Unified modeling of the mammalian and fish proton-dependent oligopeptide transporter PepT1

Maria Daniela Renna,1 Rachele Sangaletti,1 Elena Bossi,1,2 Francesca Cherubino,1 Gabor Kottra4 and Antonio Peres1,2,*

1Laboratory of Cellular and Molecular Physiology; Department of Biotechnology and Molecular Sciences; 2Center for Neurosciences; University of Insubria; Varese, Italy; 3Fondazione Maugeri IRCCS; Tradate, Italy; 4Molecular Nutrition Unit; Technische Universität München; Freising, Germany

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Electrophysiological and biophysical analyses were used to compare the partial and complete transport cycles of the intestinal oligopeptide transporter PepT1 among three species (seabass, zebrafish and rabbit). On the whole, the presteady-state currents of the fish transporters were similar to each other. Rabbit PepT1 differed from the fish transporters by having slower-decaying currents, and the charge vs. potential (Q/V) and time constant vs. potential (τ/V) curves shifted to more positive potentials. All of the isoforms were similarly affected by external pH, showing acidity-induced slowing of the transients and positive shifts in the Q/V and τ/V curves. Analysis of the pH-dependence of the unidirectional rates of the intramembrane charge movement suggested that external protonation of the protein limits the speed of this process in both directions. The complete cycle of the transporter was studied using the neutral dipeptide Gly-Gln. Michaelis-Menten analysis confirmed that, in all species, acidity significantly increases the apparent affinity for the substrate but does not strongly impact maximal transport current. Simulations using a kinetic model incorporating the new findings showed good agreement with experimental data for all three species, both with respect to the presteady-state and the transport currents.

Introduction

In many species, amino acid absorption in the intestine occurs mostly via PepT1, an ion-coupled cotransporter belonging to the solute carrier family SLC15A.1 In contrast to other cotransporter families, SLC15A peptides are proton-dependent and Na⁺-independent, that is, their function does not rely on the electrochemical gradient of sodium ions. Acidic pH appears to enhance the uptake of substrates1-3 due to the presence of an acidic microenvironment in the vicinity of the absorptive enterocytes generated by the activity of the Na⁺/H⁺ exchanger in the apical membrane.4,5

Mutational studies have provided significant evidence for the interaction of protons with the transporter protein. Histidine 57 (His57) in the second transmembrane domain of PepT1 is required for the transporter to be functional,5-8 which suggests that protonation of this residue is a necessary step in the transport cycle. Tyrosine residues (Tyr56 and Tyr64) around His57 also have been shown to stabilize proton binding8 in rabbit PepT1.

The enhancing effect of acidic pH on substrate uptake shows, however, some apparently contradictory aspects. In fish species and in rabbits, electrophysiological measurements have indicated a substantially pH-independent maximal rate of transport10,11 or even a potentiating effect of alkaline pH.12,13 Uptake experiments with human PepT1 in an extended pH range have shown a bell-shaped activity-pH relationship.14 A similar trend has been found for the maximal transport current in the rabbit isoform.15 From a structural point of view, the level of homology among the various transporter isoforms of mammalian and fish species is about 56% amino acidic identity and 72% similarity. However, these values can reach 72% and 90%, respectively, when considering only the human, rabbit, zebrafish and seabass transporters and limiting the analysis to the transmembrane domains involved in pore formation (TM 1, 3, 5, 7, 8 and 10) or to residues facing the pore (TM 2 and 4).16

Similar to the majority of ion-coupled, electrogenic cotransporters,17-19 PepT1 exhibits not only steady currents associated with the substrate transport but also presteady-state currents elicited by membrane voltage steps in the absence of substrate. In all species thus far studied (i.e., human, rabbit and seabass), this type of current is affected by external pH, although the kinetic properties vary somewhat among different species and significant variability has been reported for human PepT1.3,10,20,21

Functional kinetic models have been proposed for human PepT1,3,21,22 but no attempts have been made to verify their applicability to transporters from other species. Hence, the main purpose of the present work was to devise a kinetic model for PepT1 that can describe, with only minor adjustments of parameters, the...
different characteristics of the isoforms of different species, with respect to both presteady-state and transport-associated currents.

**Results**

**Presteady-state currents in the different species.** The PepT1s of rabbit (rbPepT1), seabass (sbPepT1) and zebrafish (zfPepT1) exhibit presteady-state currents in the absence of organic substrate. **Figure 1** compares the currents induced in each isoform by voltage pulses in the absence and presence of saturating Gly-Gln at pH 6.5. Although we performed experiments in a more extended range (6.0–8.0), pH 6.5 seems to most approximate real physiological conditions, at least in rabbit PepT1 (based on the pH of the luminal surface of the mammalian small intestine).23

Although the presteady-state currents of rbPepT1 and sbPepT1 have already been described and analyzed,10,20 information on zfPepT1 is lacking. As shown in **Figure 1** (top row), the two fish species, exhibited larger currents for hyperpolarization than for depolarizations, while the transients from rbPepT1 are approximately symmetrical around the -60 mV holding potential. Adding saturating amounts of organic substrate (3 mM Gly-Gln) elicited steady transport currents (**Fig. 1**, bottom row) and, as commonly observed in most electrogenic transporters, abolished the presteady-state currents.

The presteady-state currents were separated from the fast peaks due to the endogenous oocyte capacity, using two different procedures (see Materials and Methods). In the first, we fit a double exponential function to currents recorded in the absence of substrate and regarded the slow component as the presteady-state current. In the second, we subtracted the current traces in the presence of saturating substrate from those in its absence and fit a single exponential to the difference. In both cases, steady transport currents were subtracted before fitting the exponentials.

As previously observed,10,24 the two methods gave virtually identical results. This is relevant not only to better understand the transporter mechanism, but also important because of the intrinsic limitations of the two-exponential method. Namely, the separation of two exponentials by fitting is impossible when the two time constants are close in value. Because the time constants for charging the oocyte membrane capacity are generally around 1 ms, the two-exponential method becomes unreliable when the decay time constant of the presteady-state currents approaches this value (i.e., 2–3 ms). This is indeed the case for both fish species at the most positive potentials explored (see below). In addition, both rabbit and seabass PepT1 exhibit an acceleration of the presteady-state current decay at alkaline pH.10,20 For these reasons, we used the subtraction method to isolate these currents. Because the presteady-state currents of seabass and zebrafish are very similar to each other and the results from seabass PepT1 have already been published,10 only data from zebrafish are shown here.

The voltage dependences of the decay time constant \( \tau \) and the intramembrane charge movement \( Q \) obtained from the isolated presteady-state currents are shown in **Figure 2**.

Several observations can be made when comparing the time constant vs. potential (\( \tau/V \)) and charge vs. potential (\( Q/V \)) curves of the rabbit and fish PepT1. First, as already mentioned, at pH 6.5, the rbPepT1 transients are more symmetrical around the holding potential; this is confirmed by the position of the bell-shaped \( \tau/V \) curve with a maximum at about -90 mV. For the fish transporters, only the right half of the bell-shaped curve is visible in the voltage range that can reasonably be explored; the seabass transporter appears to have a maximum at about -140 mV.10

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**Figure 1.** Comparison of PepT1 presteady-state currents from three species. Voltage pulses lasting 200 ms and spanning the -140 to +20 mV interval were applied from \( V_h = -60 \) mV and at pH 6.5. In the absence of organic substrate (top row), all three transporters display slowly decaying transients in response to “on” and “off” voltage steps. The bottom row shows the steady currents induced in the same oocytes by the addition of 3 mM Gly-Gln and the simultaneous disappearance of the presteady-state currents. Note the faster decay of the transient currents in the fish transporters and their absence in the depolarizing direction.
The equilibrium distribution of the charge moved during the presteady-state currents can be described with the Boltzmann equation:

$$Q = \frac{Q_{\text{max}}}{1 + \exp\left(\frac{V - V_{0.5}}{\sigma}\right)}$$

(1)

where $Q_{\text{max}}$ is the maximal moveable charge; $V_{0.5}$ is the voltage at which half of the charge is moved (that is, the midpoint of the sigmoidal); and $\sigma = kT/q\delta$ represents a slope factor, in which $q$ is the elementary electronic charge, $k$ is the Boltzmann constant, $T$ is the absolute temperature and $\delta$ is the fraction of electrical field over which the charge movement occurs. Fitting the $Q/V$ curves to this equation gives the parameters summarized in Table 1.

### Table 1. Boltzmann equation parameters

|                  | Rabbit     | Seabass    | Zebrafish |
|------------------|------------|------------|-----------|
| **pH**           | 6.5        | 7.5        | 6.5       | 7.5       | 6.5 | 7.5 |
| $Q_{\text{max}}$ (nC) | 33.2 ± 1.9 | 31.5 ± 1.2 | 10.2 ± 0.2 | 6.8 ± 0.9 | 11.0 ± 0.3 | 9.9 ± 1.1 |
| $V_{0.5}$ (mV)   | -41.4 ± 2.5 | -100 ± 2.3 | -98.7 ± 1.3 | -122.0 ± 0.6 | -108 ± 1.6 | -119 ± 7.2 |
| $\sigma$ (mV)    | 42.9 ± 3.1 | 39.5 ± 1.7 | 23.1 ± 0.7 | 23 ± 2.7 | 31.1 ± 0.9 | 33.5 ± 3.3 |

whereas the maximal $\tau$ for the zebrafish isoform may be at even more negative voltages. Similarly, the $Q/V$ curves for rbPepT1 show a clear sigmoidal shape, whereas in the fish transporters only the rightmost part of a sigmoid is detectable. Therefore, the properties of the fish PepT1s are shifted to more negative potentials compared to those of the rabbit isoform.

Furthermore, the decay rates of rbPepT1 transient currents are much slower than those of both fish transporters, with sbPepT1 faster than zfPepT1.\(^{10}\)

**Effects of external pH on the presteady-state currents.** Alkalization of the external medium has been shown to produce negative shifts in the $\tau/V$ and the $Q/V$ curves in rabbit and seabass PepT1.\(^{10,20}\) This effect was also seen in zfPepT1. Figure 2 compares these results among the fish and rabbit isoforms.
those showing a pH-dependent increase in both unidirectional rate constants. To incorporate the slowing effects of protonation on both unidirectional rate constants, some changes were made to existing models. As shown in Figure 3, the intramembrane charge movement occurs between states 1 and 6 and the rearrangement of the empty transporter displaces an intrinsic net negative charge in the membrane field. This transition is bracketed by two proton-bound states (T2 and T7) effectively modulating the T1 ↔ T6 transition (see below).

Extracellular oligopeptides bind to state T2, leading to state T3 and causing a partially voltage-dependent conformational rearrangement from T3 to T4, a state from which the organic substrate is released intracellularly (T5). The subsequent release of H+ leads to the inward-facing, empty transporter (state T6).

The introduction of the protonated state T7 is justified by the need to reproduce the slowing action of external protons on the inward rate of charge movement, observed especially in the fish isoforms (i.e., the transition T6 → T1). External protons "sequester" the transporter in this state, effectively slowing down the return from the inward- to the outward-facing conformation. The transition to state T7 may represent a proton binding to an allosteric site, as has already been suggested, to account for the smaller transport current observed at acidic pH in the zebrafish PepT1.

The set of differential equations describing the complete scheme can be written as:

\[
\begin{align*}
\frac{dT_1}{dt} & = - (k_{12} + k_{14}) T_1 + k_{23} T_2 + k_{41} T_4 \\
\frac{dT_2}{dt} & = k_{12} T_1 - (k_{21} + k_{23}) T_2 + k_{32} T_3 \\
\frac{dT_3}{dt} & = k_{24} T_2 - (k_{32} + k_{34}) T_3 + k_{45} T_4 \\
\frac{dT_4}{dt} & = k_{35} T_3 - (k_{45} + k_{45}) T_4 + k_{55} T_5 \\
\frac{dT_5}{dt} & = k_{45} T_4 - (k_{54} + k_{54}) T_5 + k_{55} T_6 \\
\frac{dT_6}{dt} & = k_{65} T_5 - (k_{65} + k_{65} + k_{65}) T_6 + k_{76} T_7 \\
\frac{dT_7}{dt} & = k_{67} T_6 - k_{76} T_7
\end{align*}
\]

The higher value of \(\sigma\) in the rabbit form suggests that charge movement may occur over a smaller fraction of the membrane electrical field in rabbit than in the two fish species.

The sigmoidal Q/V curve may represent the steady-state distribution of the transporter molecules between two conformations with the center of charge in two different locations of the membrane electrical field. The charge movement process may be described with the simple reaction:

\[
\frac{Q_{\text{in}}}{Q_{\text{out}}},
\]

where \(Q_{\text{in}}\) and \(Q_{\text{out}}\) are the amount of charge at an inner and outer position, respectively, in the membrane electrical field. It is easy to derive \(Q_{\text{in}}\) and \(Q_{\text{out}}\) from the experimental Q/V and \(\tau/V\) curves, because:

\[
\tau = \frac{1}{Q_{\text{in}} + Q_{\text{out}}} \quad \text{and} \quad \frac{Q_{\text{in}}}{Q_{\text{max}}} = \frac{Q_{\text{in}}}{Q_{\text{out}} + Q_{\text{in}}} = \frac{Q_{\text{in}}}{Q_{\text{max}}},
\]

where \(Q_{\text{max}}\) is the maximal moveable charge, obtained from the saturating values of the Q/V sigmoidal curve.

In all transporters, alkalinization sped up both the inward and outward rates, although the effect on this last parameter was much larger (Fig. 2, bottom row). Notably, the voltages at which the in and out rates crossed showed the same shifts as the \(V_{0.5}\) values.

These observations indicate that protonation of unidentified amino acids slows down the rates of the charge-moving conformational changes induced by membrane potential. Indeed, the much slower value of the decay time constant for rbPepT1 may be explained by its higher isoelectric point; at the same pH, it will be protonated to a higher degree than will sbPepT1 and zfPepT1. Its movements in response to voltage changes will occur less frequently for this reason.

Modeling PepT1 presteady-state currents. We devised a kinetic scheme to simulate the observed results—particularly those showing a pH-dependent increase in both unidirectional rate constants. To incorporate the slowing effects of protonation on both unidirectional rate constants, some changes were made to existing models. As shown in Figure 3, the intramembrane charge movement occurs between states 1 and 6 and the rearrangement of the empty transporter displaces an intrinsic net negative charge in the membrane field. This transition is bracketed by two proton-bound states (T2 and T7) effectively modulating the T1 ↔ T6 transition (see below).

Extracellular oligopeptides bind to state T2, leading to state T3 and causing a partially voltage-dependent conformational rearrangement from T3 to T4, a state from which the organic substrate is released intracellularly (T5). The subsequent release of H+ leads to the inward-facing, empty transporter (state T6).

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The set of differential equations describing the complete scheme can be written as:

\[
\begin{align*}
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\frac{dT_3}{dt} & = k_{24} T_2 - (k_{32} + k_{34}) T_3 + k_{45} T_4 \\
\frac{dT_4}{dt} & = k_{35} T_3 - (k_{45} + k_{45}) T_4 + k_{55} T_5 \\
\frac{dT_5}{dt} & = k_{45} T_4 - (k_{54} + k_{54}) T_5 + k_{55} T_6 \\
\frac{dT_6}{dt} & = k_{65} T_5 - (k_{65} + k_{65} + k_{65}) T_6 + k_{76} T_7 \\
\frac{dT_7}{dt} & = k_{67} T_6 - k_{76} T_7
\end{align*}
\]
are the zero-voltage rates in the voltage-dependent transition $T_1 \leftrightarrow T_6$. In the last column the higher value of $\delta = \delta_{in} + \delta_{out}$ for the fish transporter is in agreement with the smaller value of $\sigma$ in Table 1.

Table 2. Model kinetic parameters in absence of substrate

| Rate | $k_{21}$ | $k_{32}$ | $k_{34}^0$ | $k_{45}^0$ | $k_{56}$ | $k_{54}$ | $k_{65}$ | $\delta_{34}$ | $\delta_{56}$ |
|------|---------|---------|------------|------------|---------|---------|---------|-------------|-------------|
| Units | M$^{-1}$s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | M$^{-1}$s$^{-1}$ | M$^{-1}$s$^{-1}$ | s$^{-1}$ |
| Rabbit | 9 x 10$^9$ | 750 | 172 | 10 | 1 x 10$^7$ | 1.5 x 10$^7$ | 5 x 10$^6$ | 400 | 0.45 | 0.26 | 0.71 |
| Fish | 3.5 x 10$^9$ | 1050 | 15 | 1 x 10$^4$ | 1.5 x 10$^7$ | 400 | 0.39 | 0.48 | 0.87 |

$k_{24}^0$ and $k_{56}^0$ are the zero-voltage rates in the voltage-dependent transition $T_3 \leftrightarrow T_4$. $\delta_{in}$ = 1 - $\delta$ represents the fraction of electrical field covered by proton movement before neutralization of the intrinsic negative charge.

where $T_1$–$T_7$ are the probabilities that the transporter is in a given state and the $k$ values are the rate constants, either dependent or independent of concentration and voltage (Tables 2 and 3).

The unidirectional rate constants of Figure 2 were fitted with growing and decaying exponentials in the form $outrate = outrate_0 \exp(\delta_{out} qV/kT)$ and $inrate = inrate_0 \exp(-\delta_{in} qV/kT)$, where $outrate_0$ and $inrate_0$ are the zero-voltage rates; $q$, $k$ and $T$ have the same meaning as in Equation 1; and $\delta_{out}$ and $\delta_{in}$ (with $\delta_{out} + \delta_{in} = \delta$ in Eqn. 1) are the asymmetric fractional dielectric distances.

Rates $k_{21}$, $k_{24}$, $k_{12}$, $k_{56}$, $k_{57}$, and $k_{26}$ were determined based on the effects of voltage and external pH on the unidirectional rate constants of Figure 2. Assuming that the binding/unbinding of protons in transitions $T_1 \leftrightarrow T_2$ and $T_6 \leftrightarrow T_7$ is much faster than in $T_1 \leftrightarrow T_6$, the unidirectional rate constants of charge movement $outrate$ and $inrate$ can be written as:

\[
outrate = f_{21} k_{16}^0
\]
\[inrate = f_{70} k_{61}^0\]

where

\[
f_{21} = \frac{k_{21}}{k_{12} + k_{21}}
\]
\[
f_{70} = \frac{k_{70}}{k_{67} + k_{70}}
\]

are modulating factors that depend on the external proton concentration. Because $k_{21}$ and $k_{70}$ (but not $k_{12}$ and $k_{67}$) increase with acidity, the modulating factors will reduce the values of both $outrate$ and $inrate$.

The values of $k_{21}$ and $k_{70}$ were adjusted to account for the effects of internal pH, assuming a fixed value of 7.5. Because the parameters of the two fish species were similar, they were handled together and a “generalized” fish transporter was modeled (Table 2).

Figure 4 shows the results of the simulations for rabbit and the generalized fish transporter. Clearly, the simulations are in very good qualitative and, for rabbit PepT1, also quantitative agreement with the experimental results shown in Figure 2.

Transport currents of PepT1. The transport currents generated by rabbit, zebrafish and seabass PepT1 have been previously studied. These three isoforms show a similar trend; in all cases, acidic external pH causes substrate affinity to increase, but either decreases or does not affect the maximal current. These features are illustrated in Figure 5 for the rabbit transporter; very similar curves can be observed for the zebrafish and seabass isoforms. In human PepT1, on the other hand, high acidity increases both substrate affinity and $I_{max}$. This effect has also been reported for the zebrafish transporter, but not for the seabass or human transporters.

The decrease in $I_{max}$ at acidic pH in rabbit and fish transporters is unexpected given that the inwardly directed proton electrochemical gradient increases. This effect, however, might be explained by a decreased cycling rate related to the slowing of the charge movement caused by protonation. In the case of inward substrate transport, the transport cycle is circulated only in a clockwise direction, i.e., the transition of the empty transporter occurs in the $T_6 \rightarrow T_1$ direction, showing large pH dependency (Fig. 2, bottom row).

Another interesting feature apparent in Figure 5 is the presence of a small outward current, especially at low substrate concentration and alkaline pH. This effect has also been reported for the zebrafish transporter, but not for the seabass or human transporters.

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A useful index of the overall transport efficiency is given by the ratio $I_{max}/K_{cat}$, which is the slope of the Michaelis-Menten relationship at zero substrate concentration. The largest difference in efficiency, in favor of the more acidic solution in the physiological range of membrane potential, was observed in the rabbit transporter; there was no significant difference in the zebrafish. This is in line with the acidic pH of the small intestine of rabbit but alkaline pH in zebrafish.

Transport current modeling. Kinetic models for human and rabbit PepT1 have been proposed; however, none have been tested thoroughly on rabbit or fish transporters to verify their ability to simulate the main aspects of transporter functioning. Therefore, we tested the presteady-state current model developed above to determine its ability to simulate the characteristics of the
transport-associated currents. Only the essential states necessary to complete the transport cycle were added, namely states T3 and T4, corresponding to the outward- and inward-facing conformations with bound substrate. Because the best fit of the experimentally observed charge movement required transition T1 ↔ T6 to occur over 71% of the membrane electrical field for rabbit and 87% for fish (Table 2), the remaining fractional voltage-dependence for protons was attributed to the T3 ↔ T4 transition.

The additional kinetic parameters necessary to account for substrate binding and translocation are shown in Table 3; the parameters used to simulate the presteady-state currents (Table 2) were not modified. In particular, $k_{23}$ and $k_{32}$ were chosen to give a value of $K_{05}$ consistent with the experimental observations at pH 6.5 and $k_{34}$ and $k_{43}$ were set to be equal to each other at 0 voltage and were significantly faster than the other rates. Similarly $k_{45}$ was set at a high value, while $k_{54}$ was derived from the other rates to satisfy the principle of detailed balance.28

Figure 7 illustrates simulations showing the model’s ability to reproduce the main findings for transport-associated currents in the different species, that is, the effects of external pH on the Michaelis-Menten parameters $I_{\max}$ and $K_{05}$. These parameters were obtained by applying the same procedure used for the experimental data: the $I/V$ curves corresponding to various substrate concentrations (0.03–10 mM) were generated by numerical simulations and then $I_{\max}$ and $K_{05}$ were obtained by fitting the dose-current curves at each potential with a Michaelis-Menten equation. The left column in Figure 7 simulates the rabbit transporter. At alkaline pH, $I_{\max}$ appears to remain constant11 or to slightly increase12 while apparent affinity decreases (higher $K_{05}$). Both experimental observations are reproduced by the model, although the $K_{05}$ simulations are not completely satisfactory (Fig. 5), possibly because “zero trans” conditions were assumed in calculations, whereas substrates can be expected to accumulate in the intracellular compartment near the membrane in real measurements. The central column of the figure represents the generalized fish transporter (Tables 2 and 3). Again, the model reproduced the unique features of this transporter, namely the increase in $I_{\max}$ at alkaline pH and the affinity decrease common to all species. To stress the versatility of the model, we have also tried to simulate an opposite effect on $I_{\max}$, such as the increase observed with acidity in human PepT1:3 this is shown in the right column of Figure 7, together with the usual effect on $K_{05}$. This last series of simulations was obtained using the rabbit kinetic parameters with a single change: a reduction of $k_{67}$ from 5 x 10^8 to 1 x 10^7 (Table 2) is sufficient to produce an opposite effect of pH on $I_{\max}$.

Discussion

PepT1, the oligopeptide transporter responsible for most of the amino acid intake in the intestine of several vertebrates, has been
Figure 5. Transport-associated current from rabbit PepT1. Top row: I/V relationships of the transport-associated currents (substrate present minus substrate absent) at pH 7.5 (A) and 6.5 (B). Data points represent the mean ± SE from six oocytes (two batches). For each oocyte, a normalization to the current at -140 mV in pH 7.5 was performed before averaging. Bottom row: values of $I_{\text{max}}$ (C) and $K_{0.5}$ (D) obtained by fitting the concentration-current relationships from (A and B) with the Michaelis-Menten equation. Bars are the errors from the fitting procedure.

Figure 6. Transport efficiency. Plots of the ratio of relative $I_{\text{max}}/K_{0.5}$ for rabbit (A) from data in Figure 5, seabass (B) from data in reference 10, and zebrafish (C) from our unpublished data. In the physiological range of potentials, efficiency is equal or better in the more acidic pH.
cloned and functionally tested in many species, including human, rabbit, zebrafish, chicken and seabass. Its transport activity has generally been reported to be pH-dependent, with lower external acidic pH having a potentiating effect on the uptake of radioactively labelled substrate. However, electrophysiological results have been contradictory in various species, ranging from an acidity-induced increase in \( I_{\text{max}} \) to no change or a decrease in seabass, zebrafish and rabbit isoforms. More consistent effects are seen in the apparent affinity for substrate, which appears to increase with acidification in all cases.

Although an increase in transport-associated current with lower pH is easily understood as a consequence of a stronger protonic electrochemical gradient, an increase in current at higher pH is harder to explain.

The presteady-state currents of many ion-coupled transporters in the absence of organic substrate arise from the ion-transporter interaction and are qualitatively and quantitatively related to transport activity. Therefore, we analyzed in parallel the properties of such currents in three isoforms showing partially different pH dependency, with the aim of gathering additional information to explain the observed differences.

PepT1 is capable of transporting neutral as well as negatively or positively charged oligopeptides. In this study, which compared the behavior of PepT1 isoforms from different species, only the neutral dipeptide Gly-Gln was used to avoid excessive complications in the system. Subsequent studies should overcome this limitation.

**Comparison of the presteady-state currents in the different species.** An analysis of the presteady-state currents with respect to the voltage dependence of the decay time constant and the amount of displaced charge showed that the two types of curves are positioned differently (at the same external pH) on the voltage axis. The rabbit curves are positively shifted and the zebrafish curves are negatively shifted, with the seabass curves located close to the zebrafish ones. In addition, the decline in the presteady-state current following voltage jumps is faster in the zebrafish and seabass transporters than in the rabbit isoform. These results are in good agreement with the different isoelectric points of the three proteins: 7.47, 6.68 and 6.00 for rabbit, seabass and zebrafish, respectively (reviewed in ref. 25 and our calculation). This suggests that rabbit PepT1 may be protonated more easily than the fish isoforms. If this protonation includes the residues involved in establishing states T2 and T7 in our model, it may explain the slower rates of charge movement observed at the same pH in the rabbit protein.

**Effect of external pH on unidirectional rates.** As previously reported for the rabbit and seabass transporters, the charge movement characteristics of all three proteins are affected.
by external pH through shifts along the voltage axis and an acceleration of the decay at alkaline pH. In the present work, we extended the analysis to calculate the unidirectional rate constants of charge movement in the membrane electrical field and their dependence on external pH.

The results, shown in Figure 2, indicate that an increase in external protons slows down both inward and outward charge movements in all of the examined species. These observations make it unlikely that the presteady-state currents are primarily caused by the movement of external protons in the membrane electrical field and instead suggest that the currents are mostly due to the rearrangement of intrinsic transporter charges. These results are in agreement with models proposed for rabbit and for human PepT1, in which the presteady-state currents are generated largely by the movement of intrinsic transporter charges (negative, on the whole). In these models, inward and outward rates are slowed down by the binding of internal and external protons that trap the transporters in “occluded” states.

Because these models do not account for the slowing down of the inward charge movement by external protons, we modified them by adding a new state (T7 of Fig. 3) in which the “inward facing” transporter can bind external protons. Trapping of the transporter in state T7 by external protonation may then explain the decrease in inward rate of (positive) charge movement (transition T6 → T1) caused by acidic pH. The voltage- and pH-dependence of the unidirectional rates of charge movement was best fitted with a single conformational change of the intrinsically charged transporter over 71% (rabbit) or 87% (fish) of the membrane electrical field. For this reason, a partial voltage dependence encompassing the residual fraction of the field was introduced in the transition between the outward- and inward-facing conformations of the fully loaded transporter (T3 ↔ T4).

The ability of the model to reproduce the voltage dependence of the displaced charge and the decay time constant, as well as the effects of changing external pH, was verified by simulating the presteady-state currents in the rabbit and a generalized fish PepT1 using kinetic parameters derived from experimental observations. The results in Figure 4 show that the kinetic scheme of Figure 3 is capable of reproducing the properties of both the rabbit and fish PepT1 with only a minor adjustment of the parameters (Table 2).

Transport currents. The model of Figure 3 was further tested to verify its ability to simulate the transport-associated currents. The comparison was limited to the currents generated by the neutral dipeptide Gly-Gln at pH 6.5 and 7.5, which should represent the physiological conditions of the unstirred microenvironment in which PepT1 operates, at least in mammals. Because the model was constructed from observations of the presteady-state currents and the parameters were optimized to simulate the characteristics of these currents, the behavior of the transport currents cannot be completely reproduced for two main reasons. First, different substrates may present specific interactions with the transporter that are difficult to incorporate in the model. Secondly, the model does not include the effects of the variable percentage of charged vs. uncharged species of substrate at different pH. In spite of these drawbacks, Figure 7 shows that simulations of mammalian and fish PepT1s using the additional parameters of Table 3 can reproduce the basic experimental observations. There is less agreement than that obtained for the presteady-state currents and it is mostly limited to qualitative aspects such as changes in the relative amplitudes of $I_{\text{max}}$ at different pH values and the common higher affinity at the more acidic pH. The general trend of the voltage dependence of affinity is mimicked, although there are significant quantitative discrepancies.

As mentioned in the Introduction, the pH dependence of the maximal transport current of human PepT1 is the opposite of that of rabbit and fish. In an analysis by Fujisawa and coworkers, the pH dependence of human PepT1 was bell-shaped, with maximal transport rate at pH 5.5. Although the decreasing transport at high pH values is caused by a reduced transmembrane pH gradient, the decrease at low pH results from a change in the transporter protein itself caused by protonation of a histidine residue near the substrate binding domain. The model presented in this work similarly predicts that $I_{\text{max}}$ should decrease at both extreme acidic and alkaline pH. Depending on the pH at which the maximal transport rate occurs and the width of the bell-shaped $I_{\text{max}}$ vs. pH curve (possibly related to slight structural differences in the functionally important regions; see Introduction), this feature can account for all observed species-specific differences in the pH dependencies, including the increase in $I_{\text{max}}$ with acidity found in the human PepT1. The observed differences in the pH dependencies may be related to the actual pH values found in the small intestine. Although slightly acidic values are found in mammals because there is no luminal expression of the sodium-proton exchanger (NHE3), the microenvironment of zebrafish enterocytes should be alkaline, thus allowing optimal conditions for peptide transport. Interestingly, the type-II Na+-phosphate cotransporter cloned from the zebrafish intestine also exhibits maximal transport activation at alkaline extracellular pH.

Conclusions. This paper proposes a unified kinetic scheme that explains the main electrophysiological properties of PepT1 transporters in the presence and absence of organic substrate and their pH dependence. The model also can describe the behavior of different isoforms from mammalian and fish species.

Interestingly, the present results and analysis suggest a dual role for protons in the operation of PepT1. Protons are fundamental to neutralizing the transporter during the inward substrate translocation and their release in the cytosol uncovers the net negative charge of the empty transporter, which then undergoes an energy-dissipating working stroke to return to the outward-facing conformation (transition T6 → T1 in Fig. 3). However, protonation of the transporter breaks the transport cycle and counteracts the potentiating effects of external acidity on the turnover rate. As demonstrated by our model, a different balance between the two roles played by protons may generate opposite effects on the maximal transport velocity, as has been experimentally observed in human PepT1 vs. rabbit or fish transporters.

The existence of two apparently contrasting actions of external protons does not negatively impact the overall efficiency of substrate uptake, because turnover rate and affinity are generally inversely related in transport processes. Indeed, using the ratio of $I_{\text{max}}$ to $K_{50}$ as an index of the efficiency of transport, both experimental data and our model show that PepT1 is well optimized.
to meet its function across species and expected physiological pH conditions.

**Materials and Methods**

**Oocyte expression.** To prepare the mRNA for oocyte injection, the cDNA encoding PepT1 transporters, cloned into the pSPORT-1 vector (Invitrogen), was linearized with NotI for rbPepT1 and with HindIII for sb and zf PepT1. Subsequently, cRNA was synthesized in vitro in the presence of Cap Analog and 200 units of T7 RNA polymerase. All enzymes were supplied by Promega Italia, Milan, Italy.

*Xenopus laevis* frogs were anesthetized in MS222 (tricaine methansulphonate) 0.10% (w/v) solution in tap water and portions of the ovary were removed through an abdominal incision. The experiments were performed according to institutional and national ethical guidelines. The oocytes were treated with collage-

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pher to its function across species and expected physiological pH conditions.

**Electrophysiology and data analysis.** Measurements of the currents generated by the transporters in controlled voltage conditions were performed using the classical two-electrode voltage clamp (TEVC) technique (GeneClamp, Axon Instruments Foster City, CA USA or Oocyte Clamp OC-725B, Warner Instruments, Hamden, CT USA). Glass microelectrodes, filled with KCl 3 M and with tip resistances between 0.5 and 2 MΩ were used. Bath electrodes were connected to the experimental chamber via agar bridges (3% agar in 3 M KCl). Voltage pulses to test potentials from -140 to +20 mV were applied in 20 mV increments from a -60 mV holding potential. The current signal was filtered at 1 kHz before sampling at 2 kHz.

Two methods were used to isolate the presteady-state currents resulting from the presence of the transporter from the endogenous capacitive transients: (i) the transient currents recorded in response to membrane voltage jumps were fitted with the sum of two exponentials, a fast one attributed to the charging of the oocyte membrane linear capacity and a slow one attributed to the charge movement associated with rearrangement of the transporter; and (ii) currents in the presence of saturating substrate were subtracted from those in its absence at the same potentials, followed by zeroing of the steady transport currents. The result of measuring time function was assumed to represent the intramembrane charge movement and was fitted with one exponential. These two methods have been shown to give substantially equivalent results. The fit results were used to calculate the potential dependency of the time constant (τ/V) and of the charge movement (Q/V).

Data were analyzed using Clampfit 8.2 (Axon Instruments) and figures were prepared with Origin 5.0 (Microcal Software Inc., Northampton, MA USA). Numerical simulations were performed using Berkeley Madonna 8.3.14 (Berkeley Madonna Inc.).

**Solutions.** The external control solution had the following composition (mM): NaCl, 98; MgCl₂, 1; and CaCl₂, 1.8. Heps 5 mM was used to buffer the solution to pH 7.5 and MES 5 mM was used to buffer to pH 6.5. The final pH values were adjusted with HCl and NaOH. Gly-Gln dipeptide at 3 mM was used as the organic substrate for saturating transport activity.

Experiments were conducted at room temperature (20–25°C).

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