Biophysical Evidence for His^{57} as a Proton-Binding Site in the Mammalian Intestinal Transporter hPepT1

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Purpose. The objective of this study was to provide direct evidence of the relative importance of the His^{57} residue present in transmembrane domain 2 (TMD 2) and the His^{121} residue in TMD 4 as proton-binding sites in human PepT1 (hPepT1) by using a novel mutagenesis approach.

Methods. His^{57} and His^{121} in hPepT1 were each mutated to alanine, arginine, or lysine individually to obtain H^{57}A-, H^{57}R-, H^{57}K-, H^{121}A-, H^{121}R-, and H^{121}K-hPepT1. H^{7}A-hPepT1 was used as a negative control. [^{3}H]Glycylsarcosine (Gly-Sar) uptake was measured 72 h posttransfection using HEK293 cells individually transfected with these mutated proteins. Steady-state I/V curves (−150 mV to +50 mV, holding potential −70 mV) were obtained by measuring 5 mM Gly-Sar–induced currents in oocytes expressing H^{57}R- and H^{57}K-hPepT1. Noninjected oocytes and wild-type hPepT1 (WT-hPepT1)–injected oocytes served as negative and positive controls, respectively.

Results. At pH 6.0, H^{57}K-, H^{57}R-, H^{121}K-, and H^{121}R-hPepT1 led to a 97%, 90%, 45%, and 75% decrease in [^{3}H]Gly-Sar uptake into HEK293 cells, respectively. At pH 7.4, uptake in cells transfected with H^{57}K- and H^{57}R-hPepT1 was not significantly different from that at pH 6.0, whereas cells expressing H^{121}R- and H^{121}K-hPepT1 showed 56% and 65% decrease, respectively, compared to that at pH 6.0. In oocytes expressing H^{57}R-hPepT1, steady-state currents induced by 5 mM Gly-Sar increased with increasing pH (I_{max} = 300 nA at pH 8.5), suggesting the binding of protons to H^{57}R. No such trend was observed in oocytes injected with H^{57}K, H^{121}R, and H^{121}K cRNA.

Conclusions. H^{57}R-hPepT1 is able to bind protons at a relatively basic pH, resulting in facilitation of transport of Gly-Sar by hPepT1 at higher pH. Our novel approach provides direct evidence that His^{57} is a principal proton-binding site in hPepT1.

KEY WORDS: proton-binding site; dipeptide transporter; histidine; site-directed mutagenesis; two-electrode voltage clamp; *Xenopus* oocyte; HEK293 cells.

INTRODUCTION

The human intestinal dipeptide transporter hPepT1 is a proton-coupled peptide transporter that is mainly expressed on the apical membrane of the intestinal epithelial cells. It exhibits broad substrate specificity by facilitating the uptake of nutritional di- and tripeptides and a variety of peptidomimetics (1) and has received considerable attention as a drug carrier system over the past several years. Substrate transport is proton coupled, and both, protons and the substrate are driven down an electrochemical proton gradient. The basic structural requirements for a substrate that helps in its recognition by PepT1 have been documented by Daniel *et al.* (2,3). Various efforts have also been made to determine the structure–function relationships of PepT1, including the nature of its substrate-binding and proton-binding sites. Site-directed mutagenesis studies have identified tyrosine 56 and tyrosine 64 in transmembrane domain (TMD) 2 (4), tyrosine 167 in TMD 5 (5), tryptophan 294 in TMD 7 (6), and glutamic acid 595 in TMD 10 (6) to be critical for hPepT1 transport activity.

Several transport systems for which H^+ is a cotransported ion are known to contain specific histidyl residues that are essential for their activity. Examples include the Na^+/H^+ exchanger (7), the organic cation/H^+ antiporter (8), and the folate transporter (9) in mammals. The importance of the histidyl groups in PepT1 has been evaluated by diethylpyrocarbonate (DEPC) modification (4,10–13) and site-directed mutagenesis studies (4,12,14). Miyamoto *et al.* first indicated that active dipeptide transport was inhibited by DEPC, a histidine-modifying reagent, in rabbit renal brush border membrane vesicles (11). DEPC was also shown to inhibit substrate uptake by rat PepT1 (12,13), rabbit PepT1 (4), and hPepT1 (10). This led to the suggestions that the histidyl residues in PepT1 might function as proton-binding sites (4), as residues supplying a proton to the side chain of an acidic dipeptide (4), as a binding site for a substrate α-amino group (13), and/or as recognition site for a free carboxyl group or a carbonyl of a peptide bond (15).

The histidine residues located at positions 57 and 121 in TMD 2 and TMD 4, respectively, are conserved in the rat, rabbit, and human PepT1 and are reported to be critical for transport activity and H^+/substrate recognition in hPepT1 (12,14). Terada *et al.* showed that histidine mutations H^{57}Q and H^{121}Q in rat PepT1 completely abolished uptake of substrate (12). However, only mutations at His^{57} (H^{57}N, H^{57}Q, and H^{57}R) and not at His^{121} (H^{121}C, H^{121}N, H^{121}Q, and H^{121}R) abolished uptake of Gly-Sar in rabbit and human PepT1 (4,14). It has been well known that His^{57} and His^{121} are critical residues for PepT1 transport function, but their exact role still remains unclear. Hediger *et al.* showed that His^{57} and His^{121} in rabbit PepT1 are intimately involved in the binding of the coupling ion H^+ and the recognition of a transportable peptide substrate, respectively (4). However, their method of using adjunct amino acid mutations around His^{57} and His^{121} did not provide a direct evidence of these residues serving as binding sites for protons or substrate.

The purpose of our study was to provide direct evidence of the relative importance of the His^{57} residue present in TMD 2 and the His^{121} residue in TMD 4 as proton-binding sites in human PepT1 (hPepT1) by using a novel mutagenesis approach. To that end, we generated cationic amino acid mutants of His^{57} (H^{57}K and H^{57}R) and His^{121} (H^{121}K and H^{121}R) and evaluated their proton-binding capacity at various pH values (5.0–8.5). The rationale was that arginine (pK_a = 12.5) and lysine (pK_a = 10.5) residues can bind and release

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protons similarly to histidine (side chain $pK_a = 6.0$) as the pH approaches the pK_a of the side chain and thereby facilitate uptake of substrate. A histidine residue that is a proton-binding site should increase or maintain substrate uptake with increasing pH when mutated to Arg or Lys.

METHODS AND MATERIALS

Materials

[^{3}H]Glycyl sarcosine (4 Ci/mmol) was purchased from Moravek Chemical Co. (Brea, CA). Cell culture media and supplies were obtained form GIBCO (Grand Island, NY). Collagenase A was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were available commercially and of reagent grade.

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 2306 *Kpn*I/*Bam*HI fragment into the multiple cloning sites of pcDNA3. The pcDNA3-hPepT1 plasmid is under the control of cytomegalovirus (CMV) promoter and was used as a template for all the mutagenesis reactions. The standard site-directed mutagenesis protocol provided by the manufacturer was followed using the Gene Editor™ site-directed mutagenesis kit (Promega Corporation, Madison, WI). The mutagenic primers are shown in Table I. All mutations were confirmed by DNA sequencing (Genemed Synthesis Inc., San Francisco, CA).

Transient Transfection and Uptake Studies

HEK293 cells were transfected with plasmid as described previously (5,6). After 72 h post-transfection, the growth medium was removed from the transfected cells and 0.5 ml of uptake buffer (25mM MES (pH 6.0) or HEPES (pH 7.4 or pH 8.5)/Tris, 140mM NaCl, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, and 5mM glucose) containing 150nM ^{3}H-Gly-Sar (0.6μCi/mL) was added to each well at 37°C for 5 min. After washing three times with ice-cold uptake buffer, the cells were lysed using 1% SDS. An aliquot (20 μl) was taken for protein assay using a Bio-Rad DC kit (Bio-Rad Laboratories, Hercules, CA), while the remainder of the cell lysate was used for liquid scintillation counting.

cRNA Synthesis

The wild-type and mutant plasmids were linearized with the restriction enzyme BamHI (Invitrogen, Carlsbad, CA), and the cDNA inserts were transcribed *in vitro* by T7 RNA polymerase in the presence of m7G(5′)ppp(5′)G RNA cap analogue using the Ambion MEGAscript kit (Ambion Inc., Austin, TX).

Isolation of *Xenopus* Oocytes

Oocytes were isolated as reported previously (16). Approximately 50 ng (1 ng/nl) of wild-type or mutant hPepT1 cRNA were injected into oocytes 1 day after isolation and were incubated at 18°C. The oocytes were used for electrophysiologic studies 4–7 days after cRNA injection.

A two-microelectrode voltage-clamp system (17) was used to measure the steady-state currents associated with wild-type or mutant hPepT1 expressed in oocytes. Oocytes were superfused at room temperature first with a pH 7.5 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/Tris), followed by the appropriate pH buffer (pH 5.0–8.5, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 3 mM HEPES, 3 mM MES, and 3 mM Tris) and then by Gly-Sar solutions of various concentrations made in the same buffer. Test solutions were always washed out by superfusing the oocyte with Gly-Sar–free, choline chloride–containing medium, pH 7.5. The holding membrane potential was –70 mV. For determination of the I/V (current/membrane potential) relationship, step changes in membrane potential (V_m) were applied from +50 to –150 mV in 20-mV increments, each for duration of 100 ms using a voltage clamp amplifier (OC-725) controlled by the computer software program pCLAMP 8.1 (Axon Instruments, Foster City, CA). The currents were averaged over three sweeps and low-pass filtered at 500 Hz by an eight-pole Bessel filter. Steady-state data were fitted to the following equation:

$$I = I_{max}S/(K_{0.5} + S)$$

where I is the Gly-Sar–evoked current (i.e., the difference between the steady-state currents measured in the presence and absence of Gly-Sar), I_{max} is the derived current maximum, S is the concentration of Gly-Sar or H^+, and $K_{0.5}$ is the concentration of Gly-Sar or H^+ at which the current is half maximal.

Immunolocalization

The procedure for immunofluorescence microscopy staining has been described in detail previously (5). Affinity-purified anti-hPepT1 primary antibodies and FITC-conjugated secondary antibodies were incubated with the samples at a dilution of 1:500 for 2 h and 1 h, respectively.

Data Analysis

Results are expressed as mean ± SEM. Statistical differences among multiple (≥3) different group means were determined by one-way analysis of variance with Dunnett test using commercial software (Instat, GraphPad Software, San Diego, CA). $p < 0.05$ was considered significant.

RESULTS

Functional Analysis of Wild-Type and Mutated hPepT1

As depicted in Fig. 1, the functional activities of wild-type and seven mutated hPepT1s expressed in HEK293 cells were evaluated by measuring [^{3}H]Gly-Sar uptake. His^{57} and His^{121} were mutated to alanine ($H^{57}A$, $H^{121}A$), arginine ($H^{57}R$, $H^{121}R$), or lysine ($H^{57}K$, $H^{121}K$). In addition, His^7 was changed to alanine (H^7A), and H^7A-hPepT1 exhibited activity similar to that of WT-hPepT1 at pH 6.0. This suggested that H^7 was not critical for hPepT1 activity and hence could serve as a negative control. We found that the mutants $H^{57}A$-, $H^{57}R$-, and $H^{57}K$-hPepT1 significantly ($p < 0.01$, $n \geq 4$) decreased uptake of [^{3}H]Gly-Sar by 90–100% as compared to WT-hPepT1 at pH 6.0. Similarly, $H^{121}A$-, $H^{121}K$-, and $H^{121}R$-hPepT1 significantly ($p < 0.01$, $n \geq 4$) decreased [^{3}H]Gly-Sar uptake at pH 6.0, but to a lesser extent. For example, we

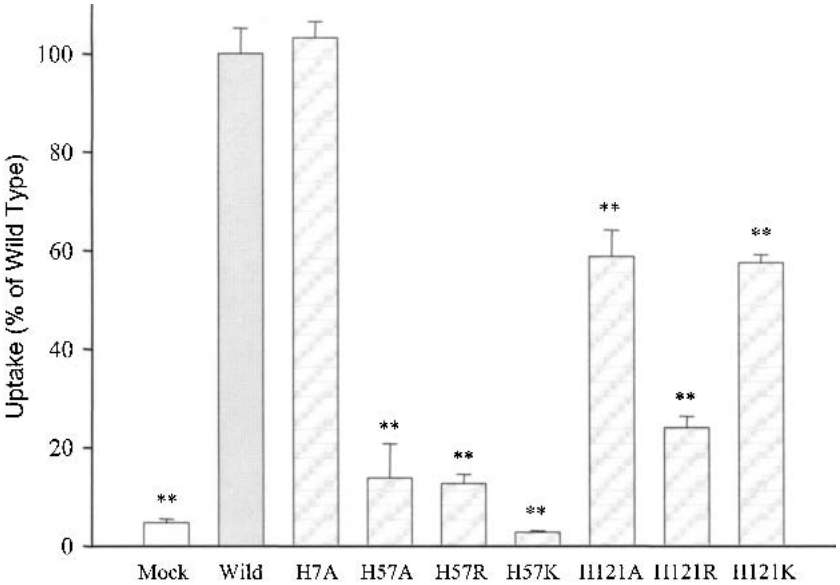

Fig. 1. Uptake of [^{3}H]Gly-Sar via the wild-type or in the various mutated hPepT1. HEK293 cells were transfected with WT-hPepT1, pcDNA3 vector alone, or one of the mutated hPepT1s. Uptake of [^{3}H]Gly-Sar (0.6 μCi/ml, 150 nM) was measured at pH 6.0 for 5 min. Values represent mean ± SEM for at least four determinations. **$p < 0.01$ compared to control.

found that H^{57}A-hPepT1 decreased hPepT1 uptake activity by 90%, whereas the same mutation of H^{121} (i.e., H^{121}A-hPepT1) decreased uptake by only 40%. These results indicated that H^{57} is a very crucial histidyl residue for hPepT1 activity, which is consistent with previously reported data (4,12,14).

We next measured uptake of [^{3}H]Gly-Sar by H^{57}R-, H^{57}K-, H^{121}R-, and H^{121}K-hPepT1 at pH 7.4 and compared it with the uptake values observed at pH 6.0. As illustrated in Fig. 2, H^{57}K- and H^{57}R-hPepT1 showed no significant difference ($p > 0.05$, $n \geq 4$) in [^{3}H]Gly-Sar uptake observed at pH 6.0 and pH 7.4. Increasing the pH of the buffer further to 8.5 caused a slight decrease in the H^{57}R- and H^{57}K-hPepT1 uptake activity. However, it is important to note that the inherent activity of His^{57} mutants was very low, and hence the effect of pH on [^{3}H]Gly-Sar uptake was not very clear. On the other hand, H^{121}R- and H^{121}K-hPepT1 showed significantly ($p < 0.05$, $n \geq 4$) decreased uptake of [^{3}H]Gly-Sar at pH 7.4 compared to pH 6.0. The uptake activities of H^{121} mutants at pH 8.5 were significantly ($p < 0.05$, $n \geq 4$) lower than that observed at pH 7.4. H^{121} mutants did not increase the uptake of [^{3}H]Gly-Sar with increasing pH from 6.0 to 7.4, suggesting that H^{121} is not involved in proton binding by hPepT1.

These results suggest a role for His^{57}, but not for His^{121}, in proton binding to hPepT1. Because the inherent activity of His^{57} mutants was very low, and the effect of pH on [^{3}H]Gly-

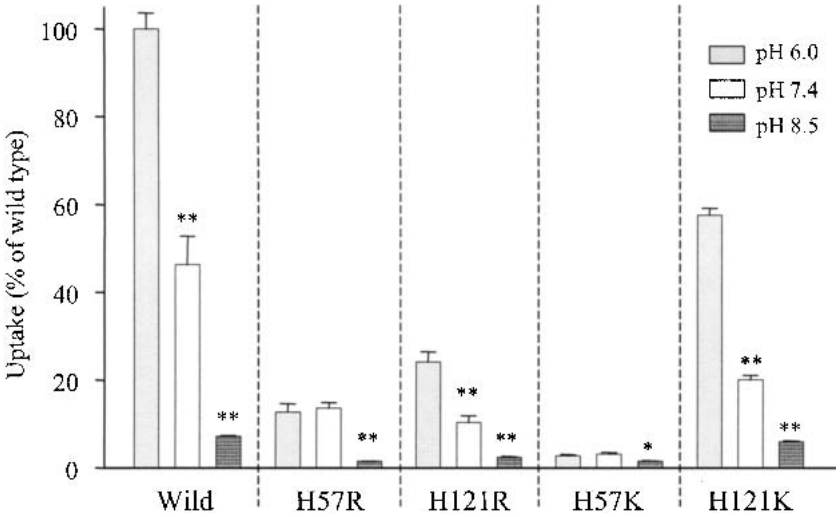

Fig. 2. Comparison of pH dependence of the uptake of [^{3}H]Gly-Sar (0.6 μCi/ml, 150 nM) in wild-type and mutated hPepT1s. Uptake of [^{3}H]Gly-Sar (0.6 μCi/ml, 150 nM) was measured at pH 6.0, pH 7.4, or pH 8.5 for 5 min. Values represent mean ± SEM for at least four determinations. *$p < 0.05$, **$p < 0.01$ compared to control.

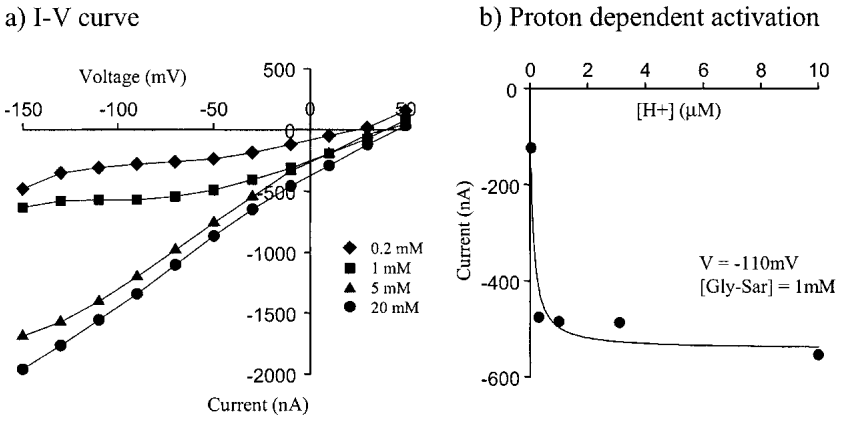

Fig. 3. Voltage-dependent and proton-dependent currents evoked by Gly-Sar in WT-hPepT1–injected oocytes. Currents evoked by 0.2, 1, 5, and 20 mM Gly-Sar in a single oocyte expressing WT-hPepT1 were measured (left panel). Currents evoked by 1 mM Gly-Sar at various external proton concentrations were measured at pH 5.0, 6.0, 7.4, and 8.5 in a single WT-hPepT1 cRNA-injected oocyte (right panel). Control values (substrate-free) were subtracted from each data point.

Sar uptake was not very clear, we performed a more detailed evaluation of the effect of pH on [^{3}H]Gly-Sar uptake in His^{57} mutants. We repeated the pH dependence experiment for His^{57} mutants using the *Xenopus* oocyte system in conjunction with the two-electrode voltage clamp technique.

Electrophysiologic Studies

To determine the proton-binding capacity of H^{57}R- and H^{57}K-hPepT1 mutants, oocytes expressing these proteins or WT-hPepT1 were studied by analyzing Gly-Sar (5 mM)–evoked currents (I) at a holding potential of –70 mV. In Na^+ medium, at pH 5, Gly-Sar invoked concentration-dependent inward currents, which bore a nonlinear dependence on membrane potential in oocytes injected with WT-hPepT1 cRNA (Fig. 3a). At pH 5, 5 mM Gly-Sar invoked a maximum current of ~1600 nA in oocytes injected with WT-hPepT1, and this current was saturated at hyperpolarizing conditions (–150 mV). As depicted in Fig. 3b, Gly-Sar–evoked current increased in magnitude as a hyperbolic function of extracellular proton concentration. At –110 mV, the apparent affinity constant of hPepT1 for $K_{0.5}$ was 91.3 nM. WT-hPepT1 activity was dramatically decreased above pH 6.0. These characteristics of WT-hPepT1 were consistent with the ones reported by Mackenzie *et al.* (18).

Oocytes injected with H^{57}K-hPepT1 did not exhibit hPepT1-like activity (Fig. 4a). Voltage-dependent inward currents were not observed with hyperpolarizing potentials when the oocyte was superfused with 5 mM Gly-Sar at pH values ranging from 5.0 to 8.5. The inward currents in the absence of substrate were much higher in magnitude compared to those in the presence of the substrate. This suggests that the addition of substrate does induce some inward currents, but the net result is positive outward currents (seen as positive "difference" values). These currents are not voltage dependent and hence are not mediated by the transporter protein. So, these inward currents are independent of the H^{57}K-hPepT1. On the other hand, oocytes injected with H^{57}R-hPepT1 ex-

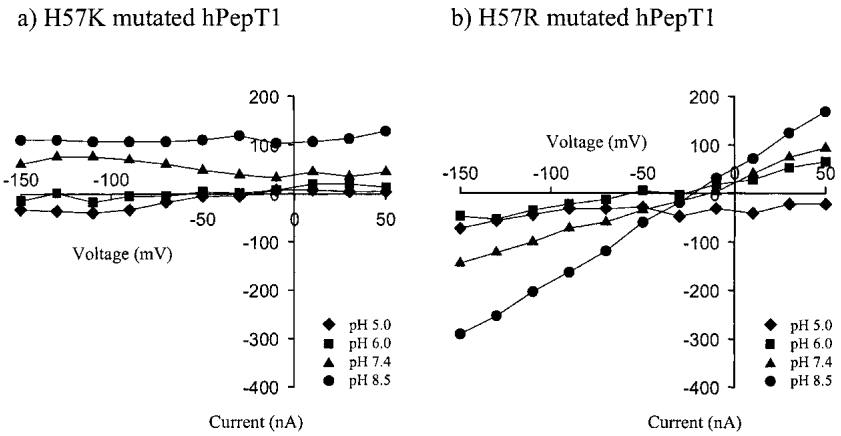

Fig. 4. Voltage-dependent Gly-Sar–evoked currents for (a) H^{57}K and (b) H^{57}R mutated hPepT1 at different pH values ranging from pH 5.0 to pH 8.5. Currents evoked by Gly-Sar were measured at pH 5.0, 6.0, 7.4, and 8.5 in a single H^{57}R-hPepT1 or H^{57}K-hPepT1 cRNA-injected oocyte. Control values (substrate-free) were subtracted from each data point.

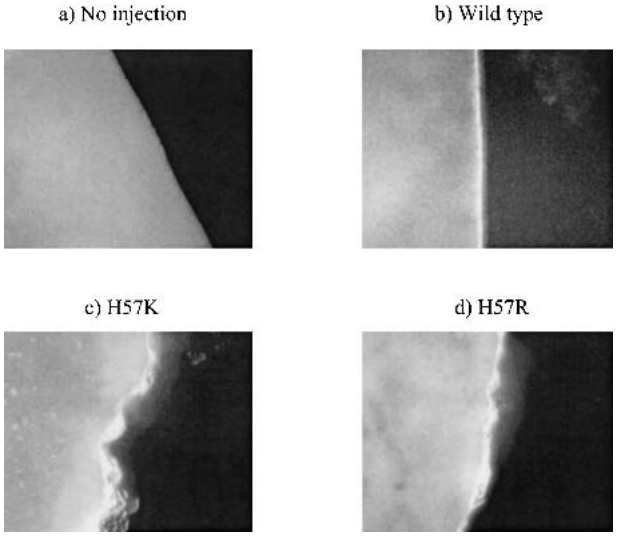

Fig. 5. Immunolocalization of WT-hPepT1, H^{57}R-hPepT1, and H^{57}K-hPepT1 injected in *Xenopus* oocytes. Oocytes expressing WT-hPepT1, H^{57}R-hPepT1, and H^{57}K-hPepT1 were processed for immunofluorescence using purified rabbit anti-hPepT1 primary antibody and FITC-labeled secondary antibody, both at a dilution of 1:500. Immunostaining result with a noninjected oocyte is shown as a negative control.

hibited voltage-dependent 5 mM Gly-Sar–induced inward currents when the pH of the superfusing solution was gradually increased from 5.0 to 8.5. At pH 5, the Gly-Sar–induced currents were negligible with essentially no voltage dependence (Fig. 4b). At –110 mV, the magnitude of these currents jumped from –43.75 nA (negative sign suggests that the currents are inward) at pH 5.0 to –100 nA at pH 7.4 (128% increase over pH 5.0) and then to –203.13 nA at pH 8.5 (364% increase over pH 5.0). These currents were also voltage dependent. At pH 8.5 the maximum current invoked by 5 mM Gly-Sar was –290 nA at –150 mV membrane potential. These results suggested that side chain nitrogen of arginine in the His^{57} position could work as a proton-binding site.

Immunolocalization of Wild-Type and Mutant hPepT1s

In order to confirm the plasma membrane expression of H^{57}R- and H^{57}K-hPepT1, we assayed these proteins by immunofluorescence microscopy. As shown in the Fig. 5, H^{57}R- and H^{57}K-hPepT1 and WT-hPepT1 were expressed at comparable levels at the plasma membrane, suggesting that the observed effects on uptake of Gly-Sar are a result of the effect of the single mutation at the His^{57} residue on the transport function rather than an effect on protein folding or delivery to plasma membrane.

DISCUSSION

We have provided direct evidence that His^{57} is the predominant proton-binding site in hPepT1 and that His^{121} is not. His^{57}-hPepT1 mutants (H^{57}A, H^{57}R, and H^{57}K) totally abolished uptake of [^{3}H]Gly-Sar at pH 6.0. These results are consistent with previous reports in which H^{57}Q, H^{57}N, and H^{57}R did not exhibit any uptake activities (4,12,14), suggesting that His^{57} is essential for the transport function of hPepT1 and that a positive charge is not important at position 57. The elimination of uptake in H^{57}R- and H^{57}K-hPepT1 occurs because arginine and lysine at position 57 cannot bind and release protons at pH 6.0 because of the high pK_a value of their side chain. On the other hand, the side chain pK_a of histidine is 6.0, making it feasible for histidine to conveniently bind and release protons at pH 6.0. His^{121} mutants of hPepT1 significantly decreased uptake of [^{3}H]Gly-Sar at pH 6.0 by 43% (H^{121}A), 45% (H^{121}K), and 75% (H^{121}R). This was also consistent with previous reports (4,14).

Lysine and arginine side chains can hypothetically work as a histidine if the pH of the uptake buffer is increased such that it is closer to their side chain pK_a values. This strategy provides the rationale for our study. Assuming that histidine at residue 57 or 121 is indeed a proton-binding site, the H^{57}R and H^{57}K or H^{121}R and H^{121}K mutations should either increase or maintain their [^{3}H]Gly-Sar uptake at increasing pH. As demonstrated in Fig. 2, both H^{57}R- and H^{57}K-hPepT1 did not show a significant difference in [^{3}H]Gly-Sar uptake at pH 6.0 and pH 7.4. We did see a slight decrease in their uptake activities at pH 8.5 compared to pH 7.4; however, it is worth noting that the inherent activities of these mutants were very low, and hence, the effect of pH on [^{3}H]Gly-Sar uptake was not very clear. Conversely, both H^{121} mutants (H^{121}R and H^{121}K) significantly decreased [^{3}H]Gly-Sar uptake at pH 7.4 compared to pH 6.0. They showed a further decrease in uptake activity when the pH of the uptake buffer was increased from 7.4 to 8.5. Using hPepT1-injected *Xenopus* oocytes, Chen *et al.* demonstrated that H^{121}R mutation decreased the transmembrane current evoked by Gly-Leu and Gly-Lys when pH of the perfusing medium was increased from 5.0 to 8.5 (4). It was also reported that K_m for Gly-Lys and Gly-Glu was drastically decreased by H^{121}R, suggesting that H^{121} is involved in substrate recognition (4). These studies combined

Table I. Mutagenic Primers for Histidine Mutants

WT	G	TCC	AAA	TCA	**CAC**	AGT	TTC	TTT	GGT	TAT	CCC	CTG
H^7A	G	TCC	AAA	TCA	**GCC**	AGT	TTC	TTT	GGT	TAT	CCC	CTG

WT	ACC	GCC	ATC	TAC	**CAT**	ACG	TTT	GTG	GCT
H^{57}A	ACC	GCC	ATC	TAC	**GCT**	ACG	TTT	GTG	GCT
H^{57}R	ACC	GCC	ATC	TAC	**CGT**	ACG	TTT	GTG	GCT
H^{57}K	ACC	GCC	ATC	TAC	**AAG**	ACG	TTT	GTG	GCT

WT	AGC	CTT	CCT	GTG	**CAC**	GTG	GTG	CTG	TCC
H^{121}A	AGC	CTT	CCT	GTG	**GCC**	GTG	GTG	CTG	TCC
H^{121}R	AGC	CTT	CCT	GTG	**CGC**	GTG	GTG	CTG	TCC
H^{121}K	AGC	CTT	CCT	GTG	**AAG**	GTG	GTG	CTG	TCC

with our data suggest that His^{121} is not involved in proton binding in hPepT1.

Gly-Sar transport via WT-hPepT1 is electrogenic and coupled to an inward H^+ current. Our electrophysiologic profiles, which indicated that transport current in wild-type hPepT1 increased with increasing substrate concentration and drastically decreased above pH 6.0, are consistent with the data reported by Mackenzie *et al.* (18). Further, we found that $H^{57}R$ evoked increasing steady-state currents with gradually increasing pH (pH 5.0–8.5). Arginine (side chain $pK_a = 12.5$) has a nonprotonated side chain: protonated side chain ratio of 1:10,000 at pH 8.5 as compared to 1:3,160,000 at pH 6.0. However, the ratio of nonprotonated to protonated side chain nitrogen of histidine is 1:1 at pH 6.0. This factor combined with low H^+ ion concentration at higher pH values leads to a maximum current (I_{max}) of –203.13 nA at pH 8.5 at –110 mV holding potential. This I_{max} is very low compared to WT-hPepT1. These results suggested that an arginine residue at position 57 is able to bind and release protons as the pH approaches the pK_a of the side chain. On the other hand, $H^{57}K$ did not show any hPepT1-like activity even with increasing pH. Immunostaining data confirmed membrane expression of $H^{57}K$-hPepT1 in oocytes at levels comparable to wild-type hPepT1. The reason $H^{57}K$ did not show the same tendency as $H^{57}R$ is still unclear. This difference between $H^{57}R$ and $H^{57}K$ may lie in the side-chain structure differences between arginine and lysine. Further studies are required to provide an explanation for these differences. Preliminary computer modeling has raised the possibility of the lysine being folded in the membrane and being inaccessible to the protons in the solution. Further studies are required to confirm this possibility.

In summary, we have provided direct biophysical evidence suggesting that His57 is the crucial residue serving as the predominant proton-binding site in hPepT1. The His^{121} residue does not appear to be involved in proton binding. Our data gave an insight to understand transport mechanisms of hPepT1. However, it remains uncertain as to why only His^{57} works as a proton-binding site in hPepT1. One possible explanation is an interaction with adjacent amino acids, which may affect the real pK_a value of side chain of histidine. Computer modeling may help understanding His^{57} function in hPepT1.

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