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Research Paper

A Charge Pair Interaction Between Arg282 in Transmembrane Segment 7 and Asp341 in Transmembrane Segment 8 of hPepT1

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Purpose. To determine whether R282 in transmembrane segment 7 (TMS7) of hPepT1 forms a salt bridge with D341 in TMS8.
Methods. Mutated hPepT1 transporters containing point mutations at R282 and/or D341 were transiently transfected into HEK293 cells. Their steady state expression and functional activity were measured using immunoprecipitation and ^{3}H-gly-sar uptake, respectively. Gly-sar uptake by cysteine mutants (R282C and D341C) was also measured in the presence and absence of cysteine-modifying MTS reagents.
Results. The reverse-charge mutants R282D-hPepT1 and D341R-hPepT1 showed significantly reduced gly-sar uptake, but the double mutant (R282D/D341R-hPepT1) has functionality comparable to that of wild-type hPepT1. Gly-sar uptake by R282C-hPepT1 is reduced, but pre-incubation with 1 mM MTSET, a positively charged cysteine-modifying reagent, restored function to wild-type levels. Similarly, pre-incubation of D341C-hPepT1 with 10 mM MTSES, a negatively charged cysteine-modifying reagent, increased gly-sar uptake compared to unmodified D341C-hPepT1. In contrast, MTSET modification of D341C-hPepT1 (giving a positive charge at position 341) resulted in significant reduction in gly-sar uptake, compared to D341C-hPepT1.
Conclusion. Our results are consistent with a salt bridge between R282 and D341 in hPepT1, and we use these and other data to propose a role for the R282-D341 charge pair in the hPepT1 translocation mechanism.

KEY WORDS: charge pair; dipeptide; hPepT1; mutagenesis; salt bridge; transporter.

INTRODUCTION

The human dipeptide transporter, hPepT1, has attracted attention over the past decade as a route for transport of peptides and peptidomimetics across the intestinal lumen. This transporter protein is also an excellent target for peptide-based prodrugs designed to increase the oral bioavailability of poorly absorbed compounds. For example, Anand *et al.* (1,2) have shown that dipeptide ester prodrugs of acyclovir have high affinity towards hPepT1, Knutter *et al.* (3) have synthesized dipeptide derivatives that were shown to be competitive inhibitors of PepT1 with an apparent binding affinity of 5–10 μM, and Amidon and Walgreen (4) found that 5′-amino acid esters of antiviral nucleosides are absorbed by hPepT1. A peptide mimic such as 4-aminophenylacetic acid (4-APAA), which lacks a peptide bond, is also a PepT1 substrate (5). The structural requirements for compounds to have significant affinity towards hPepT1 have been reviewed by Brandsch *et al.* (6). In parallel with these substrate studies, effort has been directed at elucidating the tertiary structure of hPepT1 (7–9) and determining the nature of the substrate-binding site (10–13) and proton-binding site (14–17).

cDNA encoding hPepT1 was cloned in 1995 and showed that the transporter contains 708 amino acids (18). Hydropathy analysis (19) predicted 12 putative α-helical transmembrane segments (TMS) with both the N and C terminals located on the cytoplasmic side of the plasma membrane, and this topology has subsequently been confirmed by Covitz *et al.* (20). We have proposed a simplified computer model of hPepT1, initially based on limited mutagenesis data and helix amphipathicity (7). Although this model lacks atomic detail, the general feature of amphipathic α-helical transmembrane segments in contact with an aqueous translocation channel has largely been supported by subsequent experimental data (8,9). Specifically we have used a chemical modification strategy to evaluate the relative orientation, functional importance, and solvent accessibility of specific α-helical transmembrane segments in hPepT1, and shown that TMS5 and TMS7 both form part of the substrate translocation pathway (8,9). For TMS5, the cytoplasmic half of the helix seems to be highly solvent

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accessible and Y167, N171, and S174 probably play a role in substrate binding (8). TMS7 appears to be solvent accessible along most of its length, but again the cytoplasmic half of the helix is particularly so. We also speculated that the extracellular end of TMS7 might shift during a conformational change of the protein, providing the basis for channel opening and substrate translocation (9).

Arginine at position 282 in TMS7 is well conserved across PepT1 in different species and we have previously suggested that it might form a charge-pair interaction with D341 in TMS8 (9). Furthermore, Meredith (21) has reported that R282 plays a key role in the rabbit PepT1 (rbPepT1) translocation mechanism: an R282E mutation in rbPepT1 uncouples the co-transport of protons and peptides by rbPepT1 and creates a peptide-gated cation channel, suggesting that R282 plays a role in the proton-coupling pathway (21). In the current study we present evidence for the presence of an R282-D341 charge-pair interaction in hPepT1 based on the functional activities of proteins carrying single amino acid mutations of R282 and D341 and, most importantly, on the restoration of wild-type hPepT1 function by an R282D/D341R double mutation.

MATERIALS AND METHODS

Materials

^{3}H glycyl-sarcosine (4Ci/mmol) was purchased from Moravek Chemical Co. (Brea, CA). Cell culture media and supplies were obtained from GIBCO (Grand Island, NY). The MTS reagents were purchased form Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada).

Site-Directed Mutagenesis

The hPepT1 cDNA (kindly provided by Dr. Matthias A. Hediger) was subcloned into the eukaryotic expression plasmid pcDNA3 (Invitrogen, Carlsbad, CA) by ligating the 2,306 *Kpn*I/*Bam*HI fragment into the multiple cloning sites of pcDNA3. This pcDNA3-hPepT1 plasmid is under the control of the cytomegalovirus promoter, and was used as a template for all the mutagenesis reactions, unless specified otherwise, following the standard site-directed mutagenesis protocol provided by the manufacturer for the Gene Editor™ site-directed mutagenesis kit (Promega Corporation, Madison, WI). The mutated cDNA was transformed into an *E. 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. The transformed cells were then plated onto ampicillin LB plates and incubated overnight. Individual colonies obtained on the ampicillin LB plates were amplified further. The plasmid extracted from each colony was then subjected to DNA sequencing analysis to verify the mutations (Genemed Synthesis Inc., San Francisco, CA).

Transient Transfection of the Mutants in HEK293 Cells

The transfection of hPepT1 in HEK293 cells (ATCC no. CRL-1573) has been reported previously (7). These cells were used for the expression of wild-type hPepT1 and the mutated transporters because of their low endogenous dipeptide transport activity. The uptake of ^{3}H-gly-sar in wild-type hPepT1-transfected HEK293 cells was 100 times higher than that in mock-transfected HEK293 cells (7). The HEK293 cells were split into 60 × 15 mm dishes and grown overnight (>20 h) at 50–75% confluence. The medium was then removed and 2 ml transfection solution (2 ml DMEM with 20 μl DEAE-dextran (25 mg/ml) and 10 μl chloroquine (20 mM)) was added to each dish. The mixture was then incubated at 37°C for 2 h. The transfection solution was previously prepared in a stock of 20 ml which was then divided into 10 portions (2 ml each), 1 μg of DNA was added to each portion and the mix was incubated at 37°C for 10 min. After removing the transfection solution, 2 ml of 10% DMSO in DPBS (sterile PBS) was added to each dish. Following an incubation period of 2 min at room temperature, the DMSO solution was removed and replaced by 4 ml fresh DMEM in each dish. The cells were grown overnight and then each dish was split into 6 wells of a 12-well plate.

Immunoprecipitation and Western Blots

Cell lysates were pre-cleared with sepharose CL-2B cross-linked 2% beaded agarose (Sigma-Aldrich Corp., St. Louis, MO) combined with affinity purified anti-hPepT1 antibody and the immunoprecipitates were collected with protein A sepharose (Sigma-Aldrich Corp.). Further details of the protocol will be subsequently published (C. Chu, C. T. Okamoto, and V.H.L. Lee, in preparation). The immunoprecipitates were fractionated by electrophoresis on 8% precast polyacrylamide electrophoresis gels (Gradipore, Inc., Hawthorne, NY), blotted onto Trans-Blot® transfer medium pure nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), probed with affinity purified anti-hPepT1 primary antibody and visualized with secondary antibody and chemiluminescence (22).

Gly-Sar Uptake Studies

The transfected cells, which adhered to the wells, were washed with the transport medium (MES-Tris, pH 6 buffer) prior to the uptake measurements. Each well was then incubated for 10 min at 37°C with a solution containing [^{3}H] gly-sar (0.5 μCi/ml. After washing thrice in ice-cold MES-Tris (pH 6.0) buffer, the cells were lysed in 1 ml 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 wild type hPepT1-transfected HEK293 cells were used as negative and positive controls, respectively. The procedure for evaluating the effect of 1 mM MTSET or 10 mM MTSES on the uptake activity of single cysteine mutants of hPepT1 has been described previously (8).

Statistical Analysis

Gly-sar uptake data were compared between the wild-type hPepT1 and each mutated transporter using a Student's

t-test, with $p < 0.05$ considered to be significant. Gly-sar uptake data for each MTSET- or MTSES-modified mutated transporter were compared with the equivalent unmodified mutated transporter, again using a Student's *t*-test.

RESULTS

We have shown (9) that TMS7 forms an integral part of the hPepT1 substrate translocation pathway and postulated that R282 of TMS7 may form a charge pair with D341 in TMS8. To examine the putative charge-pair interaction we generated hPepT1 proteins with point mutations at positions 282 or 341 and a doubly mutated R282D/D341R-hPepT1, in which the charges at positions 282 and 341 are reversed. The doubly mutated protein was generated using R282D-hPepT1 as template cDNA. The mutated transporters were transiently transfected into HEK293 cells and their expression levels were measured 72 h post transfection by immunoprecipitation followed by western blot analysis. The steady state protein expression levels of the mutated proteins in HEK293 cells were comparable to that of wild-type hPepT1 (Fig. 1).

Functional activities of the mutated proteins were evaluated using gly-sar uptake, and are expressed relative to wild-type hPepT1 activity, which was taken to be 100%. The uptake activities for transporters carrying mutations of R282 are shown in Fig. 2. The protein in which positively charged R282 is replaced with a neutral amino acid (R282A-hPepT1) retained 75.2 ± 13.9% of wild-type gly-sar uptake ($p > 0.05$; $n = 4$). Substitution of R282 by another positively charged amino acid (R282K–hPepT1) resulted in 108.6 ± 3.9% gly-sar uptake ($p < 0.05$; $n = 6$), whereas its substitution with a negatively charged amino acid (R282E-andR282D-hPepT1) decreased gly-sar uptake to 57.3 ± 3.8% and 82.4 ± 5.1%, respectively, compared to wild-type hPepT1 ($p < 0.05$; $n = 6$–8) (9). The data for R282E-hPepT1 are in agreement with those for R282E-rbPepT1 (21).

The uptake activities obtained with D341 mutant transporters are also shown in Fig. 2. Replacement of negatively charged D341 with a neutral amino acid (D341A–hPepT1) resulted in 71.7 ± 29.3% of the wild-type gly-sar uptake, but

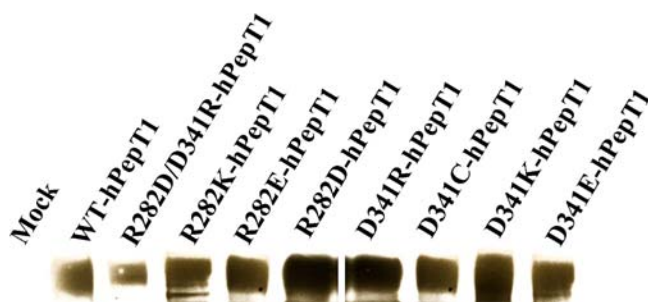

Fig. 1. Immunoprecipitation and western blot analysis of mutated hPepT1 transporters expressed in HEK293 cells. Seventy-two-hour post-transfection, the transfected HEK293 cells were subjected to immunoprecipitation followed by western blot analysis using affinity purified rabbit anti-hPepT1 primary antibody, and were then visualized with secondary antibody and chemiluminescence (22). This analysis was performed in duplicate for each transporter, and representative data are shown.

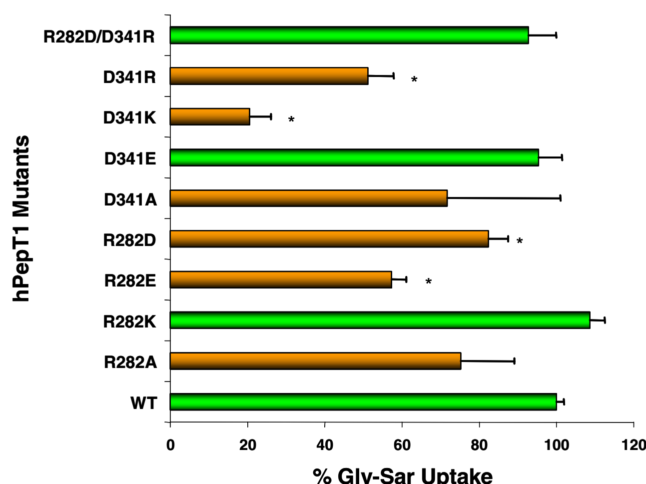

Fig. 2. Percentage gly-sar uptake of hPepT1 transporters with mutations at R282, D341 and R282/D341. ^{3}H-gly-sar uptake (0.5 µCi/ml, 10 min at 37°C) was measured 72 h post-transfection in HEK293 cells transfected with individual mutants. The data are shown as the mean ($n = 4$–8) % gly-sar uptake for each mutated transporter protein, where 100% is the wild-type hPepT1 uptake. The background uptake values of mock-transfected HEK293 cells were subtracted from the measured data. *$p < 0.05$ compared to wild-type hPepT1 uptake.

the change from wild-type hPepT1 was not significant ($p > 0.05$; $n = 4$). Preserving the negative charge at position 341 by substituting D341 with a glutamic acid (D341E-hPepT1) produced a protein that retained wild-type hPepT1 activity (95.3 ± 6.1%, $n = 4$), but replacing D341 with a positively charged amino acid (D341K- or D341R-hPepT1) significantly reduced gly-sar uptake to 20.5 ± 5.6% and 51.2 ± 6.6%, respectively, compared to wild-type uptake ($p < 0.05$; $n = 5$–8).

The above results show that charge reversal at either position 282 or position 341 substantially decreases hPepT1 function. However, as shown in Fig. 2, concomitant charge reversal at these positions to create the double mutant protein, R282D/D341R-hPepT1, resulted in recovery of gly-sar uptake activity to close to that of wild-type hPepT1. This is a significant piece of evidence for the presence of an R282-D341 charge pair in hPepT1. The double mutation results in the swapping of the two charges at positions 282 and 341; thus, the putative charge-pair interaction is retained. The R282D/D341R-hPepT1 double mutant has a gly-sar uptake activity of 92.7 ± 7.1%, similar to that of wild-type hPepT1. This activity is significantly greater ($p < 0.05$; $n = 3$–5) than that of D341R-hPepT1 (51.2 ± 6.6%) and higher than that of R282D-hPepT1 (82.4 ± 5.1%).

The results of uptake by R282C and D341C–hPepT1 in the absence and presence of the cysteine-modifying reagents MTSET and MTSES are shown in Fig. 3. MTSET and MTSES are water-soluble, lipid-insoluble molecules that are positively and negatively charged, respectively; these reagents have been widely used to probe solvent accessibility of amino acids in transmembrane domains (23–27). A single cysteine mutation at position 282 (R282C-hPepT1) reduced hPepT1 uptake activity (Fig. 3), although the change was not statistically significant ($p > 0.05$; $n = 4$). However, incubation of R282C-hPepT1 with 1 mM MTSET (positively charged) caused a 23% increase in gly-sar uptake, compared to that of

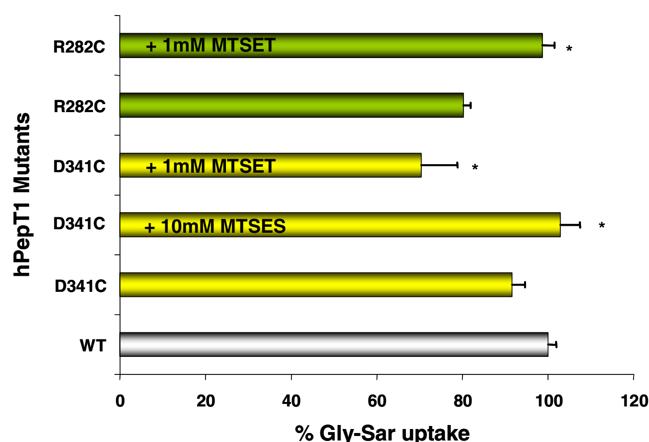

Fig. 3. Effect of MTS reagents on ^{3}H gly-sar uptake of R282C- and D341C-hPepT1. ^{3}H gly-sar uptake (0.5 µCi/ml, 10 min at 37°C) was measured 72 h post-transfection in HEK293 cells transfected with wild-type hPepT1, R282C-hPepT1 or D341C-hPepT1, with or without pre-incubation with MTS reagents (1 mM MTSET or 10 mM MTSES). The data are shown as the mean (n = 4–6) % gly-sar uptake for each mutated transporter protein. The background uptake values of mock-transfected HEK293 cells were subtracted from the measured data. *$p < 0.05$ for the difference in uptake in the presence and absence of the cysteine-modifying reagent.

unmodified R282C-hPepT1 (Fig. 3; $p < 0.05$; $n = 4$), and restored the uptake activity to wild-type levels (9). Gly-sar uptake by D341C-hPepT1 is similar to that of the wild-type protein (Fig. 3), but reaction of D341C with 1 mM MTSET (positively charged) reduced gly-sar uptake to 76% that of unmodified D341C-hPepT1 (Fig. 3; $p < 0.05$; $n = 5$). However, reaction of the cysteine at position 341 with the negatively charged cysteine modifying reagent MTSES (10 mM) caused a 12.5% increase in gly-sar uptake activity compared to unmodified D341C-hPepT1 (Fig. 3; $p < 0.05$; $n = 5$).

DISCUSSION

TMS7 is an important component of hPepT1 from both structural and substrate interaction perspectives (9). Three hydrophobic amino acids near the exoplasmic end of TMS7 (F293, L296, and F297) may anchor TMS7 to either the lipid bilayer or another hydrophobic TMS, and the cytoplasmic end of TMS7 forms part of the substrate translocation pathway (9). R282 is located in the exoplasmic region of TMS7, and cysteine modification data and geometrical considerations led us to propose that R282 may be involved in a charge-pair interaction with D341 in TMS8 (9). The current study provides evidence for the presence of this charge pair (salt bridge) in hPepT1.

Mutation of either R282 or D341 to an oppositely charged amino acid causes a significant loss of function. If R282 and D341 were functionally independent of each other in the wild-type protein, their concomitant mutation might be expected to further reduce the loss of function of the single mutant proteins. However, the R282D/D341R double mutant has uptake function similar to that of the wild-type protein. A plausible explanation for this result is the presence of a salt bridge between positions 282 and 341. We note that this charge-reversal mutagenesis strategy has been used to demonstrate that H338 of the rat vesicular acetylcholine transporter forms an ion pair with D398 (28). Similarly, Dunten *et al.* (29) have shown that D237 and K358 in the lactose permease of *Escherichia coli* form a salt bridge; an observation that has subsequently been validated by X-ray crystallography (30).

The presence of an R282-D341 salt bridge in hPepT1 accounts for the uptake data for proteins with R282 and D341 single point mutations. R282K-hPepT1 remains fully functional since the positive charge at position 282 is maintained, but substitution of R282 with negatively charged amino acids (R282E-andR282D-hPepT1) significantly decreases gly-sar uptake. These data suggest that a positive charge at position 282 is important for hPepT1 function. Further, it seems likely that, based on the proposed charge-pair interaction between R282 and D341, the presence of a negative charge at position 282 will result in repulsion with D341, perhaps leading to the reduced function.

Point mutations of D341 result in a similar predictable pattern. Replacing the negatively charged D341 with a positively charged amino acid residue (D341R- or D341K-hPepT1) significantly reduces gly-sar uptake due to repulsion between the side chain of R282 and the positive charge at position 341, whereas conservation of the charge pair in D341E-hPepT1 preserves wild-type function. Mutation of D341 to a positive amino acid reduces the uptake activity to a greater extent than mutation of R282 to a negative amino acid: D341R and D341K- hPepT1 exhibit an average gly-sar uptake of about 35%, compared to wild-type uptake, whereas the average uptake of R282D-andR282E-hPepT1 is about 70%. Since lysine and arginine side chains are larger than glutamate and aspartate side chains, the distance between R282 and D341R or D341K is likely to be smaller than the distance between D341 and R282D or R282E. Hence, repulsion in the proteins with positive charges at positions 282 and 341 (D341R and D341K) is likely to be greater than that in proteins with negative charges at these positions (R282D and R282E). This difference in charge repulsion may account for the greater loss in uptake activity for D341R- and D341K-hPepT1.

Gly-sar uptake by R282C - and D341C - hPepT1 and the influence of MTSET and MTSES (positively and negatively charged cysteine-modifying reagents, respectively) on uptake by these proteins is also consistent with the presence of an R282 - D341 salt bridge in the wild-type protein. Mutation at either position to a neutral amino acid (cysteine or alanine) does not significantly alter function, suggesting that such mutations can be tolerated. Modification of R282C with MTSET, a water-soluble, membrane-impermeable species that carries a permanent positive charge due to a tetraalkylammonium ion, results in an increase in gly-sar uptake. Exposure to MTSET typically results in either no change (interpreted as the cysteine mutation being inaccessible to solvent) or a reduction in activity (caused by MTSET reaction with the cysteine and subsequent blockade of the substrate pathway) (31). We have previously attributed the increased gly-sar uptake upon MTSET reaction with R282C-hPepT1 to restoration of the charge-pair interaction with D341 (9). Similarly, reaction of the D341C with the negatively charged cysteine-modifying reagent, MTSES

causes a 12.5% increase in gly-sar uptake compared to unmodified D341C-hPepT1. Again, this may be due to re-establishment of the charge-pair interaction with R282. In contrast, MTSET reaction with D341C-hPepT1 results in a decrease in gly-sar uptake, with the resultant repulsion between positive charges at positions 282 and 341 giving a protein with activity similar to D341R- and D341K- hPepT1. We note that we have shown previously that the uptake activity of wild-type hPepT1 is reduced by only 15% by MTSET (8), despite the presence of 11 endogenous cysteines. The functional significance of the increased uptake induced by MTSET treatment of R282C-hPepT1 and MTSES treatment of D341C-hPepT1 is further emphasized by the behavior of the wild-type protein with MTSET (8), since the same decrease in uptake should also be present for the mutant proteins.

Interactions between charged amino acid residues within transmembrane helices have been shown in a number of proteins, such as the sulfate transporter SHST1 (32), the human reduced folate carrier hRFC (33), the HCN2 pacemaker channel (34), the rat vesicular acetylcholine transporter (28), and the vesicular monoamine transporter (35). These charge pairs play a variety of roles in integral membrane proteins, including stabilizing the association between the transmembrane segments involved in the interaction (33), reducing the free energy of membrane partitioning (36), and promoting high affinity substrate recognition (35). The R282-D341 charge pair in hPepT1 is not absolutely essential for substrate transport, since proteins with single point mutations at these positions are still functional. Furthermore, Meredith has shown that R282 in rbPepT1 does not interact with the substrate (21). Given the strong conservation of R282 across species and the similarity in the uptake activity of R282E-hPepT1 in our study with that of R282E-rbPepT1 (21), it is likely that R282 in the human protein also does not interact directly with the substrate. However, the R282- D341 charge pair may play a role in maximizing the efficiency of substrate translocation, as suggested in the model presented in the following paragraph.

In the model proposed in Fig. 4, the resting state of hPepT1 includes an intact charge pair formed between R282 in TMS7 and D341 in TMS8. Concomitant substrate and proton binding cause a conformational change in the protein that weakens the R282–D341 charge-pair interaction; this change may also be associated with opening of the substrate translocation pathway. This pathway is formed by TMS3 (unpublished data), TMS5 (8), TMS7 (9), and other as yet unidentified TMS domains. Based on Meredith's (21) observation that mutation of R282 uncouples the co-transport of protons and peptides in rbPepT1, we speculate that the translocated proton may interact with the R282–D341 charge pair during substrate transport. This may first occur through protonation of D341 (step a in Fig. 4) accompanying a conformational change in the protein, thereby disrupting the electrostatic association between D341 and R282. Subsequent transfer of a proton (step b in Fig. 4) from R282 to the substrate carboxylic acid group, which is an important component of hPepT1 substrates (37), and then transfer of the acidic proton of D341 to the transiently

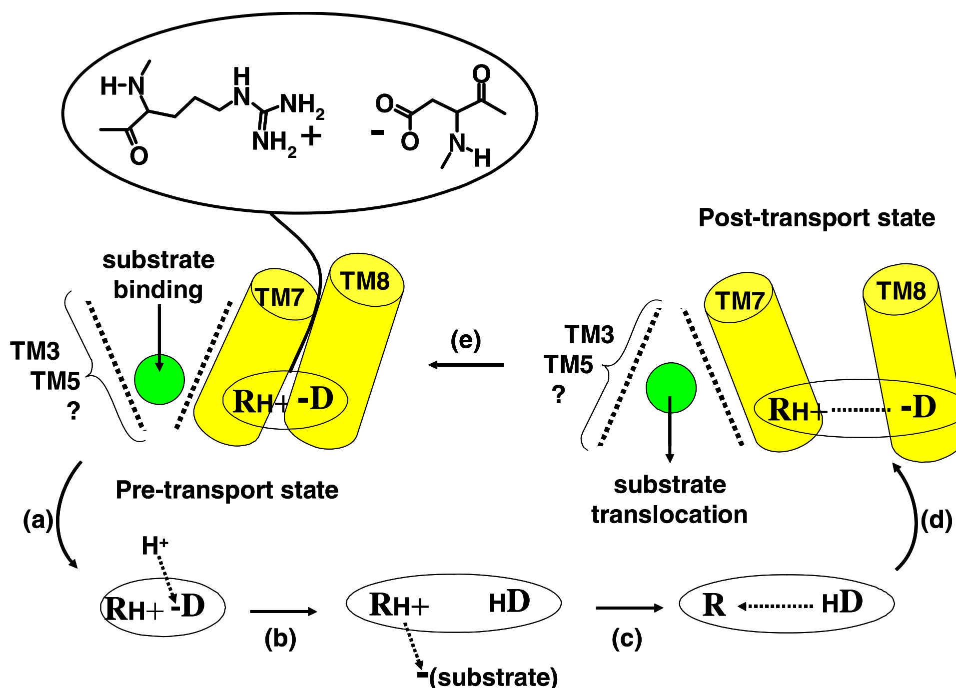

Fig. 4. A potential role for the R282-D341 charge pair during substrate transport by hPepT1. In the proposed model, R282 and D341 form a charge pair in the pre-transport conformation of the protein. During substrate transport and in the post-transport state the charge pair is proposed to be broken transiently, and re-establishment of the electrostatic association between R282 and D341 may provide a driving force for the protein to return to the pre-transport state in step (e). Steps (a–d) are discussed in the text.

neutral R282 with accompanying substrate translocation (steps c and d in Fig. 4) leads to the post-transport conformation of the protein. The continued separation of the charged forms of R282 and D341 is energetically unfavorable, and the driving force for restoration of the charge pair may contribute to reversion of the protein to its original conformation, in which it is ready to begin another transport cycle.

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