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

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Background: Proton-coupled oligopeptide transporters (POTs) facilitate di- and tripeptide uptake.

Results: Both glutamates of the highly conserved POT motif EXXERFXYY are required simultaneously for substrate accumulation. Arginine swaps interaction between the glutamates and interacts with another conserved motif, FYING, to facilitate larger structural change.

Conclusion: Two conserved motifs interact to facilitate structural changes upon substrate and proton binding.

Significance: Our results have contributed to understanding the mechanism of POTs.

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, E1XXE2RFXY (from here on referred to as E1XXE2R), 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 E1XXE2R, E1XXE2Y, E1XXQ2Y, or Q1XXE2Y were able to accumulate peptide, whereas those with E1XXQ2R, Q1XXE2R, 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-E2 to R-E1, which is consistent with previous structural studies. Molecular dynamics simulations of the motif variants E1XXE2R and E1XXQ2R 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 these 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 E1XXE2R located on Helix

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3 The abbreviations used are: POT, proton-coupled oligopeptide transporter; MD, molecular dynamics; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; β-Ala-Lys(AMCA), β-Ala-Lys-N7-αmino-4-methylcoumarin-3-acetic acid; pHext, outer pH; Gk, G. kaustophilus.
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FIGURE 1. Overall architecture and conformations of POTs. A, crystal structure of the inward open hPepT12-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 EXXERFXYY motif (green) and the FYING motif (wheat). The substrate is represented as a black sphere with spikes, and the peptide is represented as a black rhombus. B, schematic representation of possible conformational changes in POTs during proton/peptide translocation; the proton is represented as a green wheat, and the peptide is represented by a yellow wheat. C, multiple sequence alignment of E1, E2, and FY motifs using mafft from GkPOT (Q5KYD1), YjdL (P39276), YdgR (P77304), YbgH (P75742), hPepT1 (P46059), PepTso (Q8EHE6), PepTst (Q5M4H8), and NRT1.1 (Q05085). I (Fig. 1A) and the FYXXINXG motif (from here on referred to as FYING) located on Helix V (14, 15, 28). Some evidence has linked E2,XXER, in particular E2, to interaction with the co-transported proton (15, 29). To understand the role of E1,XXER in greater detail, we engineered this motif by introducing conserved 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 E1,XXER motif. Furthermore, the rearrangement in E1,XXER 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 Q1,XXQ2,Y (15, 29). Overexpression of transporter variants, all carrying a C-terminal His<sub>6</sub> tag, was performed as described previously (6, 18, 20, 29, 30). Briefly, plasmids pTTQ18-yjdI/yydr 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 A<sub>600</sub> 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 A<sub>600</sub> 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 Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 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-His<sub>6</sub> 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 CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 5 mM glucose, pH 5.5, 6.5, or 7.5, to an A<sub>600</sub> of 10. 900 μl of the cell suspension was 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.
and data were analyzed using GraphPad Prism (18, 20, 29). To estimate the level of accumulation, the concentration of the β-Ala-Lys(AMCA) inside the cell was calculated by using OD-specific total cell volumes of 3.6 μl·ml⁻¹·OD⁻¹ (33). Saturation ratios were estimated by extrapolating from hyperbolic curve fitting. If the [substrate_in]/[substrate_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 was kept unprotonated. Glu 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 K in the NVT ensemble with restraints on heavy atoms of the solute in 12 ps. Subsequently, 12 ps was run at T = 10 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 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 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 E1XXER motif, which reads QXXE2Y. 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 (pHₐ) values, uptake of a non-hydrolyzable fluorescent dipeptide β-Ala-Lys(AMCA) (41) was followed at pHₐ, 5.5, 6.5, and 7.5, respectively, for 30 min, and the fluorescence values were converted to a [substrate_in]/[substrate_out] ratio. A premise to perform this calculation is that no quenching of β-Ala-Lys(AMCA) fluorescence occurs inside the cells. To test this phenomenon, cells that had taken up β-Ala-Lys(AMCA) were lysed, and the cleared lysate was measured for fluorescence and compared with non-lysed cells. No significant fluorescence quenching of β-Ala-Lys(AMCA) was observed (Fig. 2A). Accordingly YjdL-WT was able to accumulate substrate to 10, 11, and 5 at a pHₐ of 5.5, 6.5, and 7.5, respectively (Fig. 2A). 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 pHₐ, 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 pHₐ, 5.5. All variants were found to be expressed as judged from three independent Western blot analyses (Fig. 2B).

E₁ and E₂ Have No Significant Influence on Substrate Accumulation in QXXE₂Y—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 QXXQ₂Y, E₁XXQ₂Y, and E₂XXE₂Y were tested, and the foremost objective of these analyses was to investigate whether a
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Both E1 and E2 Are Essential for Accumulation in a Conventional E1XXER—Single site changes in the YjdL motif Q1XXE2Y of Y to R and Q1 to E resulted in the conventional E1XXER 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 E1XXER, all motif variants in which E1 and/or E2 was changed to Q, i.e. E1XXQ2R, Q1XXE2R, and Q1XXQ2R, were tested. E1XXQ2R and Q1XXER 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 Q1XXQ2R 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 E1 and E2 simultaneously. When only E1 or E2 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 Q1XXE2Y instead of an E1XXE2R motif YjdL readily accumulates substrate. Introduction of a conventional motif into YjdL retains its ability to accumulate. Although E to Q changes in Q1XXE2Y only affect the level of accumulation, in E1XXE2R motif, 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 E2 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 E1 according to the protonation state of E2.

Arginine Is Able to Change Conformation and Interact with E1—To gain a detailed understanding of the conformational mobility of residues in the E1XXE2R 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 E1XXE2R and the same structure with the motif changed to E1XXQ2R. The distances between R and E1 and E2, respectively, were monitored during the MD simulations. Arginine maintained a close interaction with E2 in the E1XXR simulations (Fig. 4A). However, in the E1XXQ2R simulation, arginine shifted its conformation toward E1 to form a salt bridge (Fig. 4B). Arginine is in contact (within a 5-Å distance) with Glu32 (E1), Glu35 (E2), and Ile165 in GkPOT and most bacterial POT structures. Ile165 belongs to the FYING motif. During the conformational shift of arginine in the E1XXQ2R simulation, the interactions with Ile165 present of tyrosine, accumulation is observed for all motifs; however, E1 and particularly E2 enhance the level of saturation. The importance of E2 is even more evident at pH 6.5 where a saturation ratio of approximately unity was observed for Q1XXQ2Y, thus showing inability to accumulate substrate (Fig. 3D). Interestingly, E1 was able to restore accumulation at either pH 5.5 or 6.5 in the absence of E2 (Fig. 3, B–D, and Table 1).

FIGURE 3. Accumulation profiles of motif variants Q1XXE2Y (A), E1XXQ2Y (B), E1XXE2R (C), Q1XXQ2Y (D), E1XXE2R (E), E1XXQ2R (F), Q1XXE2R (G), and Q1XXQ2R (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).

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
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. E1XXE₂Y is located in

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<td>Q₁XXE₂Y</td>
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<td>5.3±0.3</td>
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<td>Q₁XXQ₂Y</td>
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<td>E₁XXE₂R</td>
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FIGURE 4. Molecular dynamics simulations probing the effect of E₂ to Q change. The shortest side chain N–O distances (Å) from Arg36 to Glu32 (gray) and Glu35 (black), respectively, as a function of time in GkPOT-WT (E₁XXE₂R) (A) and E35Q-GkPOT (E₁XXQ₂R) (B) are shown. Insets represent the position of arginine at 0 and 35 ns.

FIGURE 5. Movement of Helix V as a consequence of salt bridge swapping. A, the movement in Helix V of E35Q-GkPOT accompanying the shift of Arg36 on Helix I. The helices of E35Q-GkPOT at 35 ns (wheat) were aligned to GkPOT-WT at 35 ns (white). B, the root mean square deviation (RMSD) (Å) of the Cx atom of Tyr162 on Helix V plotted as a function of time in GkPOT-WT (gray) and E35Q-GkPOT (black).
close proximity of the substrate-binding pocket in all structures (Fig. 1A). In most POT structures with a prototypical E1XXE2R motif that have been crystallized at near neutral pH (14, 26, 28), arginine is found to interact with E2 potentially through a salt bridge. Among the motif variants that we tested, such an interaction would be possible in Q1XXE2R and E1XXE2R; however, only E1XXE2R was able to accumulate substrate. A different arginine conformation within the motif was found at low pH in the structure of nitrate/auxin transporter NRT1.1 (43) showed an interaction between arginine and E2. Such an interaction would be possible in motif variants containing E1 and arginine, i.e. E1XXQ2R and E1XXE2R motifs; however, E1XXQ2R was not able to accumulate. Taken together, our data combined with current structural knowledge strongly suggest that substrate accumulation among POTs with a conventional E1XXE2R motif is only possible when arginine is able to swap between E1 and E2. Protonation of E2 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 E2. It should also be mentioned here that introducing substantial changes, such as the E1XXE2R motif, in YjdL can have effects on its activity and result in slower rates and final levels of accumulation. The reasons behind this could be a suboptimal local environment affecting accommodation of the introduced residues. Nonetheless, our MD simulations on GkPOT support the observation that arginine swaps interactions in the protonated E2, mimicking E1XXQ2R but not E1XXE2R.

The presence of tyrosine instead of arginine in the YjdL motif (Q1XXE2Y) 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 6.5. The importance of E2 is evident from the fact that Q1XXQ2Y is not able to accumulate at pH 6.5 and suggests a role in the proton translocation pathway as discussed above. However, E35Q is not strictly required for accumulation as E1XXQ2Y 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, E1XXE2Y and E1XXE2R 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.

\[ \text{Arginine swapping} \]

**Implications for POTs with a Prototypical E1XXE2R 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 E2, and E1 may be bound to a nearby lysine residue (Figs. 6A and 7A). (ii) Upon peptide binding, arginine becomes sandwiched between the carboxyl group of E2 and the peptide C terminus (Fig. 6B). These interactions are supported by the crystal structure of PepTSt 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-E1 salt bridge, would in itself lead to weakening of the arginine-E2 interaction, enhancing the proclivity for E2 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 E1 (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 PepTSt::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, His57 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 E2.

**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 E1XXE2R maintains a salt bridge to E2. Our MD simulation, based on an unliganded GkPOT structure with an E1XXQ2R motif (E35Q variant), prompted a shift of the arginine conformation to form interactions with E1 (Fig. 4). Simultaneously, we observed an ~5-Å shift of Helix V away from the central cavity,

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**FIGURE 6. Schematic representation of salt bridge swapping in the E1XXE2R 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.
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 simulation (Fig. 7B). Observing a conformational change during the MD simulation indicates that the initial system is conformationally 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. 5A), 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 conformation (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 the E1XXE2R arginine and FYING isoleucine can be highlighted (Fig. 5). It can be speculated that this tight interaction between E1XXE2R and FYING ensures active proton-coupled 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...
der Waals interactions (Fig. 8A). Both are conserved among peptide-transporting POTs (26, 44, 47), and Tyr40 is involved in interactions with the peptide substrate C terminus. Mutations at this position also abolish the activity of hPepT1 (46). Tyr40 furthermore interacts tightly with Helix V. A shift of arginine to E1 and Helix V away from the active site during the transport cycle would affect the position of Tyr40. During the MD simulation of the E1,XXQ2R-containing GkPOT structure, the Tyr40 conformation changed significantly compared with the corresponding E1,XXE2R simulation (Fig. 8A). Although the E1,XXE2R Tyr40 conformations represent a peptide-bound state, the E1,XXQ2R 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, Tyr39 and Tyr40 interact with Arg43 (Fig. 8A). Arg43 has been proposed to be important for changing the conformation from inside open to the occluded state by forming a salt bridge with Glu310 from the C-terminal domain (14) (Fig. 8B). During our simulations, we observed an increased flexibility of Arg43 in E1,XXQ2R as shown by the Arg43 to Glu310 distance (Fig. 8C). Thus, the changed conformation of Tyr40 destabilizes the Arg43 to Glu310 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 E1,XXE2R motif.

FIGURE 8. Further destabilization of the binding site as a consequence of E2 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 Tyr40. B, crystal structure of GkPOT displaying the salt bridge between N- (wheat) and C- (blue)-domains, i.e. between residues Arg43 and Glu310 (yellow). C, the shortest side chain N–O distance (Å) between Arg43 and Glu310 as a function of time in GkPOT-WT (black) and E35Q-GkPOT (gray).
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homologue of the mammalian oligopeptide-proton symporters, PepT1 and PepT2. EMBO J. 30, 417–426


