; and D498A, forward, 5’–CTGGGCCCTGCTTGCACCCGGCCGCTTCTTCATCAC-3’ and D498A, reverse 5’–GTATGAGAAGACCGCCGCGAAGGCCCAG-3’. Plasmids were stably transfected into MDCK1 cells as described previously (14).

**T₃ Uptake Assays—**One day before the experiments, 200,000 cells/well were seeded into 12-well plates. [125I]T₃ (PerkinElmer Life Sciences) was purified from iodide ions by adsorption chromatography and finally resuspended in DMEM-F12 (1:1) without serum (14). For competitive inhibition studies, cells were exposed to 10 nM [125I]T₃ tracer in medium for 3 min in the presence or not of 10 μM of parent ion in first quadrupole. With the 4000QTRAP triple-quadrupole tandem mass spectrometer (LC-MS/MS) (17, 18). The cells were incubated for 3 min in DMEM-F12 (1:1) with a final concentration of 50 μM. Then the medium was removed, and the cells were washed once with ice-cold PBS and harvested.

**LC-MS/MS Instrumentation and Detection—**LC-MS/MS analyses were performed using a Shimadzu UFLC system (Shimadzu Scientific Instruments, Columbia, MD) and a 3500QTRAP triple-quadrupole tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a TurboIonSpray interface. The chromatographic column and the autosampler were operated at 40 and 10 °C, respectively. The detection was performed using positive electrospray ionization for T₃ and T₃AM and negative electrospray ionization for TRIAC in the selected reaction monitoring mode. For determination of apparent Kₘ values of wild-type MCT8, and the mutants R445A and D498A was directly measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (17, 18). The cells were incubated for 3 min in DMEM-F12 (1:1) with a final concentration of 50 μM. Then the medium was removed, and the cells were washed once with ice-cold PBS and harvested.

**Device-specific LC-MS/MS parameters** obtained from Henning (Berlin, Germany), and naringenin was purchased from Sigma-Aldrich (Taufkirchen, Germany). The uptake of T₃, T₃AM, and TRIAC by wild-type MCT8 and the mutants R445A and D498A was directly measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (17, 18). The cells were incubated for 3 min in DMEM-F12 (1:1) with a final concentration of 50 μM. Then the medium was removed, and the cells were washed once with ice-cold PBS and harvested.

**LC-MS/MS Instrumentation and Detection—**LC-MS/MS analyses were performed using a Shimadzu UFLC system (Shimadzu Scientific Instruments, Columbia, MD) and a 4000QTRAP triple-quadrupole tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with TurboIonSpray interface. The chromatographic column and the autosampler were operated at 40 and 10 °C, respectively. The detection was performed using positive electrospray ionization for T₃ and T₃AM and negative electrospray ionization for TRIAC in the selected reaction monitoring mode. The mass spectrometric working parameters were: Turbolon spray voltage (internal standard), 5500 V; curtain gas (CUR), 30 p.s.i.; collision gas (CAD), 5 p.s.i.; nebulizer gas (GS1), 60 p.s.i.; heater gas (GS2), 50 p.s.i.; entrance potential, 10 V; source temperature, 400 °C; dwell time, 90 ms.

**Chromatographic separation of all substances was achieved using** a Synergi Polar-RP 80-Å column (150 × 2 mm; Phenomenex, Aschaffenburg, Germany) and an analytical Guard Cartridge System (4.0 × 2.0 mm, Phenomenex) using a gradient elution program at a flow rate of 300 μl/min. Simultaneous detection of all substances in the same biological sample was performed using a modified LC-MS/MS method as described before (18). With the 4000QTRAP triple-quadrupole tandem mass spectrometer, we reduced the time per run to 25 min using a gradient elution program: mobile phase A (water/acetonic acid, 95:5:0.6), mobile phase B (water/acetonic acid 95:5:0.6), 0 – 2 min, 2% B; 2 – 12 min, 40% B; 12 – 15 min, 60% B; 15 – 15.5 min, 90% B; 15.5 – 16.5 min, 90% B; 16.5–17 min, 2% B; 17 – 19.95 min, 2% B. The device-specific mass spectrometric parameters for analytes are presented in Table 1. Data processing was performed using Bio Analyat version 1.5. software (Applied Biosystems). Liquid-liquid extraction of all compounds for LC-MS/MS analysis was performed as described previously (17). For LC-MS/MS analysis, 4 pmol of deuterated 3-T₃AM-d₄ was used as internal standard.

**Table 1. Data processing was performed using Bio Analyst version 1.5. software (Applied Biosystems).**
Surface Biotinylation and Western Blotting—Surface biotinylation was performed on MDCK1 cells as described previously (14). Briefly, equal amounts of 5 µg of biotinylated protein (or 80 µg of total cellular protein fraction) were separated on SDS gels, transferred onto nitrocellulose membranes, and probed with an MCT8 antibody (Atlas, Stockholm, Sweden). An anti-
body directed against β-actin (Rockland, Gilbertsville, PA) was used for loading control. Every experiment was performed at least twice with similar results.

Bioinformatics and Molecular Homology Modeling—All amino acid sequence alignments (Fig. 1 and supplemental Fig. S1) were produced using a combination of automatic and manual procedures. For automatic alignments, ClustalW (19) software was applied with the Blossum62 amino acid similarity matrix. Few manual refinements were performed including Gap-introduction at loop regions, but not in transmembrane helices. Alignments were produced with the BioEdit software package.

Human MCT family members were aligned to analyze shared and divergent features in amino acid composition (Fig. 1). The potential dimensions of transmembrane helices (TMH) were predicted based on observable helices in the crystal structure of the glycerol-3-phosphate transporter (GlpT, PDB code 1PW4 (20)), another member of the MFS superfamily (supplemental Fig. S1), which can be used as a structural template for homology models (see section below). From this alignment, it becomes obvious that MCT family proteins share high sequence and most likely structural similarity, despite some regions of flexibility like the third intracellular loop (ICL3).

The high sequence similarity among MCT proteins suggested to us to propose a unifying numbering system for this protein subfamily. The rationale of this numbering scheme is adopted from the description by Ballesteros and Weinstein (21) for family A G-protein-coupled receptors. We use the numbering convention x.50 for the most conserved amino acids in each TMHx. For example, the most highly conserved residue in TMH1 is defined as 1.50, and in TMH2, it is defined as 2.50, etc. We suggest the following amino acids as highly conserved for all MCT (Fig. 1): Trp1.50 (MCT8 Trp175) at TMH1, TMH2 Pro2.50 (MCT8 Pro233), TMH3 Gly3.50 (MCT8 Gly258), TMH4 Gly4.50 (MCT8 Gly276), TMH5 Gly5.50 (MCT8 Gly312), TMH6 Gly6.50 (MCT8 Ser351), TMH7 Pro7.50 (MCT8 Pro412), TMH8 Arg8.50 (MCT8 Arg449), TMH9 Gly9.50 (MCT8 Gly472), TMH10 Gly10.50 (MCT8 Gly499), TMH11 Pro11.50 (MCT8 Pro536), TMH12 Phe(Tyr)12.50 (MCT8 Phe554).

The crystal structure of the inward facing conformation of GlpT from Escherichia coli was used as a structural template for an MCT8 homology model (20). The alignment between human MCT8 and GlpT amino acid sequences reveals a similarity score of 25.5% (supplemental Fig. S1). With small deviations, the helix dimensions observed in the GlpT crystal structure are applicable for human MCT8. The reliability of this structural template is supported by examples of modeling for other members of the MFS-like GLUT1 (22) or MCT1 (23, 24).

helices in MCT8 comprise the following residues: TMH1, Phe166–Leu202; TMH2, Gln212–Thr239; TMH3, Cys244–His260; TMH4, Arg271–His296; TMH5, Gln299–Met326; TMH6, Thr336–Thr353; TMH7, Gly401–Glu429; TMH8, Val435–Leu458; TMH9, Ile461–Met477; TMH10, Phe484–Leu512; TMH11, Ala521–Gly548; and TMH12, Ala553–Asp589.

Gaps of missing residues in the loops of the template structure were closed by the Loop Search tool implemented in Sybyl 7.35 (Tripos Inc., St. Louis, MO). Comparison with loop conformations extracted from the PDB by the SuperLooper bioinformatics software were also performed to find superimposing loop structures from the PDB as criteria for reliability. The quality and stability of the model were validated by checking the geometry using PROCHECK (25).

Side chains and loops of the homology model were subjected to conjugate gradient minimization (until converging at a termination gradient of 0.05 kcal/(mol × Å)) and molecular dynamics simulation (5 ns) by fixing the backbone H-bonds of the TMH. Finally the model was minimized without any constraints. Structure images were produced using PyMOL software (51).

RESULTS

Structural Determinants of Substrate Molecules Determined by Competition with [125I]T3—The only substrates of MCT8 so far identified are iodothyronines (4). A large number of iodothyronines and related molecules are available to probe structural determinants for transport by MCT8. The basic structure of thyroid hormones is the thyronine backbone carrying iodine atoms at one or several of four possible positions (Fig. 2A). The thyroid produces mainly 3,3',5'-triiodothyronine (thyronine, T3), which can be converted by the action of iodothyronine deiodinases into any iodothyronine (26). Moreover, thyronamines (TAM), decarboxylated iodothyronine derivatives, and iodothyroacetic acids are naturally occurring metabolites and exert biological and pharmacological effects (27, 28). We tested a total number of 23 iodothyronine derivatives for their interference with transport of the paradigmatic MCT8 substrate, T3. Among L-(iodo)thyronines, only T3, T3, rT3 (3,3',5'-triiodothyronine), and 3,3'-diiodothyronine (3,3'-T2) competed with T3 for transport (Fig. 2B). We concluded that MCT8 requires for transport at least one iodine atom at the three positions in both aromatic rings.

In addition, L-T3 transport is inhibited only by L-iodothyronines, whereas their D-isomers had no effect (Fig. 2C). These results support and extend similar studies of rat MCT8 by Friesema et al. (4).

FIGURE 1. Alignment of MCT subspecies. The alignment includes members of the human MCTs to extract shared and divergent features of amino acid composition. Especially conserved residues are marked in colors, according to their biophysical properties (blue, positively charged; red, negatively charged; green, hydrophobic; orange, hydrophilic; black, proline), indicating amino acids of functional or structural importance for several or all subspecies. From this alignment, it becomes obvious that the MCTs share high sequence and most likely also structural similarity despite regions of flexibility like the third intracellular loop (ICL3). Asp439 of MCT8 is boxed in red and can also be found in MCT10, another T3-transporting MCT family member. Also boxed are the potential dimensions of the TMHs based on the x-ray structure of an E. coli glycerol-3-phosphate transporter, another member of the major facilitator superfamily. We suggest a unifying numbering system for the MCTs indicated by the italic numbers above each TMH box. This position identifier scheme uses a highly conserved residue in each transmembrane helix as a common reference for all members of the MCT family. For example, the highly conserved tryptophan in TMH1 is defined as 1.50, and the highly conserved proline from TMH2 is defined as 2.50. The first number is related to the particular helix, and the second number after the dot determines the specific position in relation to the conserved residue. This procedure might be helpful to compare insights from different MCT species. For specific comparison between MCT subtypes, we provide amino acid numbering for each MCT in the right column.
Structural-Function Relationships in MCT8

A) Thyronine (Tₐ) 3-iodothyronine (3-T₁) 3'-iodothyronine (3'-T₁)

3,3'-diiodothyronine (3,3'-T₂) 3,5-diiodothyronine (3,5-T₂) 3',5'-diiodothyronine (3',5'-T₂)

3,5,3'-triiodothyronine (T₃) 3',5'-triiodothyronine (rT₃) 3,5,3',5'-tetraiodothyronine (T₄)

B) % inhibition +/- SE

C) % inhibition +/- SE

D) % inhibition +/- SE

E) % inhibition +/- SE

F) % inhibition +/- SE

G) % inhibition +/- SE

Naringenin

BSP

L-Tyr  L-phe L-Trp

DMSO F21388 Naringenin BSP
We then asked whether TAM also compete with T3 for transport by MCT8. In comparison with L-iodothyronines, TAM lack the amino acid carboxyl group and the chiral center.

Inhibition by a 1000-fold excess of any TAM was negligible with the single exception of 3-iodothyronamine (3-T3, AM), an endogenous metabolite known to exert profound pharmacological effects (27, 29). However, even at 10 μM, 3-T3, AM achieved a maximal inhibition of only about 30% of T3 uptake (Fig. 2D) (30).

Our next question was whether the amino group of the backbone represents an important structural determinant. In contrast to earlier findings with rat Mct8 (4), N-acetylation of iodothyronines is apparently tolerated by human MCT8 because NAc-T3 and NAc-T4 both exhibited inhibitory effects similar to the non-acetylated compounds (Fig. 2E). TRIAC and 3,3’,5,5’-tetraiodo-thyroacetic acid (TETRAC) were entirely devoid of inhibitory potential in the competition assay (Fig. 2E).

Our conclusions regarding substrate recognition were supported by the lack of inhibition by 3,5-diiodo-thyropropionic acid (DITPA, Fig. 2E), a thyromimetic compound that was used to pharmacologically circumvent the transport deficiency in Mct8-deficient mice (31). Unlike the closely related MCT10, MCT8 does not accept aromatic amino acids (Fig. 2F).

Finally, we tested polyphenolic flavonoids F21388 and narigenin for inhibition of T3 transport. These compounds were previously shown to bind to transthyretin and compete for T4 binding. Both flavonoids were inactive in this uptake assay in contrast to the known MCT8 inhibitor, bromsulphthalein (BSP, Fig. 2G) (4).

Measuring the Uptake of MCT8 Substrates with LC-MS/MS—Competition assays are an easy and common way to determine structural determinants for receptor binding or transport. However, this type of experiment represents an indirect approach. We took advantage of our LC-MS/MS assays for iodothyronines and TAM (17, 18) and directly measured uptake of unlabeled ligands into MCT8-transfected MDCK1 cells. In a first step, we validated the LC-MS/MS method in direct comparison with the established [125I]T3 uptake assay (Fig. 3, A and B). The Km values determined by both methods, 4.7 and 7.5 μM, are within the range reported for rat, 4–8 μM (4). We then tested T3, AM and TRIAC. Even at 50 μM, both compounds were not transported by MCT8 (Fig. 3C). Thus, the direct assay supports that MCT8 requires the intact L-amino acid backbone for substrate recognition.

Two Charged Amino Acids Are Spatially Located at the Potential Substrate Transport Channel—We were interested in the identification of amino acids in MCT8, which are involved in substrate recognition. Structure-function analysis of the substrate suggested a requirement for both amino and carboxylic functional groups. Therefore, we focused on charged amino acids located in the transmembrane helices. The MCT sequence alignment revealed that all MCTs carry a conservedarginine in TMH8 (Arg445, position 8.50). In MCT1, the corresponding amino acid (Arg106) is described to be essential for transporter function (23). We built a homology model for MCT8 based on the x-ray structure of GlpT (Fig. 4A). Our model is in a conformation open to the intracellular side (Fig. 4B). In this model, the charged side chain Arg445 points into the interior space between the transmembrane helices. Moreover, Arg445 protrudes into a solvent-filled cavity, which we presume is the substrate channel (Fig. 4B). This arginine likely interacts within the interior region of the transporter with a negative charge from the substrate. The MCT8 model further suggests a salt bridge between Arg445 and Asp498 (TMH10). Thus, both charged residues might be involved in essential helix interactions or in substrate recognition.

Mutations in Arg445 and Asp498 Lead to Total Loss of Transport Activity—To test the significance of the two charged amino acids, Arg445 and Asp498, for substrate transport, we...
replaced both amino acids with alanine, established stable cell clones, and tested their activity. Alanine replacement removes charges while retaining the propensity of a peptide to form helices. Both mutants were expressed at lower levels than wild type but were exposed to the plasma membrane, as shown by surface biotinylation (Fig. 5A). Strikingly, both mutants entirely lacked any [125I]T3 uptake activity (Fig. 5B). If the functional role of these charged amino acids relied solely on interaction with the amino acid backbone of the iodothyronine substrate, alanine mutants should accept T3AM or TRIAC at least at high concentrations. We tested this hypothesis directly using the LC-MS/MS assay. Both alanine mutations entirely abrogated transport of T3, and the mutant MCT8 accepted neither TRIAC nor T3AM (Fig. 5C). This finding is compatible with the assumption that an essential salt bridge between basic Arg445 and acidic Asp498 may be formed during the transport cycle.

DISCUSSION

As a first step to gain insight into structural features, substrate interaction, and transport mechanism of thyroid hormone transporters, we systematically delineated the conserved structural elements in MCT8 substrates and tried to identify amino acids in MCT8 potentially interacting with substrate. A homology model for MCT8 was built based on the structure of GlpT, a bacterial glycerol-3-phosphate transporter (20). Combining modeling and biochemical approaches, we identified two essential charged amino acids in TMH8 and TMH10 located in close proximity to the presumed substrate transport channel.

Substrate Determinants Required for Recognition by MCT8—Using 23 iodothyronine and related compounds, we systematically investigated their structure-function relationships to extract key features of MCT8 substrates. Unlike MCT10 (4, 5), MCT8 does not transport aromatic amino acids, suggesting specificity for the thyronine structure. Moreover, human MCT8 is sensitive to the number and distribution of iodine atoms attached to the thyronine backbone. Our data support and extend earlier work done in Xenopus oocytes (4) and show that MCT8 substrates have to carry at least an iodine atom in the three positions of both aromatic rings. In addition, an L-amino acid moiety is essential. In contrast to the findings on rat Mct8 expressed in Xenopus oocytes (4), human MCT8 expressed in MDCK1 cells tolerated N-acetylation of iodothyronines and was not inhibited by D-isomers and TRIAC. Neither naringenin nor F21388 interfered with T3 transport by MCT8. Hence, MCT8 has higher specificity for iodothyronines than type I-deiodinase (32–34), transthyretin (32), and T3-receptors (35, 36).

Amino Acids in MCT8 Involved in Both Substrate Recognition and Transport—Our alignment of MCT family amino acid sequences revealed Arg445 (position 8.50) as conserved among

FIGURE 4. Homology model of the human MCT8. A, crystal structure of the glycerol-3-phosphate transporter (PDB code 1PW4, white backbone), another member of the major facilitator superfamily, which was used as a structural template. Two arginines and a histidine (blue sticks, dotted circle, GlpT amino acids Arg45-TMH1, His165-TMH5, His169-TMH5, Arg269-TMH7) are known to be related to glycerol-3-phosphate (G3P) binding, which is mediated by the phosphate moiety of glycerol-3-phosphate (20). This binding site is located in the middle of the transmembrane region of the GlpT and is part of a central cavity that represents the presumed substrate transport channel. B, lengths of specific loops and helices may vary in detail between the MCT8 model when compared with the crystal structure of GlpT (A), but the overall topology, especially in the transmembrane region, is assumed to be similar. The two charged amino acids Arg445 in blue (TMH8) and Asp498 in red (TMH10) are located in the central transmembrane part of the MCT8. In the top view with clipped extracellular loops, the arrangement of the helices to each other is visualized and shows TMH8 and TMH10 in close proximity to where these charged residues are located. Both amino acids are in close spatial distance, and they are flanking the potential channel for substrate-transport (light blue circle in the top view). This spatial region superimposes with the known binding region for glycerol-3-phosphate in the GlpT (panel A). Therefore, orientations of these residues are predestined for both helix justification and/or substrate binding. Arginine 445 and aspartate 498 are suggested to interact via H-bond (dotted line). Ntt, N-terminal tail; Ctt, C-terminal tail.


all MCT family proteins (Fig. 1). This high conservation indicates an important and specific function for this amino acid, and indeed, in our homology model, Arg^{445} protrudes into a central cavity in the protein in between the helices (Fig. 4B), which we assume is representing the substrate channel in accordance with the GlpT structure (20). This channel assignment is in agreement with the hypothesis that cavities within membrane gates are encircled by polar amino acids (37). Moreover, this is in agreement with recent results on MCT1, where the involvement of arginine Arg^{450} (TMH8, position 8.50) for transport activity was found, which corresponds to Arg^{445} (TMH8, position 8.50) in MCT8. In addition, on MCT1, the negatively charged residue Asp^{453} at the same TMH8 (position 8.46) was also identified to contribute in the transport mechanism (23, 24, 38). Guided by our MCT8 model we found on MCT8 mutants R445A, D498A, and empty vector were exposed to 10 nM \[^{[125]I}\]T_3 for 1–30 min. The radioactivity associated with empty vector-transfected clones was subtracted as background. Data represent of at least two independent \[^{[125]I}\]T_3 uptake experiments performed in triplicate. Error bars indicate S.E.

TABLE 2

| Structure-Function Relationships in MCT8 |
|----------------------------------------|
| Naturally occurring side-chain substitutions at the MCT8 |
| We extracted only those known pathogenic mutations from the literature with single side-chain substitutions (deletions or insertions are not included) because our purpose was to map the positions of such mutations to the three-dimensional MCT8 homology model (Fig. 6) to reveal insights into their spatial neighborhood to residues Arg^{445} and Asp^{498}. The mutations are helpful to evaluate function of wild-type amino acids, also with respect to their potential contact partners. Finally the combination of such sequence-structure- (mal)function relationships is a reliable procedure to identify and to describe hot-spots of transport mechanisms. ECL, extracellular loop. |
| |
| Structural localization | Substitution | Position | References |
|-------------------------|--------------|----------|------------|
| TMH1                    | H192R        | 1.67     | (39) (Review: Ref. 13) |
| TMH1                    | S194F        | 1.69     | (8, 14, 42) |
| TMH2                    | G221R        | 2.38     | (8, 43, 44) |
| TMH2                    | A224V/A224T  | 2.41     | A224V: Refs. 6, 14, and 42 |
| TMH2                    | V235M        | 2.52     | (8, 14, 42) |
| ECL2/TMH4               | R271H        | 4.39     | (14, 42, 45) |
| TMH4                    | G282C        | 4.50     | (44, 46) |
| TMH5                    | P321L        | 5.59     | (43) |
| ICL3/TMH7               | G401R        | 7.39     | (47) |
| ECL4                    | L434W        | 8.50     | (8, 14, 42) |
| TMH8                    | R445C        | 8.50     | (39) (Review: Ref. 13) |
| TMH8                    | D453V        | 8.58     | (44) |
| TMH9                    | L471P        | 9.49     | (6, 14, 42) |
| ICL5                    | L512P        | 9.76     | (7, 14) |
| TMH11                   | P537L        | 11.49    | (44, 48) |
| TMH12                   | G558D        | 12.54    | (14, 44, 49) |
| TMH12                   | G564R        | 12.60    | (50) |
| TMH12                   | L568P        | 12.64    | (8, 14, 42) |

FIGURE 5. MCT8 surface translocation and time course of \[^{[125]I}\]T_3 uptake by MCT8 mutants R445A and D498A, A, MDCK1 cells were stably transfected with wild-type MCT8, R445A, D498A, and empty vector. Whole cellular lysates (left panels) were compared by Western blotting for MCT8 with the biotinylated and affinity-purified plasma membrane protein fraction (right panels). Immunoblotting against \(\beta\)-actin was used as loading control (bottom panels). B, for time course experiments, stably transfected MDCK1 cells with MCT8 mutants R445A, D498A, and empty vector were exposed to 10 nM \[^{[125]I}\]T_3 for 1–30 min. The radioactivity associated with empty vector-transfected clones was subtracted as background. Data represent of at least two independent \[^{[125]I}\]T_3 uptake experiments performed in triplicate. Error bars indicate S.E. C, determination of uptake of 50 \(\mu\)M \(T_3/\text{AT}, \) and TRIAC by MCT8 mutants R445A and D498A by LC-MS/MS. Data are representative of one out of at least two independent \(T_3/\)uptake experiments performed in triplicate. Error bars indicate S.E.
Structure-Function Relationships in MCT8

We conclude from our MCT8 homology model with highlighted amino acid positions (green) of reported pathogenic side-chain substitutions (Table 2) that the spatial region surrounding Asp^{408} and Arg^{625} is characterized by high sensitivity for transport. The close spatial distances to amino acids like Asp^{453} or His^{192}, which are also located at the potential transport cavity, are remarkable. However, pathogenic mutations are identified in most of the helices, and some mutations may cause protein-folding defects.

R445A mutant. We conclude that our model can serve as a basis for further investigations into structure-function relationships in MCT8 and may further allow the rational interpretation and prediction of structural changes caused by pathogenic mutations.

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