Salt Bridge Swapping in the EXXERFXY Motif of Proton-coupled Oligopeptide Transporters*

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Proton-coupled oligopeptide transporters (POTs) couple the inward transport of di- or tripeptides with an inwardly directed transport of protons. Evidence from several studies of different POTs has pointed toward involvement of a highly conserved sequence motif, E1XERFXY (from here on referred to as E1XXER), located on Helix I, in interactions with the proton. In this study, we investigated the intracellular substrate accumulation by motif variants with all possible combinations of glutamate residues changed to glutamine and arginine changed to a tyrosine, the latter being a natural variant found in the Escherichia coli POT YjdL. We found that YjdL motif variants with E1XXER, E1XXER, E2XXEY, or Q1XXEY were able to accumulate peptide, whereas those with E1XXQXR, Q1XXER, or Q1XXQ2Y were unable to accumulate peptide, and Q1XXQ2R abolished uptake. These results support a mechanism that involves swapping of an intramotif salt bridge, i.e. R-E1 to R-E1, which is consistent with previous structural studies. Molecular dynamics simulations of the motif variants E1XXER and E1XXQXR support this mechanism. The simulations showed that upon changing conformation arginine pushes Helix V, through interactions with the highly conserved FYING motif, further away from the central cavity in what could be a stabilization of an inward facing conformation. As E2 has been suggested to be the primary site for protonation, these novel findings show how protonation may drive conformational changes through interactions of two highly conserved motifs.

Peptide uptake energized by a proton electrochemical gradient is present in all kingdoms of life. In most organisms uptake of these vital nutrients is facilitated by proton-coupled oligopeptide transporters (POTs)3 (Transporter Classification Database number 2.A.17). Di- and tripeptides (1–5) and in rare cases single amino acids (6) are natural POT substrates. Several animal POTs have shown ability to take up a number of drugs such as angiotensin-converting enzyme inhibitors and prodrugs exemplified by valacyclovir (7–9). In plants, the substrate specificity of POTs has diversified to include nitrate (10), dicarboxylates (10), glucosinolates (11), and plant hormones such as auxin (12, 13). Thus, as a family, the POTs cover an exceptionally wide substrate space. The three-dimensional structures of POTs follow the fold observed for other major facilitator superfamily members (14–17). A large cavity is located between two 6-helix domains (Fig. 1A), which interact to form an outside occluded or inside facing conformation (Fig. 1B). Mutagenesis and substrate analogue studies on bacterial (18–20), plant (21, 22), fungal (23), and mammalian (9, 24, 25) POTs pinpoint important regions with respect to substrate recognition and affinity. Recent advances in the determination of three-dimensional structures (14, 15) have verified these observations and added novel details to the POT mechanism. Collectively, these studies show that peptide termini are important for peptide affinity (14, 17, 26), and primarily residues of the N-terminal domain bind the C-terminal carboxylate group, whereas residues from both N- and C-terminal domains bind the N-terminal amino group. Peptide side chain specificity, if present, is determined by residues from the C-terminal domain (14, 17, 26).

Compared with peptide recognition, much less is known about residues important for interactions with the proton and for conformational transitions accompanying proton and peptide binding. Several well characterized major facilitator superfamily members are proton-coupled; however, they do not depict consensus in proton binding and coupling mechanisms (27). POTs are identified by the presence of two sequence motifs that are highly conserved: the E1XXER located on Helix

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3The abbreviations used are: POT, proton-coupled oligopeptide transporter; MD, molecular dynamics; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propan-1,3-diol; β-Ala-Lys(AMCA), β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid; pHw, outer pH; Gk, G. kaustophilus.
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**FIGURE 1. Overall architecture and conformations of POTs.** A, crystal structure of the inward open PepTSo{Ala–Ala–Ala complex (Protein Data Bank code 4TPJ) (42). The N- and C-terminal domains are colored white and blue, respectively. Side chains shown as sticks correspond to the E1XXE2RXXY motif (green) and the FYING motif (wheat). The substrate is represented as yellow sticks. B, schematic representation of possible conformational changes in POTs during proton/peptide translocation; the proton is represented as a black sphere with spikes, and the peptide is represented as a black rhombus. C, multiple sequence alignment of E1XXE2RXXY and FYXXINXXG motifs using mafft from GkPOT (Q5KPYD1), YjdL (P39276), YdgR (P77304), YbgH (P75742), hPepT1 (P46059), PepTSo (Q8EHE6), PepTSt (QSM4H8), and NRT1.1 (Q05085). I (Fig. 1A) and the FYXXINXXG motif (from here on referred to as FYING) located on Helix V (14, 15, 28). Some evidence has linked E2XXE2R in particular E2 to interaction with the co-transported proton (15, 29). To understand the role of E2XXE2R in greater detail, we engineered this motif by introducing consensus residues into a naturally occurring unusual motif variant found in YjdL, a POT from *Escherichia coli*. The transport capabilities of the resulting proteins were investigated by accumulation assays in *E. coli* and interpreted in the context of available POT structures and complementary molecular dynamics (MD) studies. Collectively, the results imply that a prototypical POT requires both E1 and E2 to facilitate substrate transport and that this proceeds via a mechanism that involves protonation triggered salt bridge swapping in the E1XXE2R motif. Furthermore, the rearrangement in E1XXE2R seems to induce a larger structural change as a result of cross-talk with the FYING motif.

**Materials and Methods**

**Protein Expression**—All mutated variants of YjdL and YdgR were synthesized and subcloned by GenScript except YjdL Q14XXQ2 (15, 29). Overexpression of transporter variants, all carrying a C-terminal His6 tag, was performed as described previously (6, 18, 20, 29, 30). Briefly, plasmids pTTQ18-yjdL/ydgR and their mutants were transformed into *E. coli* BL21 (DE3)pLysS cells. A single isolated colony of transformants was inoculated in 3 ml of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and incubated overnight. The overnight cultures were diluted to 1:50 in 10 ml of LB medium containing similar antibiotics as mentioned earlier. The cultures were grown to an A600 of 0.6–0.8 and induced by addition of 1 mM isopropyl β-d-1-thiogalactopyranoside. The cells were harvested after 3 h by centrifugation.

**Western Blotting**—Western blotting was performed as described previously (20, 29, 30). Briefly, harvested cells were resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, 1% N-dodecyl-β-d-maltopyranoside, pH 7.5 supplemented with one Complete protease inhibitor tablet (Roche Applied Science)/10 ml) to an A600 of 10. The cell suspension was incubated on ice for 30 min followed by sonication and immediate centrifugation at 12,600 × g for 15 min at 4 °C. The clarified, solubilized lysate was then separated by SDS-PAGE (NuPAGE® Novex® 10% Bis-Tris gel). The gel was blotted onto a PVDF membrane using an XCell II module (Invitrogen), and then the blotted membrane was incubated with blocking buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 3% bovine serum albumin (BSA), 0.5% Tween 20) at 4 °C overnight. The membrane was incubated for 1 h each at room temperature with mouse anti-His6 and HRP-conjugated rabbit anti-mouse antibodies (IBA) followed by SuperSignal® West Pico chemiluminescent substrate (Pierce). The signals were detected using a MicroChem imaging system (DNR Bio-Imaging Systems). Band quantification was performed using ImageJ.

**Accumulation Assay**—The uptake assay was performed as described previously (15, 30–32). Briefly, the harvested cells were resuspended in uptake buffer containing 50 mM MES, 50 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose, pH 5.5, 6.5, or 7.5, to an A600 of 10. 900 μl of the cell suspension was added to 100 μl of β-Ala-Lys-N’-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys(AMCA)) at a final concentration of 0.5 mM. At regular time intervals (1, 3, 5, 8, 10, 15, 20, and 30 min), 100 μl of the cell suspension was collected and added to 500 μl of ice-cold uptake buffer to discontinue uptake. Cells were spun down rapidly, and the pellet was washed twice with 200 μl of ice-cold uptake buffer. The pellets were resuspended in 100 μl of uptake buffer, and the fluorescence was measured on a Safire 2 fluorometer (excitation at 340 nm and emission at 460 nm). Background uptake ability was measured using *E. coli* BL21(DE3)pLysS cells harboring the pTTQ18 vector. Assays were repeated three times,
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and data were analyzed using GraphPad Prism (18, 20, 29). To estimate the level of accumulation, the concentration of the \(\beta\text{-Ala-Lys(AMCA)}\) inside the cell was calculated by using OD-specific total cell volumes of 3.6 \(\mu\text{L}\cdot\text{ml}^{-1}\cdot\text{OD}^{-1}\) (33). Saturation ratios were estimated by extrapolating from hyperbolic curve fitting. If the \([\text{substrate}_{\text{in}}]/[\text{substrate}_{\text{out}}]\) ratio is greater than 1, we refer to it as accumulation. If it is below 1 and greater than background, we refer to it as uptake. The ratios of the variants that are referred to as accumulating are significantly different from those referred to as non-accumulating \((p < 0.05)\). Variants that are able to perform uptake as described above are significantly different from uptake by empty vector \((p < 0.05)\).

Molecular Dynamics Simulations and Analyses—The Schrödinger Software Release 2013-2 (34) was used to prepare the protein and run the MD simulations. The Protein Preparation Wizard was used to prepare Protein Data Bank code 4IKW for the MD simulations (35). All waters were kept during the preprocessing step. Missing side chains were added, and the buffer molecules were deleted. Hydrogen bond assignment was done using default settings, except that a pH of 6.5 was used for PROPKA. Finally, the positions of hydrogen atoms were minimized (35–37). The E35Q mutation was done in Maestro using this prepared protein structure. In addition, the protein structure with a negatively charged Glu\(^{32}\) was kept unprotonated. Glu\(^{413}\) was the only residue that was modeled in its non-default protonated state. The MD simulations in Desmond (version 3.5) were set up using the System Builder tool available in Maestro (38–40). The protein was placed automatically in a 1-palmitoyl-2-oleoylphosphatidylcholine membrane where the transmembrane atoms were positioned based on the helices. Otherwise, the system was built using default settings, \(i.e.\) with SPC water molecules and neutralized. The OPLS 2005 force field was applied for both a subsequent minimization with 2000 steps and the MD run. The equilibration was carried out using the standard equilibration protocol. In brief, the system was first minimized with restraints on solute and then without any restraints. The system was heated to \(T = 10\,\text{K}\) in the NVT ensemble with restraints on heavy atoms of the solute in 12 ps. Subsequently, 12 ps was run at \(T = 10\,\text{K}\) in the NPT (constant number of molecules, pressure, and temperature) ensemble with the same restraints. In the next step, 24 ps was run at \(T = 300\,\text{K}\) in the NPT ensemble still with restraints on the heavy atoms of the solute. Finally, 24 ps without any restraints were conducted. The MD was run using default settings, \(i.e.\) \(T = 300\,\text{K}\) and \(P = 1.01325\) bars in the NPT ensemble. The production run (100 ns) was initiated after another 5 ns of equilibration. Only the first 35 ns of the production run are shown in the figures as the salt bridge swap occurred between 10 and 20 ns.

Results

YjdL exhibits functional characteristics that are typical for POTs, \(i.e.\) proton-motive force-driven di- and tripeptide transport (25), despite having an unconventional \(E_1\)XXE\(_2\)Y motif, which reads Q\(_4\)XXE\(_2\)Y. Intracellular accumulation of di- and tripeptides by POTs can be considered as a characteristic feature of their activity and is directly linked to the ability of utilizing the electrochemical proton gradient. To test whether YjdL-WT was able to accumulate substrate and to find the most optimal outer pH (\(\text{pH}_o\)) values, uptake of a non-hydrolyzable fluorescent dipeptide \(\beta\text{-Ala-Lys(AMCA)}\) (41) was followed at \(\text{pH}_o\) 5.5, 6.5, and 7.5, respectively, for 30 min, and the fluorescence values were converted to a \([\text{substrate}_{\text{in}}]/[\text{substrate}_{\text{out}}]\) ratio. A premise to perform this calculation is that no quenching of \(\beta\text{-Ala-Lys(AMCA)}\) fluorescence occurs inside the cells. To test this phenomenon, cells that had taken up \(\beta\text{-Ala-Lys(AMCA)}\) were lysed, and the cleared lysate was measured for fluorescence and compared with non-lysed cells. No significant fluorescence quenching of \(\beta\text{-Ala-Lys(AMCA)}\) was observed (Fig. 2A). Accordingly YjdL-WT was able to accumulate substrate to 10, 11, and 5 at a \(\text{pH}_o\) of 5.5, 6.5, and 7.5, respectively (Fig. 3A). Hyperbolic curve fitting of the data suggested that accumulation of YjdL-WT would apparently saturate and achieve the highest level of accumulation at a ratio of 18.3 at \(\text{pH}_o\) 5.5 (compared with 6.5 and 7.5; \(p < 0.05\)). Because of this observation, all subsequent accumulation studies by various motif variants are presented primarily at \(\text{pH}_o\) 5.5. All variants were found to be expressed as judged from three independent Western blots (Fig. 2B).

\(E_1\), and \(E_2\), Have No Significant Influence on Substrate Accumulation in Q\(_4\)XXE\(_2\)Y—Having established that YjdL-WT is able to accumulate peptide substrate, we next aimed at investigating whether motif variants in which E residues were changed to Q or vice versa while keeping the Y position unchanged were affected in terms of substrate accumulation. The variants Q\(_4\)XXQ\(_2\)Y, E\(_1\)XXQ\(_2\)Y, and E\(_2\)XXE\(_2\)Y were tested, and the foremost objective of these analyses was to investigate whether a
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Both $E_1$ and $E_2$ Are Essential for Accumulation in a Conventional $E_1XXE_2$—Single site changes in the YjdL motif $Q_1XXE_2$ of $Y$ to $R$ and $Q_1$ to $E$ resulted in the conventional $E_1XXE_2$ variant. This motif variant was able to reach an accumulation level of 1.9 at 30 min and saturation ratio of 4.8, thus retaining the ability of substrate accumulation (Fig. 3E and Table 1). To analyze the role of ionizable residues in $E_1XXE_2$, all motif variants in which $E_1$ and/or $E_2$ was changed to $Q$, i.e. $Q_1XXE_2R$, $Q_1XXE_2$, and $Q_1XXQ_2R$, were tested. $E_1XXQ_2R$ and $Q_1XXE_2R$ variants exhibited significant uptake ratios of 0.7 and 1.4, respectively, after 30 min and saturation ratios of 1.1 and 1.5, respectively; thus, they were unable to accumulate (Fig. 3F and G, and Table 1). The $Q_1XXQ_2R$ variant incubation reached a ratio of 0.2 at 30 min and saturated at ratio 0.3, which was barely different from background (Fig. 3H and Table 1). Taken together, when arginine replaced tyrosine in the YjdL motif, accumulation was only observed in the presence of $E_1$ and $E_2$ simultaneously. When only $E_1$ or $E_2$ was present, an uptake significantly different from the background but without accumulation was observed. The absence of both glutamates from the motif abolishes transport.

In summary, our data show that despite having $Q_1XXE_2$ instead of an $E_1XXE_2$ motif YjdL readily accumulates substrate. Introduction of a conventional motif into YjdL retains its ability to accumulate. Although $E$ to $Q$ changes in $Q_1XXE_2$ only affect the level of accumulation, in $E_1XXE_2$, both the $E$ residues are strictly required simultaneously to retain the ability to accumulate (Fig. 3). The latter observation points toward interactions between $R$ and both $E$ residues in the motif and may provide important details of the function of the motif in POTs. Considering that $E_2$ has been suggested to be involved in protonation (15, 29), it can be speculated that $R$ would be able to shift conformation and interact with $E_1$ according to the protonation state of $E_2$.

Arginine Is Able to Change Conformation and Interact with $E_1$—To gain a detailed understanding of the conformational mobility of residues in the $E_1XXE_2$ motif, a GkPOT (Geobacillus kaustophilus) structure (Protein Data Bank code 4IKW) embedded in lipid bilayer was investigated by MD simulations. Two simulations were performed using a GkPOT structure with the prototypical $E_1XXE_2$ and the same structure with the motif changed to $E_1XXQ_2R$. The distances between $R$ and $E_1$ and $E_2$, respectively, were monitored during the MD simulations. Arginine maintained a close interaction with $E_2$ in the $E_1XXE_2$ simulations (Fig. 4A). However, in the $E_1XXQ_2R$ simulation, arginine shifted its conformation toward $E_1$ to form a salt bridge (Fig. 4B). Arginine is in contact (within a 5-Å distance) with Glu$^{32}$ ($E_1$), Glu$^{35}$ ($E_2$), and Ile$^{165}$ in GkPOT and most bacterial POT structures. Ile$^{165}$ belongs to the FYING motif. During the conformational shift of arginine in the $E_1XXQ_2R$ simulation, the interactions with Ile$^{165}$

![FIGURE 3. Accumulation profiles of motif variants $Q_1XXE_2Y$ (A), $E_1XXQ_2Y$ (B), $E_1XXE_2Y$ (C), $Q_1XXQ_2Y$ (D), $E_1XXE_2$ (E), $E_1XXQ_2$ (F), $Q_1XXE_2$ (G), and $Q_1XXQ_2$ (H). The ratio of substrate accumulated inside the cell with respect to the substrate concentration outside the cell was plotted as a function of time. The assay was performed at pH 5.5 (open squares), 6.5 (open circles), and 7.5 (open triangles). The closed symbols represent empty vector. The error bars indicate the S.E. ($n = 3$).](image-url)

given variant would be able to accumulate substrate. These variants were able to reach a ratio of 1.8, 6.8, and 9.4 respectively, after 30 min of incubation, and saturated (extrapolated from hyperbolic curve fitting) at ratios of 6.8, 10.4, and 16.8, respectively, thus maintaining an activity level that led to substrate accumulation (Fig. 3, B–D, and Table 1). Thus in the presence of tyrosine, accumulation is observed for all motifs; however, $E_1$ and particularly $E_2$ enhance the level of saturation. The importance of $E_2$ is even more evident at pH 5-6.5, where a saturation ratio of approximately unity was observed for $Q_1XXQ_2$, thus showing inability to accumulate substrate (Fig. 3D). Interestingly, $E_1$ was able to restore accumulation at either pH 5.5 or 6.5 in the absence of $E_2$ (Fig. 3, B–D, and Table 1).

Arginine Is Able to Change Conformation and Interact with $E_1$—To gain a detailed understanding of the conformational mobility of residues in the $E_1XXE_2$ motif, a GkPOT (Geobacillus kaustophilus) structure (Protein Data Bank code 4IKW) embedded in lipid bilayer was investigated by MD simulations. Two simulations were performed using a GkPOT structure with the prototypical $E_1XXE_2$ and the same structure with the motif changed to $E_1XXQ_2R$. The distances between $R$ and $E_1$ and $E_2$, respectively, were monitored during the MD simulations. Arginine maintained a close interaction with $E_2$ in the $E_1XXE_2$ simulations (Fig. 4A). However, in the $E_1XXQ_2R$ simulation, arginine shifted its conformation toward $E_1$ to form a salt bridge (Fig. 4B). Arginine is in contact (within a 5-Å distance) with Glu$^{32}$ ($E_1$), Glu$^{35}$ ($E_2$), and Ile$^{165}$ in GkPOT and most bacterial POT structures. Ile$^{165}$ belongs to the FYING motif. During the conformational shift of arginine in the $E_1XXQ_2R$ simulation, the interactions with Ile$^{165}$

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appeared to be maintained. This resulted in stabilization of Helix V residues Arg154–Pro173 in a conformation shifted away from the center of binding site (Fig. 5). Taken together, the MD simulations show that arginine is able to move away from E2 when E2 is changed to a neutral Q and form a salt bridge with E1 instead. This salt bridge swap stabilizes Helix V in an altered conformation.

**Discussion**

Recent advances in three-dimensional structure determination of POTs have opened up rationalization of functional and mutagenesis studies conducted on POTs. E1XXE2R is located in
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FIGURE 6. Schematic representation of salt bridge swapping in the E$_1$XXE$_2$R motif shown in GkPOT corresponding to the presence of substrate Ala-Ala and proton (red sphere). A, absence of both substrate and proton. B, presence of Ala-Ala alone. C, presence of both substrate and proton.

close proximity of the substrate-binding pocket in all structures (Fig. 1A). In most POT structures with a prototypical E$_1$XXE$_2$R motif that have been crystallized at near neutral pH (14, 26, 28), arginine is found to interact with E$_2$ potentially through a salt bridge. Among the motif variants that we tested, such an interaction would be possible in Q$_1$XXE$_2$R and E$_1$XXE$_2$R; however, only E$_1$XXE$_2$R was able to accumulate substrate. A different arginine conformation within the motif found at low pH in the structure of nitrate/auxin transporter NRT1.1 (43) showed an interaction between arginine and E$_1$. Such an interaction would be possible in motif variants containing E$_1$ and arginine, i.e. E$_1$XXQ$_2$R and E$_1$XXE$_2$R motifs; however, E$_1$XXQ$_2$R was not able to accumulate. Taken together, our data combined with current structural knowledge strongly suggest that substrate accumulation among POTs with a conventional E$_1$XXE$_2$R motif is only possible when arginine is able to swap between E$_1$ and E$_2$. Protonation of E$_2$ has been suggested to be part of the proton translocation mechanism by previous investigations of POTs from different organisms (26, 29, 44). Therefore, it is plausible that the arginine swapping is prompted by protonation of E$_2$. It should also be mentioned here that introducing a plausible that the arginine swapping is prompted by protonation state of the motif as all tested motif variants with tyrosine in the interhelical loop. In these structures, the arginine of E$_2$, mimicking E$_1$XXQ$_2$R, but not E$_1$XXE$_2$R.

The presence of tyrosine instead of arginine in the YjdL motif (Q$_1$XXE$_2$Y) suggests a different mechanism of sensing the protonation state of the motif as all tested motif variants with tyrosine present were able to accumulate at pH=5.5. The importance of E$_2$ is evident from the fact that Q$_1$XXQ$_2$Y is not able to accumulate at pH=6.5 and suggests a role in the proton translocation pathway as discussed above. However, E$_2$ is not strictly required for accumulation as E$_1$XXQ$_2$Y is able to restore it.

Recently Zhao et al. (44) reported motif mutations in E. coli YbgH, which is the closest homologue to YjdL with an identical motif. Some of their results are consistent with those presented here. However, E$_1$XXE$_2$Y and E$_1$XXE$_2$R were found to be completely inactive; both are clearly active in our experiments. These discrepancies may be due to differential experimental parameters; in particular, the substrate used by Zhao et al. (44) was not a peptide.

Implications for POTs with a Prototypical E$_1$XXE$_2$R Motif—Based on our results and previous structural and mutational studies, we propose the following events leading to the formation of POT-peptide-proton complex. (i) In an empty transporter, arginine is bound to E$_2$, and E$_1$ may be bound to a nearby lysine residue (Figs. 6A and 7A). (ii) Upon peptide binding, arginine becomes sandwiched between the carboxyl group of E$_2$ and the peptide C terminus (Fig. 6B). These interactions are supported by the crystal structure of PepT$_{St}$ in complex with the dipeptide AF (17) (Fig. 7A). Furthermore, it has been suggested that peptide binding occurs prior to proton binding (44). Interaction with the peptide C terminus along with the possibility of formation of an arginine-E$_1$ salt bridge, would in itself lead to weakening of the arginine-E$_2$ interaction, enhancing the proclivity for E$_2$ protonation. (iii) The nearby lysine would subsequently form a salt bridge with the peptide C terminus upon the protonation-induced swapping of the arginine to E$_1$ (Fig. 6C). This lysine is highly conserved throughout most POTs regardless of substrate specificity (18, 29) and thus can be expected to be part of a fundamental mechanism. Direct interactions between this lysine and the C terminus have been observed in the PepT$_{St}$:AF complex (17, 18) (Fig. 7A), and functional analyses of this lysine have shown that it is critical for recognition of the peptide carboxyl group (18). In the mammalian peptide transporters, for example hPepT1, His$_{57}$ has been suggested to be a proton-binding site, which binds the proton prior to peptide binding (45). We therefore speculate that in mammalian peptide transporters this histidine is protonated until the peptide has been bound after which the proton is released onto E$_2$.

Conformational Changes—A global conformational change that exposes a single substrate-binding site alternatingly to each side of the membrane is a hallmark of secondary transporters. To trigger such conformational changes in POTs, the proton and peptide have to be bound simultaneously. MD simulations of peptide-bound as well as apo-GkPOT have shown that the inward facing structures equilibrate in an overall occluded conformation (14) primarily by movement of Helices IV and V and the interhelical loop. In these structures, the arginine of E$_1$XXE$_2$R maintains a salt bridge to E$_2$. Our MD simulation, based on an unliganded GkPOT structure with an E$_1$XXQ$_2$R motif (E35Q variant), prompted a shift of the arginine conformation to form interactions with E$_1$ (Fig. 4). Simultaneously, we observed an ~5-Å shift of Helix V away from the central cavity,
thus resulting in a more open inward facing structure (Fig. 5). A
similar conformation is observed in the crystal structure of
NRT1.1 (43) where Helix V adopts a position as in the
E1XXQ2R-containing GkPOT structure during the MD simu-
lation (Fig. 7 B). Observing a conformational change during the
MD simulation indicates that the initial system is conforma-
tionally unfavorable and that it prefers to move toward a more
stable conformational state. Based on these observations, we
hypothesize that the arginine-E1 salt bridge (protonated E2), as
observed in the GkPOT MD simulation (Fig. 5), favors an
inward open conformation, whereas the arginine-E2 salt bridge
(deprotonated E2) favors the occluded conformation as
observed previously (14) or even the outward open conforma-
tion (Fig. 5).

Several residues of the E1XXE2R motif are in close contact
with residues from the FYING motif located on Helix V (Fig.
1A). The importance of the FYING motif has not yet been fully
established. However, the tyrosine of this motif was found to be
essential for hPepT1 activity (46). An underlying feature of both
motifs is that they are conserved among most POTs despite
differing substrate specificities (47) and therefore may have a
more fundamental role in the transport cycle. Coupling of the
salt bridge swap and concomitant movement of Helix V is due
to the tight interaction between R of the E1XXE2R motif and
residues of the FYING motif; in particular, the interaction
between E1XXE2R and FYING isoleucine can be
highlighted (Fig. 5). It can be speculated that this tight interac-
tion between E1XXE2R and FYING ensures active proton-cou-
pled transport by sensing the proton and translating this signal
into conformational changes. In YjdL and its closest homologue
YbgH where arginine is replaced by tyrosine, isoleucine is
replaced by glycine to accommodate the bulk of the tyrosine
side chain. Changing this glycine to valine in YjdL, as found in
the well characterized prototypical POT E. coli YdgR, results in
complete lack of activity; a change of valine to glycine in YdgR,
however, is tolerated, although some activity is lost (Fig. 7C).

Another noteworthy feature of arginine is that it interacts
tightly with Tyr39 and Tyr40 (GkPOT numbering) through van

![FIGURE 7. A, hydrogen bonding network in the PepT53/AF complex (Protein Data Bank code 4D2C) (17) between the E1XXE2R residues in Helix V and Lys126 in PepT53 (wheat) and the dipeptide AF (white). The amino acid residue numbering corresponds to PepT53, followed by GkPOT and YjdL. B, superposition of Helices I and V from the 35-ns E35Q-GkPOT (E1XXQ2R) structure in green and NRT1.1 protomer B (Protein Data Bank code 4OH3) (43) in wheat, respectively. Arginine is shown as sticks. C, accumulation profiles of V159G-YdgR (open squares), G146V-YjdL (open circles), YjdL-WT (open rhombuses), and YdgR-WT (closed rhombuses). The ratio of substrate accumulated inside the cell with respect to the substrate concentration outside the cell was plotted as a function of time. The assay was performed at pH 6.5. The open downward facing triangles represent empty vector. Error bars indicate S.E. (n = 3).](image-url)
der Waals interactions (Fig. 8A). Both are conserved among peptide-transporting POTs (26, 44, 47), and Tyr\(^{40}\) is involved in interactions with the peptide substrate C terminus. Mutations at this position also abolish the activity of hPepT1 (46). Tyr\(^{40}\) furthermore interacts tightly with Helix V. A shift of arginine to E\(^{1}\) and Helix V away from the active site during the transport cycle would affect the position of Tyr\(^{40}\). During the MD simulation of the E\(^{1}\)XXQ\(_{2}\)R-containing GkPOT structure, the Tyr\(^{40}\) conformation changed significantly compared with the corresponding E\(^{1}\)XXE\(_{2}\)R simulation (Fig. 8A). Although the E\(^{1}\)XXE\(_{2}\)R Tyr\(^{40}\) conformations represent a peptide-bound state, the E\(^{1}\)XXQ\(_{2}\)R conformations would disrupt the peptide-binding site, which is in agreement with the transporter being stabilized in an inward facing “substrate release” conformation.

On the opposite side of arginine, Tyr\(^{39}\) and Tyr\(^{40}\) interact with Arg\(^{43}\) (Fig. 8A). Arg\(^{43}\) has been proposed to be important for changing the conformation from inside open to the occluded state by forming a salt bridge with Glu\(^{310}\) from the C-terminal domain (14) (Fig. 8B). During our simulations, we observed an increased flexibility of Arg\(^{43}\) in E\(^{1}\)XXQ\(_{2}\)R as shown by the Arg\(^{43}\) to Glu\(^{310}\) distance (Fig. 8C). Thus, the changed conformation of Tyr\(^{40}\) destabilizes the Arg\(^{43}\) to Glu\(^{310}\) salt bridge to some extent, which results in further stabilization of the inward open state. The arginine swap and the interactions with FYING present a plausible mechanism that links protonation to local and overall conformational changes for POTs carrying the conventional E\(^{1}\)XXE\(_{2}\)R motif.

**FIGURE 8.** Further destabilization of the binding site as a consequence of E\(^{2}\) protonation. A, Helix I of E35Q-GkPOT after 35 ns (wheat) superimposed with Helix I of the GkPOT-WT after 35 ns (gray) indicating the change in conformation of Tyr\(^{40}\). B, crystal structure of GkPOT displaying the salt bridge between N- (wheat) and C- (blue) domains, i.e. between residues Arg\(^{43}\) and Glu\(^{310}\) (yellow). C, the shortest side chain N–O distance (Å) between Arg\(^{43}\) and Glu\(^{310}\) as a function of time in GkPOT-WT (black) and E35Q-GkPOT (gray).
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