The Extracellular pH Dependency of Transport Activity by Human Oligopeptide Transporter 1 (hPEPT1) Expressed Stably in Chinese Hamster Ovary (CHO) Cells: A Reason for the Bell-Shaped Activity versus pH

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Human oligopeptide transporter (hPEPT1) translocates di/tri-peptide by coupling to movement of proton down the electrochemical gradient. This transporter has the characteristics that the pH-profile of neutral dipeptide transport shows a bell-shaped curve with an optimal pH of 5.5. In the present study, we examined the reason for the decrease in the acidic region with hPEPT1-transfected CHO cells stably overexpressing hPEPT1 (CHO/hPEPT1). The pH profile of the transport activity vs. pH was measured in the presence of nigericin/menadione. Under this condition, the inwardly directed proton concentration gradient was dissipated while the membrane potential remained. As pH increased the activity increased, and the Henderson–Hasselbalch equation with a single pKa was fitted well to the activity curve. The pKa value was estimated to be 6.7±0.2. This value strongly suggests that there is a key amino acid residue, which is involved in pH regulation of transport activity. To identify the key amino acid residue, we examined the effects of various chemical modifications on pH-profile of the transport activity. Modification of carboxyl groups or hydroxyl groups had no significant influence on the pH-profile, whereas a chemical modification of histidine residue with diethylpyrocarbonate (DEPC) completely abolished the transport activity in CHO/hPEPT1 cells. On the other hand, this abolishment was almost prevented by the presence of 10 mM Gly-Sar. This protection was observed only in the presence of the substrate of hPEPT1, indicating that the histidine residue is located at the substrate recognition site. The pH-profile of the transport activity in CHO/hPEPT1 cells treated with DEPC in the presence of 10 mM Gly-Sar also showed a bell-shape similar to that in non-treated CHO/hPEPT1 cells. These data stressed that the histidine residue located at or near the substrate binding site is involved in the pH regulation of transport activity.

Key words  human oligopeptide transporter; pH profile; proton-dependency; histidine residue; uptake activity; Human oligopeptide transporter

It has been estimated that more than 80% of amino acids found in the intestinal lumen after a protein meal are in the form of small peptides rather than free amino acids.1) Therefore, a majority of protein digestion products are obligatorily absorbed via an intestine oligo-peptide transporter called PEPT1. Of interest, PEPT1 also mediates the absorption of a huge number of pharmacologically important compounds including peptidomimetic drugs,2,3) such as oral β-lactam antibiotics,4—6) ACE-inhibitors like captopril,7) or the peptidase inhibitor bestatine8) and even compounds without an obvious peptide bond or equivalent,9) such as δ-amino levulinic acid.10) This surprisingly high tolerance for structural diversity of the substrate by PEPT1 has been expected to be used as a channel to increase the intestinal absorption of poorly absorbable drugs.11)

The transport of substrates via PEPT1 is coupled to the downward movement of a proton in accordance with its electrochemical gradient.2,3,12) It has been demonstrated in intestinal membrane vesicles,4,13,14) Xenopus oocytes,15) and culture cells16) that the transport activity of PEPT1 shows a bell-shaped pH dependence with an optimal pH 5.5 to 6.0. This optimal pH is physiologically collaborated with an acidic unstirred water layer (pH 5.5—6.0) in the intestinal lumen.1,17) Hence, PEPT1 can function most efficiently in epithelial cells of the small intestine.

As for the bell-shaped activity of PEPT1 vs. pH, the reason why the transport activity decreases with an increase in pH is obviously the decrease in the driving force of the proton electrochemical potential gradient. On the other hand, why does the activity decrease in a more acidic region although the driving force should increase? To answer this question, we will try to obtain a relationship between the transport activity and extracellular pH. However, there is a problem to be solved: The driving force changes along with the change in extracellular pH. The pH-dependent properties of PEPT1 should be determined without changing the driving force. Here, we employ the experimental condition that the proton concentration gradient across the membrane is dissipated by the addition of monensin and nigericin18,19), then the driving force is solely the membrane potential. To perform this experiment, a cell line is necessary which shows high transport activity. First, we established stably transfected CHO cells which show high transport activities for various substrates with almost the same $K_m$ values as those obtained from those of a model human intestine epithelial cell line, Caco-2.20—22) Thus, this CHO cell line over-expressing hPEPT1 (designated as CHO/hPEPT1) is useful and convenient for transport studies of hPEPT1. In the present study, we then examined the pH-dependency of transport activity by hPEPT1 using CHO/hPEPT1 cells.

MATERIALS AND METHODS

Materials $^3$H-Glycylsarcosine ([$^3$H]-Gly-Sar) (148 GBq/
\( \mu \text{mol} \) was purchased from Moravek Biochemical, Inc. (Brea, CA, U.S.A.). \(^{3} \text{H}\)-Tetraphenylphosphonium (\( \left( ^{3} \text{H}\right)\)-TPP\(^{+} \)) (1.2 TBq/mmol) was purchased from Amersham (Piscataway, NJ, U.S.A.). Cephalaxin, cephradin, cepedi, diethylpropycarbonate (DEPC), Dullbecco’s modified Eagle’s medium (DMEM), enalapril, Ham F-12 and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDCM) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). MEM non-essential amino acids and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies (Grand Island, NY, U.S.A.). pCIneo and TransFast\(^{\text{TM}} \) transfection reagent were purchased from Promega (Madison, WI, U.S.A.). Blasticidin S (Bs) and pSV2bsr were purchased from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Tetraphenylphosphonium chloride was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were of the highest purity available.

**Cell Culture** Caco-2 cells (ATCC HTB-37) at passage 28 and CHO cells (ATCC CCL61) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Caco-2 cells were passaged in 75 cm\(^2\) culture flasks (Falcon, Becton Dickinson and Co., Lincoln Park, NJ, U.S.A.) in culture medium consisting of DMEM supplemented with 1\% MEM non-essential amino acids and fetal bovine serum (15\%), HEPES (5 mM) and NaHCO\(_3\) (2 g/l) without antibiotics. These cells were maintained at 37°C in an atmosphere of 5\% CO\(_2\). CHO cells between the 30th and 40th passage were used in this study.\(^{20,21}\) The composition of the uptake buffer was 140 mM NaCl, 3 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 5 mM glucose. HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) was used for pH 8.0—7.0 buffer; MES (2-(N-morpholino)ethanesulfonic acid) for pH 7.0—5.0 buffer; Homopipes (homopiperazine-N,N’-bis-2-(ethanesulfonic acid), for pH 5.0—3.5 buffer, respectively. These cells were maintained at 37°C in an atmosphere of 5\% CO\(_2\). Caco-2 cells between the 30th and 40th passage were used in this study.\(^{20,21}\) Cells were passaged in 75 cm\(^2\) culture flasks in Ham F-12 medium supplemented with 1\% MEM non-essential amino acids and fetal bovine serum (15\%), HEPES (5 mM) and NaHCO\(_3\) (2 g/l) without antibiotics. These cells were maintained at 37°C in an atmosphere of 5\% CO\(_2\). Caco-2 cells between the 30th and 40th passage were used in this study.\(^{20,21}\) CHO cells were passaged in 75 cm\(^2\) culture flasks in Ham F-12 medium supplemented with fetal bovine serum (10\%) and antibiotics (penicillin 100 units/ml and streptomycin 100 \( \mu \text{g/ml} \)). CHO cells were grown in Ham F-12 medium additionally containing 10 \( \mu \text{g/ml} \) Bs when co-transfecting pSV2bsr. At approximately 80\% confluence, cells were seeded using 0.02\% EDTA and 0.05\% trypsin at a density of ten-fold dilution.

**Construction of Expression Vector pCIneo/hPEPT1**

The Caco-2 cells were used between the 14th day for RNA isolation. Total RNA was prepared from Caco-2 by the SV total RNA kit (Promega, Madison WI, U.S.A.). The procedure followed the instruction provided. The cDNA gene of hPEPT1 was obtained from RNA by reverse transcription (Takara, Tokyo, Japan). The cDNA is under control of the cytomegalovirus (CMV) promoter.

**Transfection of pCIneo/hPEPT1 and Selection of Cell Line Which Stably Over-Expresses hPEPT1**

The CHO cells were plated one day before the transfection experiment so that the cells will be approximately 80\% confluent on the day of the transfection. pCIneo/hPEPT1 was transfected into CHO cells using TransFast\(^{\text{TM}} \) transfection reagent (Promega, Madison WI, U.S.A.), which is comprised of the synthetic cationic lipid, (+)-N,N’-[bis(2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy) propyl] ammonium iodide. Charge ratio of DNA to the cationic lipid was 1:2. The amount of pCIneo/hPEPT1 was 10 \( \mu \text{g} \) per 10 cm dish. pSV2 containing Bs resistance gene was simultaneously transfected at one twentieth the amount of pCIneo/hPEPT1 into CHO cells. The procedure followed the protocol provided. After a 48-h culture, the medium was replaced with a fresh medium containing 10 \( \mu \text{g/ml} \) Bs every 3 d. The individual clones containing transfected DNA were isolated and propagated between 14 and 21 d. Single colonies were selected for subsequent screening, in which the uptake activity of Gly-Sar was determined. Several stable transfecants were cloned.

**Uptake Experiment in Monolayer CHO/hPEPT1 Cells**

Cells were trypsinized when confluence was noted in the 75 cm\(^2\) flasks, and 0.3 \( \times 10^6 \) cells were seeded per well in 6-well culture plates. CHO cells were confluent on the third day, and the uptake experiments were performed the next day. The uptake of \(^{3} \text{H}\)-Gly-Sar was determined as described previously.\(^{20,21}\) The composition of the uptake buffer was 5 mM Goods buffer, 140 mM NaCl, 3 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 5 mM glucose. HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) was used for pH 8.0—7.0 buffer; MES (2-(N-morpholino)ethanesulfonic acid) for pH 7.0—5.0 buffer; Homopipes (homopiperazine-N,N’-bis-2-(ethanesulfonic acid)), for pH 5.0—3.5 buffer, respectively. The cells were dissolved in 1 ml of 1 N NaOH and then neutralized by 1 ml of 1 N HCl. The radioactivity was determined in an ACSII scintillation counting cocktail (Amersham/Pharmacia, Buckinghamshire, England) by a liquid scintillation counter (LSC 2500, Packard).

**Kinetic Analysis of Gly-Sar Uptake by CHO/hPEPT1**

The uptake data of Gly-Sar were fitted to a Michaelis Menten equation with a saturable component by an iterative nonlinear least-squares method in Origin (MicroCal, Northampton, MA, U.S.A.),

\[
u_s = \frac{V_{\text{max}}[s]}{K_m + [s]} \tag{1}
\]

where \( \nu_s \) represents the initial uptake rate of Gly-Sar; \( V_{\text{max}} \), the maximum uptake velocity; \([s]\), the concentration of Gly-Sar; \( K_m \), Michaelis constant.

The uptake data in the presence of nigericin/monensin were fitted to the Henderson–Hasselbalch equation as follows,

\[
u = \frac{V_{\text{max,o}}}{1 + 10^{pH - pK_a}} \tag{2}
\]

where \( \nu \) represents the initial rate of Gly-Sar transport; \( V_{\text{max,o}} \), the maximum value of transport rate; pH, the medium pH; \( pK_a \), the association of the proton, respectively.
RESULTS

Establishment of a Cell Line Which Stably Over-Expresses hPEPT1 To establish a system with which hPEPT1 function could be measured conveniently, pClneo/hPEPT1 was transfected into CHO cells to select a clone of the cells, which showed a stable and functional expression. The time course for the accumulation of Gly-Sar by the CHO/hPEPT1 cells is shown in Fig. 1. The accumulation by CHO/hPEPT1 cells showed more than one hundred times higher than that by the control CHO (i.e., the cells into which pClneo alone was transfected). The accumulation of Gly-Sar by CHO/hPEPT1 cells was reduced markedly by alkalizing the medium pH and completely diminished by the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone), indicating that hPEPT1 expressed in CHO/hPEPT1 cells preserved the original characteristics of the H⁺-coupled transport system. As shown in Fig.1, the accumulation of Gly-Sar was linear up to 4 min after incubation with Gly-Sar. Hence, the initial uptake rate was determined within 3 min after an onset. The relationship between the initial uptake rate and Gly-Sar concentration is depicted in Fig. 2, indicating that the uptake rate is saturated at high concentrations of Gly-Sar. The inset in Fig. 2 shows the Eadie-Hofstee plots in which the line was straight, revealing that the uptake process is comprised of a saturable process. The kinetic parameters were calculated by fitting data to Eq. 1; estimated values were: $K_m = 1.02 \pm 0.08 \text{mM} \pm \text{S.D.}$ and $V_{\text{max}} = 34.7 \pm 0.91 \text{nmol/min/mg protein} \pm \text{S.D.}$, respectively. The $K_m$ value was consistent with those reported for Caco-2 cells, and $V_{\text{max}}$ is about 35-times larger than those reported. Since the gene of hPEPT1 is stably retained in the genome of CHO cells and the transporter express functionally in the membranes, CHO/hPEPT1 cells are very useful and convenient for transport experiments of hPEPT1.

Substrate Specificity of hPEPT1 Expressed in CHO/hPEPT1 The substrate specificity of the hPEPT1 transporter in CHO/hPEPT1 was determined by the effects of a diversity of dipeptides, $\beta$-lactam antibiotics and an ACE inhibitor on the uptake of Gly-Sar. As shown in Fig. 3, the transport activities of Gly-Sar were reduced remarkably in the presence of 1 mM dipeptides Gly-X, Val-X and carnosine, whereas Gly-$\alpha$-Leu had no effect of Gly-Sar uptake (here X represents an amino acid for dipeptides used in Fig. 3). Gly or Val, a single amino acid, did not inhibit the transport activity of Gly-Sar. Five mM $\beta$-lactam antibiotics (cephradine, and cephalixin) and an ACE inhibitor (enalapril) exhibited approximately 60% inhibition of the control, which are very similar as those of Caco-2 cells. The order of inhibition by Gly-X was Leu, Phe $>$ Pro $>$ Glu and Lys, which is in accord with the order of hydrophobicity of X and is also consistent with that using Caco-2 cells reported previously. For the inhibition by Val-X peptides, a similar order of inhibition was observed, although the inhibition was more potent. These results were also observed for the Caco-2 cell line.

From observations described above, we conclude that a cell line, CHO/hPEPT1, is a powerful tool to study the transport by hPEPT1. Due to the H⁺-symporter, uptake of a sub-

![Fig. 1. Time Course of Gly-Sar Uptake by Control and hPEPT1-Expressing (CHO/hPEPT1) CHO Cells](image)

The uptake of Gly-Sar (10 $\mu$M) was measured in control and hPEPT1-expressing cells for various periods of time. Circles and squares represent CHO/hPEPT1 and control cells, respectively. The filled and unfilled symbols showed the uptakes at pH 6.0 and pH 7.5, respectively. Filled triangles showed the uptake of Gly-Sar in the presence of 40 $\mu$M CCCP by CHO/hPEPT1. Each point represents the mean ± S.E. of four to six experiments.

![Fig. 2. Concentration-Dependency of Gly-Sar Uptake by CHO/hPEPT1 Cells](image)

The uptakes of Gly-Sar were measured at 1, 2, and 3 min. Initial uptake rates were determined by linear regression analysis of the linear portion of Gly-Sar uptake vs. time. The inset shows an Eadie-Hofstee plot of Gly-Sar uptake. The values for the Michaelis-Menten constant and maximum velocity are $1.02 \pm 0.08 \text{mM} \pm \text{S.D.}$ and $34.7 \pm 0.91 \text{nmol/min/mg protein} \pm \text{S.D.}$, respectively. Each point represents the mean ± S.E. of five experiments.

![Fig. 3. Inhibitory Effects of Various Dipeptides on the Transport of Gly-Sar in CHO/hPEPT1](image)

Initial uptake rates of 10 $\mu$M Gly-Sar were determined at pH 6.0 within a 3-min incubation as described in the legend of Fig. 2, and then normalized by that in the absence of compounds. The concentrations of dipeptide and amino acid were 1 mM. The concentrations of pharmacological agents (*) are 5 mM. Each column represents the mean ± S.E. of four—five experiments.
strate gives rise to a decrease in the intracellular pH, which can be monitored using a pH-sensitive fluorescent dye. This high activity of CHO/hPEPT1 might be enough for such experiments. Another possible use of CHO/hPEPT1 might be application of an electrophysiological method. These experiments are now in progress in this laboratory.

**pH Dependency of Gly-Sar Uptake by CHO/hPEPT1 Cells**

The uptake of Gly-Sar by CHO/hPEPT1 cells was examined under varying pH from 8.0 to 3.5; results are shown in Fig. 4. The uptake of Gly-Sar was stimulated by acidification of the medium pH, but further acidification of the medium pH remarkably depressed the uptake. These observations indicate that the optimal pH is 5.5 as shown in Fig. 4. After 30 min incubation with pH 4.0 buffer, cells were incubated with pH 7.4 buffer for 5 min, followed by the uptake experiments at pH 6.0. The value of the uptake activity was reversed to that before incubation with buffer at pH 4.0 (data not shown), indicating that the abolishment of the uptake at lower pH is not due to damage of the protein and/or cell membranes, but may be the protonation of an amino acid(s) of hPEPT1.

For further investigation to identify this amino acid(s), we examined the uptake activities under varying pH in the presence of 10 mM monensin/nigericin, which dissipates an inwardly proton gradient $\text{DpH}$ but not the membrane potential. This treatment prevents the alteration of the driving force for the uptake by the change in the extracellular pH. Under this treatment, the driving force is attributed only to the membrane potential. As shown in Fig. 5, in sharp contrast to the bell-shape curve in Fig. 4, the activity increases with an increase in pH and seems saturable at higher pH. A Henderson–Hasselbalch equation with a single $\text{pK}_a$ was fitted well to this curve. The $\text{pK}_a$ value was estimated to be $6.7 \pm 0.2$. This may indicate that the protonation of carboxyl or amino group of amino acid residues can regulate the transport activity. Furthermore, we analyzed the kinetic parameters of Gly-Sar uptake at different pH under the condition of the dissipated $\text{DpH}$. The maximum values of the uptake rate showed a pH dependency, whereas the values of the Michaelis constant remained relatively unchanged irrespective of extracellular pH (Fig. 6, Table 1). The decrease in the transport activity with pH decrease might be due to the decrease in the maximum uptake rate rather than in the Michaelis constant.

We also monitored the membrane potential with the uptake of tetr phenylphosphonium cation (TPP$^+$) in the presence of 10 $\mu$M nigericin/monensin, which has been extensively used as the membrane potential probe. The uptake rates of 1 $\mu$M TPP$^+$ at various pH were as follows: 21.1 ± 0.5 at pH 7.0, 17.3 ± 0.7 at pH 6.5 and 11.3 ± 1.0 at pH 5.5 (pmol/mg protein/min). With range of 6.5 to 7.0, the membrane potential was decreased by the only 18%, whereas the...
The uptake rates of Gly-Sar were measured at various pH as described in the legend to Fig. 6. Kinetic parameters ($K_m$ and $V_{max}$) were determined by the linear regression analysis of Eadie-Hofstee plots at various pH. Mean values of five experiments were used for the linear regression analysis. Each data represents the calculated value±S.D.

### Table 1. Effect of Extracellular pH on the Kinetic Parameters of Gly-Sar Uptake by CHO/hPEPT1 under the Condition of the Dissipated ΔpH

<table>
<thead>
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<td>1.2±0.5</td>
<td>4.2±1.0</td>
</tr>
<tr>
<td>6.5</td>
<td>0.9±0.2</td>
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</tr>
<tr>
<td>7.0</td>
<td>1.2±0.1</td>
<td>11.4±0.9</td>
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Fig. 7. Effect of DEPC Modification on Gly-Sar Uptake Activity

The cells were preincubated at 25°C for 10 min with 1 mM DEPC (pH 6.0) in the absence or the presence of 10 mM Gly-Sar. After treatment of DEPC, the cells were rinsed twice with the incubation medium. Initial uptake rates were then determined as described in the legend of Fig. 2. Each point represents mean±S.E. of four experiments.

The uptake rate of Gly-Sar was decreased to 42% of that at pH 7.0. The Gly-Sar uptake was abolished at pH 5.5 although the membrane potential was decreased to 54±13%. The uptake clearance is governed by an exponential function of the membrane potential (Eq. 3 in Discussion). Even if the membrane potential was decreased by 40%, the uptake decreased minimal.

**Effect of a Histidine Residue Modifier, DEPC on the pH-Profile of the Transport Activity** A pKa of 6.7 strongly suggests the amino acid residue in question may be histidine. In addition, histidine residues in PEPT1 have been identified as key amino acid residues, which are involved in substrate recognition as well as responsible for the transport activity, especially proton-translocation. Therefore, we checked the effect of chemical modification of histidine by DEPC on the pH profile of transport activity. One mM DEPC treatment completely abolished the transport activity of CHO/hPEPT1. On the other hand, this abolishment was almost prevented by the presence of 10 mM Gly-Sar (Fig. 7). This protection was observed only in the presence of substrates of PEPT1, indicating that the histidine residue is located at the substrate recognition site. The pH-dependence of the transport activity of CHO/hPEPT1 that had been subjected to the DEPC-treatment in the presence of 10 mM Gly-Sar was also examined; the results are shown in Fig. 8. Although the optimal pH is shifted to pH 6 and the value at pH 8 is relatively larger than that in Fig. 4, the pH-profile shows a bell-shape. Note that the DEPC treatment in the presence of 10 mM Gly-Sar could modify all histidine residues except for the histidine residues located at the substrate binding-site.

Table 1. Effect of Extracellular pH on the Kinetic Parameters of Gly-Sar Uptake by CHO/hPEPT1 under the Condition of the Dissipated ΔpH

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Fig. 8. Effect of DEPC Modification in the Presence of 10 mM Gly-Sar on the pH Profile of hPEPT1 Activity

The cells were preincubated at 25°C for 10 min with 1 mM DEPC (pH 6.0) in the presence of 10 mM Gly-Sar. After treatment of DEPC, the cells were rinsed twice with the incubation medium. Cells were incubated additionally twice with the incubation medium for 5 min. Initial uptake rates were then determined as described in the legend of Fig. 2. Each point represents mean±S.E. of four experiments.

Fig. 9 pH Dependency of Gly-Sar Transport Activities under DEPC Treatment Conditions When Only the pH Gradient across the Plasma Membrane Was Dissipated

The cells were preincubated at 25°C for 10 min with 1 mM DEPC (pH 6.0) in the presence of 10 mM Gly-Sar. After treatment of DEPC, the cells were rinsed twice with the incubation medium. Cells were incubated additionally twice with the incubation medium for 5 min. After cells were incubated in buffers containing 10 μM nigericin/menensin at various pH for 5 min, initial uptake rates were determined. The uptake solution also contained 10 μM nigericin/menensin. Each point represents mean±S.E. of four—five experiments.

Under these conditions, we also estimated the pKa value of the amino acid residue being responsible for pH regulation of the transport activity. As shown in Fig. 9, a Henderson–Hasselbalch equation with a single pKa (Eq. 2) was also fitted well to pH-dependence of transport activity. The pKa value was estimated to be 6.1±0.1 (±calculated S.D.). These data stressed that the histidine located at the substrate recognition site is involved in the pH regulation of transport activity.

**Effect of Chemical Modification of Carboxyl Groups with EDCM or Hydroxyl Groups with PMSF on pH-Profile of the Transport Activity** To identify the involvement of other amino acid residues in pH-regulation of the transport activity, we determined the effect of chemical modification on pH-profile of the transport activity. As shown in Fig. 10, chemical modification of carboxyl groups or hydroxyl groups had no significant effect on pH profile of the transport activity: a bell-shaped curve with an optimal pH at 5.5.
showed about 35-fold larger than that in Caco-2 cells. 20—22) Mean rates were then determined as described in the legend of Fig. 2. Each point represents cells were incubated additionally with the incubation medium for 5 min. Initial uptake treatment of EDCM or PMSF, the cells were rinsed twice with the incubation medium. Presence of 10 mM glycine methylester or for 10 min with 1 mM PMSF (pH7.4). After 2.16,20,21) it is noted that the activity of hPEPT1 in this cells expressed in CHO/hPEPT1 cell and its features are the same as those in a model human intestine epithelial cell line, Caco-2.16,20,21) It is noted that the activity of hPEPT1 in this cells showed about 35-fold larger than that in Caco-2 cells.20—22) The CHO/hPEPT1 is a powerful tool to study the transport mechanism of hPEPT1. hPEPT1 is an electrogenic \( \text{H}^+\)/dipeptide cotransporter and the substrate of hPEPT1 should cause intracellular acidification. This activity is more than enough to cause the intracellular acidification due to the influx of the transporter which can be detected using pH-sensitive fluorescent dye.5) This activity also opens an avenue to determine the influx of the substrate with an electrophysiological technique or a whole-cell patch clamp technique.

The uptake of Gly-Sar by CHO/hPEPT1 cells was examined under varying pH from 8.0 to 3.5; results are shown in Fig. 4. The uptake of Gly-Sar was stimulated by acidification of the medium pH, but further acidification of the medium pH remarkably depressed the uptake. pH profile of the uptake activity showed a typical bell-shaped curve with the maximum uptake at pH 5.5 (Fig. 4). The feature of pH dependency in the transport activity was shown similarly by other researchers. The transport of substrate via hPEPT1 is unequivocally driven by an inward \( \text{H}^+\) electrochemical potential gradient.2,3,12) As to the bell-shaped activity of hPEPT1 vs. pH, the reason why the transport activity decreases with an increase in pH is obviously due to the decrease in the driving force of the proton electrochemical gradient. On the other hand, based on the driving force of hPEPT1, the uptake activity would increase when extracellular pH decreases. The aim of the present study is to clarify the mechanism why the decrease of the activity in a more acidic region although the driving force should increase.

To determine the pH-dependent properties of hPEPT1, we at first determined pH-dependency of the transport activity under conditions that only \( \Delta \text{pH} \) across the plasma membrane was abolished with monensin/nigericin treatment (Fig. 5). Under conditions that only \( \Delta \text{pH} \) has been dissipated, the pH profile exponentially decreased when extracellular pH was decreased. A Henderson–Hasselbalch equation with a single pKa was fitted well to this pH dependency and the pKa value was estimated to be 6.7±0.2, suggesting that a certain amino acid residue is involved in the pH-dependency of the transport activity. The decrease in the transport activity with pH decrease might be due to the decrease in the maximum uptake rate rather than in the Michaelis constant (Table 1). It seems unlikely that pH titration of the amino acid residue in hPEPT1 might cause the change in the binding affinity of substrate.

The driving forces for substrate transport via hPEPT1 will depend on the overall charge and on the protons which are translocated, as well as on the substrate gradient across the membrane. The driving force is therefore composed of components of the proton-motive force and of the substrate gradient, and translocation of substrate will proceed until the sum of the electrochemical potential for substrate and proton is equal to that of the extracellular space. A general consideration of energy conversion from the viewpoint of the theory of nonequilibrium thermodynamics gives the following equation as to the permeability clearance for influx processes:\(^{31,32)}\) (see Appendix);

\[
P_{\text{S,inf}} = P_{\text{S,eff}} 10^{\alpha \Delta \text{pH}} \exp \left( \frac{-\alpha F \Delta \varphi}{RT} \right)
\]

where \( P_{\text{S,inf}} \) and \( P_{\text{S,eff}} \) represent the permeability clearances for influx and efflux processes, respectively; \( R, T, F \) and \( \Delta \varphi \) are gas constant, absolute temperature, Faraday’s constant and membrane potential difference, respectively. \( \Delta \text{pH} \) represents the difference of pH between intracellular and extracellular pH (\( \text{pH}_{\text{in}} - \text{pH}_{\text{out}} \)). Under condition that the membrane potential difference remains unchanged, \( P_{\text{S,inf}} \) is a hybrid function of \( P_{\text{S,eff}} \) and \( 10^{\alpha \Delta \text{pH}} \). This relation means that its carrier cycling and mode of hPEPT1 is regulated by mutual interaction of inward and outward processes as well as the proton gradient across the membrane. hPEPT1 has been demonstrated extensively to be one of the bi-directional transporters by \( \text{trans}-\text{stimulation phenomena}^{6,21)}\); the transportable substrate preloaded on the “\( \text{trans} \)” side (opposite side) of the plasma membrane stimulates the transport of substrate applied on the “\( \text{cis} \)” side into the “\( \text{trans} \)” side. This \( \text{trans}-\text{stimulation} \) of the transport is feasibly explained by the increase in the availability of carriers at the outside due to the faster inward-to-outward transformation of the carriers loaded with the substrate.\(^{31)}\) Thus \( \text{trans}-\text{stimulation} \) suggests that the efflux process is also mediated by hPEPT1. The relationship between \( P_{\text{S,inf}} \) and \( P_{\text{S,eff}} \) expressed in Eq. 3 feasibly explains the \( \text{trans}-\text{stimulation} \) phenomena.\(^{31)}\) Very Recently, Fujisawa et al.\(^{31)}\) have established a HeLa cell line over-expressing...
hPEPT1 (HeLa/hPEPT1), in which we measured the transport currents due to the uptake and efflux processes using the whole-cell patch clamp technique. It has been demonstrated directly that hPEPT1 can transport dipeptides bi-directionally in an electrogenic and proton-coupled cotransport mode.

We have also determined the effect of extracellular pH (varying from 8.0 to 3.5) on the efflux activity (PS_{out}) by hPEPT1. The value of PS_{out} was decreased along with the extracellular pH decrease. Henderson–Hasselbalch equation was fitted well to the pH-dependency profile of efflux activity, indicating that there is a single residue of amino acid which also regulates the efflux activity. The pKa value was estimated to be about 6. Based on Eq. 3, which also regulates the efflux activity, indicating that there is a single residue of amino acid which also regulates the efflux activity. The pKa value was estimated to be about 6. Based on Eq. 3, PS_{out} is proportional to PS_{out} in the case of the only dissipation of the H^+ gradient across the membrane, suggesting the possibility that the single amino acid regulates the bi-directional transport activity in a pH-dependent manner. The transport activity (PS_{out}) is a hybrid of an exponential increase-function (10^{pH} K_s) and exponential decrease-function (PS_{out}) as pH decreases. Equation 3 could feasibly simulate the pH-profile of the transport activity activity in a bell shape. In other words, the bell-shape profile is caused by the balance between driving force and the extent of the regulation by a certain amino acid residue.

Which amino acid residue accounts for the bell-shaped pH-profile of the transport activity? The titration curve (Fig. 5) shows pH-sensitivity of the transport activity in the range of pH 5.5 to 7.4 and indicates the amino acid residue with pKa of 6.7 to regulate the activity. Therefore, most probable residue of the amino acid is inferred to be histidine, since the imidazole group of histidine is the only amino acid side chain affected within this pH range: At pH 5.5, the imidazole ring is protonated and positively charged, whereas at pH 7.0, it is electrically neutral. Up to date, histidyl residues have been reported extensively to be one of the most important amino acid residues in hPEPT1, which were revealed by DEPC modification and site-directed mutagenesis studies and protection in the presence of substrates and site-directed mutagenesis studies revealed that His-57 in the second transmembrane domain (TMD2) is the most likely residue involved in the H^+ binding/dissociation. The Site-directed mutagenesis studies affecting His-121 in TMD4 showed that it is the substrate-binding site. His-57 and His-121 were hypothesized to be intimately located and form the substrate binding pockets.

In order to determine whether these crucial histidine residues are involved in the regulation of the transport activity by pH, we tried DEPC modification, but the activity was completely abolished (Fig. 7), resulting in our inability to investigate the pH-dependence of the transport activity. We fortunately found that the modification in the presence of 10 mM Gly-Sar preserved the transport activity, indicating that the only crucial residues of histidine are protected by an excess of Gly-Sar. Using the cells done by this modification, the pH-dependence of the activity was measured. As shown in Fig. 8, essentially the same results were obtained as those of the intact cells (Fig. 4). We also estimated the pKa value of the amino acid residue involved in pH-dependency of the transport activity to be 6.1, which showed similar to that under normal conditions. These data stress at least a histidine residue located at or near the substrate-binding site is responsible for conferring the pH regulation. Most provable residues are thus inferred to be His-57 and/or His-121. These results cannot completely rule out a possibility that a certain histidine residue with resistant to DEPC modification might be involved in the titration curve (Fig. 8), except for these two histidine residues. However, the possibility seems unlikely because of the obvious evidence that the DEPC modification in the absence of substrate completely diminished the transport activity.

It is known well that the bell-shaped pH-rate profile of chymotrypsin-catalyzed cleavage is accounted for by the protonation of a single His residue in the catalytic triad. The catalytic triad is a hydrogen-bonding network formed by His, Asp, and Ser. His protonation in the catalytic triad formed a salt bridge between Asp and His causes the disturbance of the hydrogen network in the catalytic triad, resulting in a loss of activity. Glutamate or aspartate is a partner of histidine in a number of enzymes that have an active site motif known as catalytic triad. The positively charged histidine at lower pH may interact with a negatively charged residue, resulting in the change in the conformation of PEPT1 and inactivation of the transporter. In the range from pH 4.5 to 7.0, carboxylic group in aspartate and glutamate residues are charged negatively under hydrophilic environment, and can be considered as countercharge residues for the positive charge residue, protonated His. To identify the involvement of this salt-bridge in pH regulation of the transport activity, we determined the effect of chemical modification with EDCM and PMSF on the pH-profile of the transport activity. This EDCM can access only to hydrophilic environment and modify carboxylic group of aspartate and/or glutamate. As show in Fig. 10, the modification of carboxylic group with EDCM had no significant effect on the pH-profile: a bell-shaped feature of the transport activity with an optimum pH at 5.5. This pH titration curve indicates that carboxyl groups are not involved at all in pH-regulation of the transport activity. Chemical modification with PMSF also had no effect on the pH profile, indicating that there is no hydrogen-bonding network in the pH-dependency of the transport activity of hPEPT1 unlike in the chymotrypsin catalytic triad.

More precisely, we analyzed the kinetic parameters of Gly-Sar uptake at different pH under the condition of the dissipated ΔpH. Under the condition of the dissipated ΔpH, the only V_{max} value showed a pH dependency, whereas the K_m values remained relatively unchanged irrespective of extracellular pH (Fig. 6, Table 1). The decrease in the transport activity with pH decrease might be due to the decrease in the maximum uptake rate rather than in the affinity. Therefore, our focusing histidine residue is hard to be considered to form the substrate-binding pocket, and as a consequence, most provable histidine residue seems the proton coupling site, His-57. The question remains unsolved: How can His-57 regulate the transport activity in a pH-dependent manner? According to Figs. 5 and 9, the activity increased along with increase in pH and reached to the maximum more than pH 7.5. It is most crucial for the transport cycle that His-57 accepts H^+ molecule at the first state from somewhere. This step is indispensable to initiate the transport cycles. At lower pH, His-57 is protonated and positively charged before the first state of transport cycle, which culminates in the arrest in
the transport. It is too tough to elucidate the involvement of His-57 in pH-regulation of the transport activity, because the mutagenesis of His-57 with other amino acid residue completely loss the transport function.

It has been known that fluctuations in pH, under physiological and pathological conditions, modulate the activity of a number of transporters.\textsuperscript{42–45} Very recently, Ju et al.\textsuperscript{46} demonstrated that protonation/deprotonation of two histidine residues on the extracellular domain regulated glycine transport activity via one isofrom of glycine transporters (GLYT1), which are members of the Na\textsuperscript{+}/Cl\textsuperscript{−}-dependent neurotransmitter transporter family. Furthermore, they showed that zinc noncompetitively inhibits the glycine transport activity with its chelating with two histidine residues, of which one is overlapped with proton binding sites. By the contrast, Okamura et al.\textsuperscript{47} demonstrated that zinc also inhibited the function of PEPT1 via the interaction with histidine residues. The present data showed that some histidine residues might be involved in pH-dependency of PEPT1 transport activity. It is interesting that these two transporters within the different transporter families showed a similar feature in the regulation of transport activity by proton and zinc through histidine residues. Further investigation is worth to clarify molecular basis for the differences and relationship between zinc and proton binding site involved in the regulation of PEPT1 activity.

Taken together, there is the most feasible mechanism to explain our findings: An amino acid residue in the question is His-57 or His-121, which are located at or near the substrate binding. It is worth noting that the Henderson–Hasselbalch equation with a single pK\textsubscript{a} can simulate well the result. In addition, the protonation of this residue causes loss of the transport activity completely, which can be observed for us as the decrease of V\textsubscript{max}, rather than an increase of K\textsubscript{m}. DEPC treatment altered the maximal velocity of Gly-Sar uptake by PEPT1 but had no effect on its K\textsubscript{m}.\textsuperscript{26,27} Therefore, the histidine residue in the question is hard to be considered to form the substrate-binding pocket, and as a consequence, most provable histidine residue seems the proton coupling site, His-57. Strictly speaking, the discrimination of both histidine and identification of the amino acid residue will await for further investigations by site-directed mutagenesis studies for histidine residues as well as other amino acid residues. Here, we would stress that this paper explains the reason for the bell-shaped relationship between the activity and the extracellular pH, which is regulated by the protonation/deprotonation of some amino acid residues.

**APPENDIX**

hPEPT1 translocates the substrate by coupling to movement of proton down the electrochemical gradient.\textsuperscript{1,2} For the simplicity, we consider a transport system of hPEPT1 in which there are two driving forces, the electrochemical potential differences for substrate (X\textsubscript{S}) and H\textsuperscript{+} (X\textsubscript{H\textsuperscript{+}}) and two fluxes, the substrate flux (J\textsubscript{S}) and the proton flux (J\textsubscript{H\textsuperscript{+}}). According to the empirical evidence,\textsuperscript{32,48} the flow is a linear function of the driving force over a relatively large range of flow and force, which correspond in the case of the present study to the flux and the electrochemical potential difference, respectively. Thus, we can express the relationship between flux and driving force as the following phenomenological equations\textsuperscript{31,32,40}:

\[
J\textsubscript{S} = L\textsubscript{S}X\textsubscript{S} + L\textsubscript{SH\textsuperscript{+}}X\textsubscript{H\textsuperscript{+}} \tag{A1}
\]

\[
J\textsubscript{H\textsuperscript{+}} = L\textsubscript{H\textsuperscript{+}}X\textsubscript{S} + L\textsubscript{SH\textsuperscript{+}}X\textsubscript{H\textsuperscript{+}} \tag{A2}
\]

where L\textsubscript{S} and L\textsubscript{H\textsuperscript{+}} are straight conductance coefficients (conductance coefficients) for substrate and H\textsuperscript{+}, respectively; L\textsubscript{SH\textsuperscript{+}} is the coupling or cross conductance coefficients for substrate and H\textsuperscript{+}, respectively. Because hPEPT1 transports the neutral substrate accompanying H\textsuperscript{+} in a 1:1 stoichiometry, J\textsubscript{S} is equal to J\textsubscript{H\textsuperscript{+}}.\textsuperscript{49} According to Onsager’s reciprocal relationship,\textsuperscript{48} the matrix of coefficients for a system of flows and forces based on an appropriate dissipation function is symmetrical and the following equation holds;

\[
L\textsubscript{H\textsuperscript{+}} = L\textsubscript{SH\textsuperscript{+}} = L\textsubscript{C} \tag{A3}
\]

where L\textsubscript{C} is the coupling conductance coefficient. Under steady-state condition, substituting Eq. A3 into Eqs. A1 and A2 and rearrangement with regard to the X\textsubscript{S} term yield the following equations;

\[
X\textsubscript{S} = \frac{L\textsubscript{C}}{L\textsubscript{S}} X\textsubscript{H\textsuperscript{+}} \tag{A4}
\]

\[
L\textsubscript{C} = L\textsubscript{H\textsuperscript{+}} \tag{A5}
\]

Designating the ratio of L\textsubscript{C} to L\textsubscript{S} as \(\alpha\) and rewriting Eq. A4 with regard to the electrochemical potential difference gives the following equation;

\[
\tilde{\mu}\textsubscript{S} - \tilde{\mu}\textsubscript{S} = -\alpha(\tilde{\mu}\textsubscript{H\textsuperscript{+}} - \tilde{\mu}\textsubscript{H\textsuperscript{+}}) = -\alpha \Delta \tilde{\mu}\textsubscript{H\textsuperscript{+}} \tag{A6}
\]

where \(\tilde{\mu}\textsubscript{S}\) and \(\tilde{\mu}\textsubscript{H\textsuperscript{+}}\) represent the extracellular electrochemical potentials of substrate and proton, respectively; \(\tilde{\mu}\textsubscript{S}\) and \(\tilde{\mu}\textsubscript{H\textsuperscript{+}}\), the intracellular electrochemical potentials of substrate and proton, respectively; \(\Delta \tilde{\mu}\textsubscript{H\textsuperscript{+}}\), the electrochemical potential difference. In the present study, we used a zwitter-ionic peptide, Gly-Sar, as the electroneutral model substrate. The pK\textsubscript{a} values of the amino and carboxylic groups in Gly-Sar are 8.3 and 2.9, respectively; the electroneutral state of Gly-Sar does not change in the pH range from 4.0 to 7.5 where we examined the pH-dependence of Gly-Sar uptake (Figs. 4, 5, 8). Thus the rearrangement of Eq. A6 with regard to the electrochemical potential of the substrate yields,

\[
\tilde{\mu}\textsubscript{S} = \tilde{\mu}\textsubscript{S} + \frac{RT \ln C\textsubscript{in}}{C\textsubscript{in}} = -\alpha \Delta \tilde{\mu}\textsubscript{H\textsuperscript{+}} \tag{A7}
\]

where C\textsubscript{ext} and C\textsubscript{in} represent the extracellular and intracellular concentrations of substrate, respectively; At steady state, the following equation also holds,

\[
PS\textsubscript{eff}C\textsubscript{ext} = PS\textsubscript{eff}C\textsubscript{in} \tag{A8}
\]

where PS\textsubscript{inf} and PS\textsubscript{eff} represent the permeability clearances for influx and efflux processes, respectively. Substituting Eq. A8 into Eq. A7 and rearrangement with regard to the PS\textsubscript{eff} term yields the following equation;

\[
PS\textsubscript{eff} = PS\textsubscript{eff} \exp \left( -\frac{\alpha \Delta \tilde{\mu}\textsubscript{H\textsuperscript{+}}}{RT} \right) \tag{A9}
\]

The value of \(\alpha \Delta \tilde{\mu}\textsubscript{H\textsuperscript{+}}\) is given by the following equation;
\[ \alpha \Delta \mu_{H^+} = \alpha [RT \ln \frac{c^{in}}{c^{ext}} + F(\varphi^{ext} - \varphi^{in})] = \alpha [2.303RT\Delta H - F\Delta \varphi] \] 

where \( c^{in} \) and \( c^{ext} \) are the intracellular and extracellular proton concentrations, respectively; \( \varphi^{in} \) and \( \varphi^{ext} \) are the intracellular and extracellular membrane potentials, respectively; \( R \), \( T \) and, \( F \) are gas constant, absolute temperature and Faraday's constant, respectively; \( \Delta H \) and \( \Delta \varphi \) represent the differences of pH between intracellular and extracellular pH (\( \text{pH}_{\text{in}} - \text{pH}_{\text{ext}} \)) and membrane potential (\( \varphi^{in} - \varphi^{ext} \)), respectively. Substituting Eq. A10 into Eq. A9 gives the following equation,

\[ PS_{\text{diff}} = PS_{\text{tot}} \exp \left( \frac{-\alpha F \varphi \Delta \varphi}{RT} \right) \]  

REFERENCES