Analysis of Transmembrane Segment 7 of the Dipeptide Transporter hPepT1 by Cysteine-scanning Mutagenesis*

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To investigate the involvement of transmembrane segment 7 (TMS7) of hPepT1 in forming the putative central aqueous channel through which the substrate traverses, we individually mutated each of the 21 amino acids in TMS7 to a cysteine and analyzed the mutated transporters using the scanning cysteine accessibility method. Y287C- and M292C-hPepT1 did not express at the plasma membrane. Out of the remaining 19 transporters, three (P293C-, L296C-, and F297C-hPepT1) showed negligible glycyl-sarcosine (gly-sar) uptake activity and may play an important role in defining the overall hPepT1 structure. K278C-hPepT1 showed ~40% activity and the 15 other transporters exhibited more than 50% gly-sar uptake when compared with wild type (WT)-hPepT1. Gly-sar uptake for the 16 active transporters containing cysteine mutations was measured in the presence of 2.5 mM 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA) or 1 mM [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET) for the Arg-282 positive charge in substrate translocation. Most of the amino acids that were MTSET-sensitive upon cysteine mutation, including R282C, are located toward the intracellular end of the TMS7. Therefore, our results suggest that TMS7 of hPepT1 is relatively solvent-accessible along most of its length but that the intracellular end of the transmembrane domain is particularly so. From a structure-function perspective, we speculate that the extracellular end of TMS7 may shift following substrate binding, providing the basis for channel opening and substrate translocation.

The human dipeptide transporter, hPepT1, is a 780- amino acid protein containing 12 α-helical transmembrane segments.

It is mainly expressed in the small intestine (1), proximal tubules of the kidney (2), and in the lysosomes of liver cells (2), pancreatic cells (3), and renal cells (4). PepT1 has received considerable attention as a drug carrier system in the past several years (5–8). It assists the proton-dependent uphill apical uptake of nutritional di- and tripeptides (9, 10), various peptidomimetic drugs such as β-lactam antibiotics (11), the anticancer drug bestatin (12), and many non-peptidic compounds (13, 14). Its broad substrate specificity along with its high capacity makes it a highly sought after target to increase the bioavailability of orally administered drugs and prodrugs.

Prodrugs have been designed to resemble peptides in order to facilitate their recognition by PepT1 (13, 15). A recent study by Anand et al. (16) showed that dipeptide ester prodrugs of acyclovir have high affinity toward hPepT1 and seem to be promising candidates in the treatment of ocular and oral herpes virus infections. However, the rational design of drugs and prodrugs as substrates has been limited because the tertiary structure of the transporter has not yet been elucidated. Several attempts have been made to determine the nature of its substrate-binding site and proton-binding site (17–19), and we have proposed a rudimentary computer model for hPepT1 based on some preliminary mutagenesis results (20). According to this model, seven amphipathic α-helical transmembrane segments (TMS1-7) form an aqueous channel through which the substrate is transported (20). However, experimental validation of this model is required in order to define the fine structure of the substrate-binding site and to understand the mechanism of substrate transport.

The substituted cysteine accessibility method (SCAM) has been used successfully to study the fine structure of various ion channels and transporters and, in particular, to determine the portion of transmembrane segments that line the channel pore or solute pathway (21–24). This method involves scanning cysteine mutagenesis of a transmembrane domain followed by covalent modification with an aqueous-soluble cysteine-reactive reagent; usually a methanethiosulfonate (MTS). Substrate uptake is used as a measure of the extent of modification, and hence solvent accessibility, of a particular amino acid. This approach has been utilized to obtain critical insight into the structures of the Glut1 glucose transporter (25), dopamine D2 receptor (26, 27), and glutamate transporter gltT (28).

In performing such studies, it is first of importance to determine the reactivity of endogenous cysteines with the cysteine-

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1 The abbreviations used are: TMS, transmembrane segment; MES, 4-morpholinethanesulfonic acid; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; PBS, phosphate-buffered saline; gly-sar, glycyl-sarcosine; WT, wild type; SCAM, substituted cysteine accessibility method.
modifying reagents. We have shown previously that the 11 endogenous cysteines present in WT-hPepT1 either do not react with the MTS reagents or that their modification with the MTS reagents does not have a significant impact on hPepT1 activity (29). This makes the application of SCAM to hPepT1 particularly appropriate. Indeed, we have recently shown that TMS5 is an integral part of the hPepT1 substrate translocation pathway by using a systematic cysteine-scanning mutagenesis approach in conjunction with MTS reagents (29). These results suggested that TMS5 is slightly tilted from the vertical axis of the channel, with the exofacial half-forming a classical amphipathic α-helix and the cytoplasmic half being highly solvent-accessible (29).

To determine the contributions of TMS7 in forming a part of the hPepT1 substrate-binding region, we have used the same approach to evaluate the relative orientation, functional importance, and solvent accessibility of this transmembrane segment with respect to the aqueous channel through which the substrate is transported. Our cysteine-scanning data and further mutagenesis results for Arg-282 suggest that TMS7 is a relatively solvent-accessible transmembrane segment and is particularly so at its cytoplasmic end.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H][Gly]gly-ser-sarcosine (4 μCi/ml) was purchased from Moravek Biochemicals (Brea, CA). Cell culture media and supplies were obtained from Invitrogen. 2-Aminoethyl methanethiosulfonate hydrobromide (MTSEA) and [2-(trimethylammonium) ethyl]methanethiosulfonate bromide (MTSET) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

**Site-directed Mutagenesis**—The hPepT1 cDNA (kindly provided by Dr. Matthias A. Hedigar) was subcloned into the E. coli vector pET15b (Novagen). Primers were designed using the Gene Editor™ site-directed mutagenesis kit (Promega Corp., Madison, WI). The mutated cDNA was transformed into an Escherichia coli strain, BMH71-18 Mut.S, which is incapable of correcting mismatches. This process of amplification was repeated once more using the JM109-competent cells to enrich the mutated population using the JM109-competent cells to enrich the mutated population of the hPepT1 cDNA. Each of the 21 amino acid residues within the putative transmembrane helix 7 was individually mutated to a cysteine. Amino acids are designated by the single letter code, e.g. R278C represents the mutation of a lysine at position 278 to a cysteine. NA, not applicable.

**TABLE I**

| Mutation | Codon change |
|----------|--------------|
| Wild type | NA           |
| K278C    | AAG to TGC   |
| M279C    | ATG to TGC   |
| V280C    | GTC to TGG   |
| T281C    | AGC to TGC   |
| R282C    | AGG to TGG   |
| V283C    | GTG to TGG   |
| M284C    | ATG to TGC   |
| F285C    | TTC to TGC   |
| L286C    | CTG to TGC   |
| Y287C    | TAT to TGT   |
| I288C    | ATT to TGC   |
| P289C    | CCA to TGC   |
| L290C    | CTC to TGC   |
| P291C    | CCA to TGC   |
| M292C    | ATG to TGC   |
| F293C    | TTC to TGC   |
| W294C    | TGC to TGG   |
| A295C    | GCC to TGC   |
| L296C    | TTA to TGC   |
| F297C    | TTT to TGT   |
| D298C    | GAC to TGC   |

**Analysis of TMS7 of hPepT1 by Cysteine-scanning Mutagenesis**

A series of 21-cysteine scanning mutant cDNAs was created by oligonucleotide-mediated site-directed mutagenesis of the wild type hPepT1 cDNA. Each of the 21 amino acid residues within the putative transmembrane helix 7 was individually mutated to a cysteine. Amino acids are designated by the single letter code, e.g. R278C represents the mutation of a lysine at position 278 to a cysteine. NA, not applicable.

**Fig. 1.** Membrane localization of the mutated hPepT1 transporter proteins in transiently transfected HEK293 cells. 72 h post-transfection, the transfected HEK293 cells were subjected to immunofluorescence microscopy using affinity-purified rabbit anti-hPepT1 primary antibody and fluorescein isothiocyanate-conjugated secondary antibody, both at a dilution of 1:500. Immunostaining in wild type hPepT1-transfected cells (WT) is shown as a positive control.

**Immunoprecipitation and Western Blots**—Cell lysates were pre-cleared with Sepharose CL-2B cross-linked 2% beaded agarose (Sigma) combined with affinity-purified anti-hPepT1 antibody, and the immunoprecipitates were collected with protein A-Sepharose (Sigma). Further details of the protocol will be subsequently published. The immunoprecipitates were fractionated by electrophoresis on an 8% pre-cast polyacrylamide electrophoresis gel (Gradipore, Inc., Hawthorne, NY), blotted onto Trans-Blot® transfer medium pure nitrocellulose membranes (Bio-Rad), probed with affinity-purified anti-hPepT1 primary

**Immunofluorescence microscopy staining** has been described in detail previously (30). Briefly, transfected HEK293 cells were plated onto coverslips and cultured for 48 h. The coverslips were then incubated with 3.7% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min. After washing 3 times with PBS, the coverslips were permeabilized with 0.5% Triton X-100 for 15 min, washed once, and then blocked with 1% bovine serum in PBS at room temperature for 30 min. After washing once with 0.05% Tween 20 in PBS (PBST), the coverslips were incubated with primary antibodies for 2 h. After washing 3 times with PBST, they were incubated with fluorescein isothiocyanate-conjugated secondary antibodies for 1 h. The coverslips were washed again with PBST (twice) and PBS (once). Finally, the coverslips were mounted onto slides with anti-fade medium and examined by fluorescence microscopy.

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| A295C    | GCC to TGC   |
| L296C    | TTA to TGC   |
| F297C    | TTT to TGT   |
| D298C    | GAC to TGC   |
Inhibition Studies with Sulfhydryl Reagents — Prior to the uptake measurements, the transfected cells, adhered to the wells, were washed with the transport medium (MES-Tris, pH 6 buffer). Each well was then incubated for 10 min at 37°C with a solution containing [3H]gly-sar (0.5 μCi/ml) after preincubation with 2.5 mM MTSEA or 1 mM MTSET (Toronto Research Chemicals Inc., Ontario, Canada) for 10 min. After washing three times in ice-cold MES-Tris, pH 6.0 buffer, the cells were lysed in 1 ml of lysis buffer (1% SDS). BCA protein assay reagents were used to determine the protein content of each well, and the cell-associated radioactivity was measured in a Beckman liquid scintillation counter. Mock-transfected and WT-hPepT1-transfected HEK293 cells were used as negative and positive controls, respectively. The characteristics

**FIG. 2.** Immunoprecipitation and Western blot analysis of the mutated hPepT1 transporter proteins expressed in HEK293 cells. 72 h post-transfection, the transfected HEK293 cells were subjected to immunoprecipitation followed by Western blot analysis using affinity-purified rabbit anti-hPepT1 primary antibody, and they were visualized with secondary antibody and chemiluminescence (30).

**FIG. 3.** Percentage of gly-sar uptake activities of the cysteine-scanning mutants of TMS7 of hPepT1. [3H]Gly-sar uptake (0.5 μCi/ml, 10 min at 37°C) was measured 72 h post-transfection in HEK293 cells, individually transfected with the cysteine-scanning mutants of TMS7 of hPepT1. Results represent the % gly-sar uptake of individual mutant transporter protein when compared with wild type hPepT1 (n = 4–6). The background uptake values of mock-transfected HEK293 cells were subtracted. *, ≥25% specific activity.
of dipeptide uptake by WT-hPepT1-transfected HEK293 cells in the presence of MTS reagents were very similar to those in the absence of the MTS reagent (29).

**Computational Analysis**—A helical wheel model of the transmembrane segment 7 of hPepT1 was constructed using the Lasergene software (DNAstar, Inc., Madison, WI). The three-dimensional models were generated using in-house software (TMD) and viewed using WebLab ViewerPro 3.7 (Accelrys Inc., San Diego).

### RESULTS

We have shown previously (29) that the activity of WT-hPepT1 is not significantly changed by the presence of MTS reagents, by studying the effect of 1 mM MTSET or 2.5 mM MTSEA on gly-sar uptake by WT-hPepT1 in HEK293 cells and in *Xenopus* oocytes. Hence, WT-hPepT1 was used as a control in the current work. Each of the 21 residues within TMS7 of WT-hPepT1 was individually mutated to a cysteine (Table I). Each mutated transporter was transiently transfected into HEK293 cells, and their plasma membrane expression in these cells was evaluated using immunofluorescence microscopy.

Two of the 21 mutated transporters (Y287C- and M292C-hPepT1) did not express at the plasma membrane, as seen by a lack of fluorescence intensity on the edge of each cell transfected with these two mutants (Fig. 1). Low or negligible protein expression levels as a result of single amino acid mutation have been demonstrated before in various membrane proteins including hPepT1 (25, 28, 29). Immunoprecipitation of the 21 hPepT1 mutants followed by Western blot analysis confirmed these results, because Y287C- and M292C-hPepT1 showed insignificant steady state protein expression (Fig. 2). This suggests that single cysteine mutations at these two positions were responsible for the incorrect synthesis and/or misfolding of the mutated proteins. Due to lack of expression, HEK293 cells transfected with these mutants show negligible gly-sar uptake (Fig. 3).

The remaining 19 mutated transporter proteins exhibited membrane expression similar to WT-hPepT1 (Fig. 1). The steady state protein expression of these transporters also compares well with that of WT-hPepT1, as confirmed by immunoprecipitation followed by Western blot analysis (Fig. 2). Three of these 19 transporters (F293C-, L296C-, and F297C-hPepT1) showed negligible gly-sar uptake activity; K278C-hPepT1 showed ~40% activity, and the 15 remaining transporters exhibited more than 50% gly-sar uptake when compared with WT-hPepT1 (Fig. 3).

We assessed the solvent accessibilities of the cysteine residues in the 19 mutated transporter proteins in order to determine the residues that would likely be a part of the substrate translocation pathway. This was achieved by measuring the specific uptake activities of these proteins after preincubation with the hydrophilic sulfhydryl-specific chemical reagents MTSET (1 mM) and MTSEA (2.5 mM), and comparing these data to those measured in the absence of these reagents (Figs. 4 and 5). MTSET is completely membrane-impermeable, but MTSEA is slightly membrane-permeable and has a molecular volume about 60% that of MTSET (molecular volume = 109 Å³).

Of the 19 mutated transporter proteins, no significant difference was observed in the uptake activities of F293C-, L296C-, and F297C-hPepT1 because their inherent activities were neg-

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**Fig. 4. Effect of 2.5 mM MTSEA on [3H]gly-sar uptake activities of the cysteine-scanning mutants of TMS7 of hPepT1.** 72 h post-transfection, the transfected cells, adhered to the wells, were washed with the transport medium (MES-Tris, pH 6 buffer). Each well was then incubated for 10 min at 37 °C with a solution containing [3H-gly-sar (0.5 μCi/ml) after pre-incubation with 2.5 mM MTSEA for 10 min. After washing three times in ice-cold MES-Tris, pH 6.0 buffer, the cells were lysed in 1 ml of lysis buffer (1% SDS). BCA protein assay reagents were used to determine the protein content of each well, and the cell-associated radioactivity was measured in a Beckman liquid scintillation counter. Results represent the % gly-sar uptake of each individual mutant transporter protein when compared with wild type hPepT1 (n = 4–6). The background uptake values of mock-transfected HEK293 cells were subtracted. The white bars represent uptake activity in the absence of 2.5 mM MTSEA, and the black bars represent the uptake activities in the presence of 2.5 mM MTSEA, highly significant inhibition of uptake activity by 2.5 mM MTSEA.
The remaining 16 single cysteine-mutated transporters were active and exhibited considerable gly-sar uptake activities. Gly-sar uptake for these transporters was then measured in the presence of 2.5 mM MTSEA or 1 mM MTSET. MTSEA (2.5 mM) significantly inhibited gly-sar uptake by each of the 16 mutated hPepT1 transporters (Fig. 4). In contrast, 1 mM MTSET selectively inhibited the transport activities of 8 (K278C-, M279C-, V280C-, T281C-, M284C-, L286-, P291C- and D298C-hPepT1) of the 16 mutated hPepT1 proteins to a significant extent \( (p < 0.05, n = 3-6) \) (Fig. 5). F285C- and W294C-hPepT1 did show a slight inhibition of gly-sar uptake in the presence of 1 mM MTSET, but this inhibition was comparable with that observed with WT-hPepT1 under similar conditions.

The transport activity of R282C-hPepT1 and I288C-hPepT1 was significantly increased following incubation with 1 mM MTSET.
MTSET ($p < 0.05, n = 3–6$) (Fig. 5). The result for R282C-hPepT1 was somewhat surprising, because Arg-282 is positively charged and is expected to be in an aqueous environment. Hence, we expected that R282C would be accessible to the MTS reagents and that this would result in a significant inhibition of gly-sar uptake. However, we observed that after incubation with 1 mM MTSET, the transport activity of R282C-hPepT1 was similar to WT-hPepT1. To explore this observation further, we measured the gly-sar uptake activities of R282E- and R282K-hPepT1 (Fig. 6). We have reported previously (20) that R282A-hPepT1 showed a small reduction in gly-sar uptake, but the difference from that of WT-hPepT1 was not significant. R282K-hPepT1 showed uptake similar to that of WT-hPepT1 ($n = 3–6, p < 0.05$). In contrast, R282E-hPepT1 showed an ~43% reduction in gly-sar uptake when compared with WT-hPepT1 ($n = 3–6, p < 0.05$).

**FIG. 7.** Helical wheel model of TMS7 of hPepT1. Transmembrane segment 7 of hPepT1 as viewed from the extracellular side of the plasma membrane. Amino acids are represented by the single letter code. *, cysteine substitutions at these residues resulted in mutant transporters that were unable to express on the plasma membrane of the HEK293 cells. Arrows point to the residues that were unable to tolerate cysteine substitutions or were accessible to 1 mM MTSET.

**FIG. 8.** Proposed orientation of TMS7 in hPepT1. A lateral view of TMS7 in hPepT1 showing amino acids for which cysteine mutation eliminated transport (green), amino acids accessible to the MTS reagents (blue), amino acids that hamper membrane expression when mutated to a cysteine (white), amino acids that increase uptake activity in the presence of 1 mM MTSET when mutated to a cysteine (light blue), and non-accessible amino acids (orange). The yellow dotted line represents the vertical axis of the aqueous channel.

**FIG. 9.** Proposed formation of pseudo-arginine at amino acid position 282. The top panel shows the structure of an arginine residue. The bottom panel shows the chemical reaction between the –SH group of a cysteine and the MTSET reagent, leading to the formation of an “arginine-like” moiety.

**DISCUSSION**

Helical wheel analysis of the effects of cysteine-scanning and MTS modification in TMS7 of hPepT1 show that the amino acid residues that are sensitive to MTS modification are positioned over the majority of the circumference of the wheel (Fig. 7). This is in sharp contrast with the results that we have reported for TMS5 of hPepT1 (29), in which the MTSET-sensitive residues are gathered together on a single face of a generally amphipathic α-helix (29). The TMS7 helical wheel (Fig. 7) indicates that this transmembrane domain is not classically amphipathic. However, representation of the solvent-accessible and non-solvent-accessible residues in a lateral view of TMS7 (Fig. 8) suggests that the properties of the helix differ at its extracellular and intracellular ends. In the following paragraphs we examine the potential functional roles of amino acids in various region of TMS7.

Of the 19 mutated hPepT1s that showed membrane expression, three (F293C-, L296C-, and F297C-hPepT1) displayed negligible gly-sar uptake activity, suggesting that the amino acids at these positions were not able to tolerate mutation to cysteine. We have identified previously a number of amino acids in TMS5 of hPepT1 that showed a similar effect on mutation to cysteine (29) or alanine (30), including Tyr-167, Asn-171, and Ser-174 (unpublished data for N171A and S174A). These TMS5 amino acids are all hydrophilic, and we interpreted these data to suggest that they were lining the substrate translocation channel and interacting with the sub-

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strate. However, the TMS7 amino acids that cannot tolerate mutation to cysteine, Phe-293, Leu-296, and Phe-297, are hydrophobic. Hence, we believe that they probably play a structural role in transporter function. Model building of the orientation of these three amino acids suggests that the two methyl groups of the leucine side chain pack against the faces of the phenylalanine rings, and mutation of any one of the three will disturb the Phe-Leu-Phe hydrophobic packing interaction. We note that this sort of interaction has been observed elsewhere as in, for example, the peptide GILGFVFTL binding to the MHC protein HLA-A2 (31). In this context, mutation of Phe-5 to alanine of the peptide disrupts the peptide conformation and reduces the affinity for the protein. Hence, Phe-293, Leu-296, and Phe-297 in hPepT1 may form a similar interaction that provides a "cushion" against which other transmembrane segments may pack, and a mutation of any one of these 3 amino acids to a cysteine may cause a conformational change leading to hPepT1 inactivity.

W294C-hPepT1 showed a relatively small reduction in gly-sar uptake in the presence of MTSET, but the difference between its uptake and that of the wild type protein was significant. We have shown previously (20) that a W294A mutation in hPepT1 influenced the kinetics of gly-sar uptake and, in particular, had a significant effect on the Michaelis-Menten $K_m$ value, which was almost 10 times larger for WT-hPepT1. This is notable, because most other mutations of hPepT1 amino acids in putative transmembrane domains that influence uptake kinetics tend to show altered $V_{max}$ values (20) and do not influence $K_m$. Hence, we suggest that Trp-294 largely plays a role in maintaining the structural integrity of the protein, but probably does not interact directly with the substrate. Its location may lie at the interface between the solvent-accessible and inaccessible regions, and this is consistent with the MTSET modification data and with the three-dimensional helical structural model presented in Fig. 8. We note that W294C-hPepT1 did not show reduced uptake compared with WT-hPepT1 (Fig. 3), in contrast to W294A-hPepT1 (20). We have not followed up this observation further, but it may be that the somewhat larger size of the cysteine side chain, compared with alanine, sufficiently reflects the steric bulk of the tryptophan side chain and better maintains the correct helical packing.

The most surprising finding was that the mutation of Arg-282 to a cysteine did not have any significant effect on hPepT1 activity and that R282C did not show any significant inhibition of gly-sar uptake after incubation with the membrane-impermeable MTSET. One would expect that arginine, being a positively charged amino acid, would be facing the aqueous substrate pathway and would be reactive toward the MTS reagent when mutated to a cysteine, causing a significant inhibition of gly-sar uptake. However, the results are contradictory to these expectations. After incubation with 1 mM MTSET, R282C-hPepT1 exhibits WT-hPepT1-like gly-sar uptake (−20% increase in uptake activity, compared with R282C-hPepT1 in the absence of MTSET). We have also shown previously that R282A-hPepT1 has activity comparable with WT-hPepT1 (20). To investigate these observations further, we generated the R282K- and R282E-hPepT1 proteins. Our results showed that R282K-hPepT1 retains WT-hPepT1-like activity, whereas R282E-hPepT1 has a 43% reduction in its activity. This suggests that the positive charge is important at amino acid position 282, and we speculate that the 20% increase in uptake activity of R282C-hPepT1 in the presence of MTSET is due to the formation of a "pseudo-arginine" at position 282 by the MTSET (Fig. 9). However, we are unable to explain the increase in I288C-hPepT1 activity after incubation with 1 mM MTSET or the decrease in R282C-hPepT1 activity after incubation with 2.5 mM MTSEA.

The exact role of the positive charge of Arg-282 in the hPepT1 substrate translocation mechanism remains difficult to determine. However, it is of interest that Arg-282 in TMS7 and Asp-341 in TMS8 are likely to be spatially proximal. The extracellular loop between TMS7 and TMS8 of hPepT1 is relatively short (~30 amino acids), which suggests that TMS7 and TMS8 are proximal. Considering the vertical lengths of these 2 transmembrane segments and assuming that they are adjacent to each other, Arg-282 in TMS7 and Asp-341 in TMS8 are located approximately at the same depth from the extracellular side of the lipid membrane. Hence they should lie in close proximity to each other and may potentially form an Arg-282 to Asp-341 salt bridge. Supporting this idea is the observation that D341A-hPepT1 exhibits WT-hPepT1 like activity, similarly to R282A-hPepT1.

Helical wheel analysis of the effects of cysteine-scanning and MTS modification in TMS7 of hPepT1 are reminiscent of the findings obtained for TMS7 of the facilitative glucose transporter Glut1 (32) but sharply contrast with the results obtained for TMS5 of hPepT1 (29). The helical wheel arrangement of sulphydryl reagent-sensitive residues on TMS5 and TMS7 of hPepT1 is strikingly similar to the arrangement observed in TMS5 and TMS7 of Glut1 (32, 33). However, a lateral view of TMS7 of the two proteins suggests that there are a number of differences between hPepT1 and Glut1, and in particular, the extracellular end of TMS7 of hPepT1 appears to be rather less solvent-accessible than the same region of Glut1 TMS7. Hence, our data suggest that the structures of hPepT1 and Glut1 may be similar in a general sense and that there may be value in further comparison of the putative substrate translocation mechanisms of the two proteins. However, it remains likely that the details of these mechanisms will be significantly different. Preliminary molecular modeling of the likely interactions between hPepT1 TMS7 and TMS8, based on data from this and other mutagenesis and SCAM studies, allows us to speculate that the extracellular end of TMS7 may shift following substrate binding, providing the basis for channel opening and substrate translocation. We intend to further probe this speculative proposal by mutagenesis and SCAM analysis of other hPepT1 transmembrane domains.

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