Conserved Tyrosine in the First Transmembrane Segment of Solute:Sodium Symporters Is Involved in Na\(^+\)-coupled Substrate Co-transport\(^*\)

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Solute:sodium symporters (SSSs) transport vital molecules across the plasma membrane of all living organisms. vSGLT, the Na\(^+\)/galactose transporter of Vibrio parahaemolyticus, is the only SSS for which high resolution structural information is available, revealing a LeuT-like fold and a Na\(^+\)-binding site analogous to the Na2 site of LeuT. Whereas the core transmembrane segments (TMs) of SSSs share high structural similarity with other transporters of LeuT-like fold, TM1 does not correspond to any TM in those structural homologs and was only resolved for the backbone atoms in the initial vSGLT structure (Protein Data Bank code 3DH4). To assess the role of TM1 in Na\(^+\)-coupled substrate symport by the SSSs, here we have studied the role of a conserved residue in TM1 by computational modeling in conjunction with radiotracer transport and binding studies. Based on our sequence alignment and much topological data for homologous PutP, the Na\(^+\)/proline transporter, we have simulated a series of vSGLT models with shifted TM1 residue assignments. We show that in two converged vSGLT models that retained the original TM1 backbone conformation, a conserved residue, Tyr-19, is associated with the Na\(^+\) binding interaction network. In silico and in vitro mutagenesis of homologous Tyr-14 in PutP revealed the involvement of this conserved residue in Na\(^+\)-dependent substrate binding and transport. Thus, our combined computational and experimental data provide the first clues about the importance of a conserved residue in TM1, a unique TM in the proteins with LeuT-like fold, in the Na\(^+\)-coupled symport mechanism of SSSs.

Solute:Sodium Symporters (SSS)\(^3\) are Na\(^+\)-dependent transport proteins responsible for the absorption of nutrients, vitamins, osmolytes, and ions across the plasma membrane of all living organisms (1). Several members of the SSS family have been implicated in metabolic congenital disorders (2–4). Despite a wealth of functional data for members of the SSS family (1) originating primarily from the Na\(^+\)/glucose transporter 1 (5), the Na\(^+\)/iodide symporter (6), and the Na\(^+\)/proline transporter (PutP) (1), to date, the Vibrio parahaemolyticus Na\(^+\)/galactose symporter (vSGLT) is the only member of the SSS family for which high resolution structural information is available (8, 9). vSGLT is composed of 14 transmembrane segments (TMs), including a core of 10 TMs with LeuT-like structural fold arranged into two inverted repeats of five TMs (TMs 2–6 and 7–11) (10, 11). The crystal structure of vSGLT (Protein Data Bank code 3DH4) is in an inward-facing conformation and contains one occluded substrate molecule, galactose, and one Na\(^+\) (supplemental Fig. S1A). The galactose-binding site is composed of residues from TMs 2, 3, 7, 8 and 11, whereas Na\(^+\) is coordinated by residues from TM2 and TM9 (supplemental Fig. S1B), which can be structurally aligned to TM1 and TM8 of LeuT. In comparison, Na1 in the LeuT structure is coordinated by residues from TMs 1, 6 and 7, whereas Na2 is coordinated by residues from TM1 and TM8 (12). Therefore, the Na\(^+\)-binding site of vSGLT was deduced to have a structural environment and coordination properties similar to the Na2 site of LeuT (8). The four peripheral TMs in vSGLT anchor the N and C termini in the periplasm. Among those, TM1 was named TM(−1) by Abramson and Wright (11) because of its unique positioning with respect to the 10 TM-containing core structure.

In the first published crystal structure of vSGLT (Protein Data Bank code 3DH4), TM1 is poorly resolved (8) and was modeled as poly-Ala α-helix because only the backbone atoms of these residues were identified. It is positioned close to the Na\(^+\)-binding site formed by residues from TM2 and TM9 with only 12.5 Å between the closest modeled Ala atom in TM1 and the bound Na\(^+\), suggesting that this TM might be relevant to Na\(^+\) binding and/or translocation. Using this structure as the template, several molecular modeling studies that aimed to gain insight into the coupled translocation of Na\(^+\) and substrate, surprisingly, however, did not, considering the involvement of TM1 (13–15).

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\(^3\) The abbreviations used are: SSS, solute:sodium symporters; Mes, 2-(N-morpholino) ethanesulfonic acid; vSGLT, Vibrio parahaemolyticus sodium/galactose symporter; TM, transmembrane segment; PutP, Na\(^+\)/proline transporter; MD, molecular dynamics; RMSD, root mean square deviation.
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Although there is only limited information available for the structure/function relationship of particular residues in vSGLT (16, 17), early studies identified regions in the N-terminal region of PutP to be important for cation-coupled proline transport (18). This observation was confirmed by mutational analysis of PutP, revealing the functional importance of residues 54–58 in TM2 for cation-coupled substrate symport (19–21). However, this approach has not been applied to residues located in TM1.

To assess the role of TM1 in Na\(^+\)-coupled substrate symport, here we studied the involvement of a conserved Tyr residue in TM1 by means of comparative molecular modeling of vSGLT and PutP, in conjunction with functional studies using the experimentally well-established system PutP.

While this manuscript was being prepared, the crystal structure of the vSGLT mutant K294A (Protein Data Bank code 2XQ2) was determined in the absence of both galactose and Na\(^+\) (9). Most notably, in the new vSGLT structure, the side chains of TM1 were solved in monomer A of the crystal dimer, thus providing excellent structural validation of our simulations.

**EXPERIMENTAL PROCEDURES**

**Sequence Retrieval and Alignment**—Sequences of prokaryotic SSSs that have been functionally characterized were collected by blasting against the NCBI RefSeq protein sequences (22) and were aligned using Probcons (23).

**Construction of the Models and Simulation Systems**—Models of vSGLT were built with Modeler 9v7 (24) using the crystal structure of vSGLT (Protein Data Bank code 3DH4) (8) as the template. In the crystal structure, TM1 is composed of 17 residues and appears to be too short to conform the lipid bilayer dimension. Thus, in each model, two residues on the N-terminal side and four residues on the C-terminal side were added on TM1, resulting in a 23-residue segment. In addition, the missing loop residues between TM5 and TM6 (residues 179–185) were built and refined with the loop module of Modeler. The side chains of residues without structural template were initialized with Sccr4 (25). The model of vSGLT without TM1 was built using the same protocol but without the steps related to TM1. PutP models were built in a similar fashion with Modeler 9v7 (24), using the aligned TM2–TM13 in the vSGLT structure (Protein Data Bank code 3DH4) (8) and TM1 in our best vSGLT model, model 10 (see text), as the template.

The System Builder module of the Desmond package (26) was used to immerse each model in an explicit water-bilayer-water environment. The insertion of the protein in the membrane (totally water environment. The insertion of the protein in the membrane (Desmond 2.2 User Manual; Schrodinger, Inc.) was recommended for relatively long simulations on systems with membrane (Desmond 2.2 User Manual; Schrodinger, Inc.). To retain the Na\(^+\) in its binding site in a selected simulation (see text), harmonic distance restraints with a force constant of 2 kcal/mol were used. Throughout the simulations, a short range cut-off was set to 10.0 Å, and the smooth particle mesh Ewald method (29) was used for long range electrostatic interactions.

**Functional Assays in PutP**—Na\(^+\)-dependent or -independent transport of 2 \(\mu\)M \(^{3}\)H-Pro (1 Ci/mmol; Moravek) by intact *Escherichia coli* WG170 harboring given PutP variants or a control plasmid was measured for the indicated periods of time at 22 °C using a rapid filtration assay (30). Transport kinetics were analyzed by determining the initial rates of transport for 10 s (corrected for unspecific interaction of \(^{3}\)H-Pro with the cells at 0 s) by varying the \(^{3}\)H-Pro concentration between 0.1 and 50 \(\mu\)M. For the facilitated accumulation of 20 \(\mu\)M \(^{3}\)H-Pro (1 Ci/mmol), the cells were completely de-energized by incubation with 5 \(\mu\)M carbonyl cyanide m-chlorophenylhydrazone and 5 \(\mu\)M monensin at 22 °C for 30 min prior to the start of the uptake reaction in Na\(^+\)-free buffer (30).

Saturation binding of varying concentrations of \(^{3}\)H-Pro by PutP-WT, PutP-Y14E, and PutP-Y14F was determined by means of the scintillation proximity assay. Protein was expressed and purified as described (31) with the exception that 50 mM Tris/Mes, pH 7.5, 150 mM NaCl, 20% (v/v) glycerol, 1 mM Tri(2-carboxyethyl) phosphine (TCEP) was used as base buffer for solubilization and purification. Removal of contaminating Na\(^+\) was achieved by desalting the eluted PutP variants on Zeba spin columns (Pierce).
into 200 mM Tris/Mes, pH 7.5, 20% (v/v) glycerol, 1 mM TCEP, 0.1% (w/v) n-dodecyl-β-D-maltopyranoside (32). Binding of 1.4 and 10 μM [3H]Pro (5Ci/mmol) by 250 ng of PutP-WT, -Y14E, or -Y14F was determined with the scintillation proximity assay in the presence of varying [NaCl] (equimolar replacement of Tris/Mes). Binding was corrected for nonproximity elicited counts (cpm) determined in the presence of 400 mM imidazole. The data represent the means ± S.E. of triplicate determinations and were fitted using nonlinear regression algorithms in SigmaPlot with kinetic constants representing the mean ± the error of the fit.

The relative amount of PutP in the membrane was compared by Western blotting of SDS-PAGE-separated proteins from membrane vesicles originating from E. coli WG170, harboring each plasmid described, using mouse anti-FLAG IgG against the FLAG epitope at the C terminus of the protein followed by incubation with horseradish peroxidase-linked goat anti-mouse-IgG antibody (20). Immunoreactions were visualized with the enhanced chemiluminescence (ECL) method (SuperSignal® West Pico kit; Thermo Scientific) using with the AlphaImager HP system (Cell Biosciences). Protein was assayed as appropriate (33, 34) with bovine serum albumin as standard.

RESULTS

Alternative Assignments of Residues in TM1 of vSGLT Using MD Simulations—Because of the lack of high resolution information, TM1 in the crystal structure of the inward-occluded vSGLT (Protein Data Bank code 3DH4) is modeled as an α-helix consisting of 17 Ala residues (arbitrarily labeled as residues 3–19 in the pdb file), whereas the loop between TM1 and TM2 (residues 20–46) is not resolved (Fig. 1A). Curiously, when we assigned residues 3–19 to the Cα-trace of TM1, it was difficult to reconcile such a residue assignment (RA0) with detailed topological data described previously for the homologous PutP (31). In that study, Cys accessibility analysis was used to show that the sulphhydryl groups in single Cys mutants I3C and T5C were accessible to membrane impermeant sulphhydryl reagents, suggesting that they are highly exposed to the periplasm. However, based on the RA0, Leu-8 and Phe-10, which are aligned to Ile-3 and Thr-5 of PutP (see text) are shown as black spheres. During the evolution of the trajectory, Asp-12 escapes from a hydrophobic pocket (C) and the backbone hydrogen bond between Gly-7 and Ile-11 is lost (D).

To investigate this apparent inconsistency, we have performed computational modeling and simulations with the assumption that the proper residue assignment in TM1 would retain the backbone conformation revealed in the crystal structure. After construction and relaxation of the simulation system in which the vSGLT model with the TM1 residue assignment RA0 is embedded in an explicit lipid bilayer, we carried out a completely unconstrained MD simulation and collected two independent trajectories. As depicted in Fig. 1B, in one trajectory, at the end of 45 ns of the production run, the backbone of TM1 (consisting of residues 1–23) deviates significantly from its original conformation and is bent around Ile-11 with an angle of ~100° (Fig. 1B). This structural deformation is the result of several local rearrangements. Specifically, the charged Asp-12, which is initially positioned in a hydrophobic pocket, escapes during the simulation toward the cytoplasm (Fig. 1C); this change is accompanied by the loss of a backbone hydrogen

**FIGURE 1.** The TM1 residue assignment RA0 is not compatible with the backbone conformation in the crystal structure of vSGLT. A, sequence alignment of the functionally characterized prokaryotic SSS transporters (vSGLT, PutP, PanF, and NanP (7)). The regions solved in the vSGLT crystal structure are indicated by the black bars above the sequence (note the TM1 region is modeled as poly-Ala). The boxes enclose TM1 (the boundaries are based on our modeling results, see text) and TM2. B, side view of the vSGLT model with the RA0 equilibrated in a palmitoyloleoylphosphatidylcholine bilayer. Two inverted repeats, TMs 2–6 and TMs 7–11, are in blue and red, respectively. TM1 is colored in pink, and the other three peripheral TMs are in gray. C and D, the bending of the simulated TM1 structure (light pink) is associated with local rearrangements (C and D). The α-carbons of residues 8 and 10 that are aligned to the accessible Ile-3 and Thr-5 of PutP (see text) are shown as black spheres. During the evolution of the trajectory, Asp-12 escapes from a hydrophobic pocket (C) and the backbone hydrogen bond between Gly-7 and Ile-11 is lost (D).
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After 45 ns of production runs, these models were evaluated according to the backbone RMSD (residues 3–19; Fig. 2B) between the simulated TM1 and the \( \text{Ca} \)-traced TM1 in the \( \text{vSGLT} \) crystal structure (Protein Data Bank code 3DH4). In models 6 and 10, TM1 appeared to converge to a helical conformation closest to that in the crystal structure. Specifically, model 10 remained very close to its starting conformation, Na\(^{+}\) remains for a significant amount of time in the Na\(^{+}\)-binding site identified in the \( \text{vSGLT} \) crystal structure and slowly moves toward the intracellular side of the transporter after \( \sim 60 \) ns of simulation (supplemental Fig. S3). Consistent with previous observations (14, 15), when Na\(^{+}\) leaves the original binding site, it immediately forms a direct interaction with Asp-189, a conserved residue found to be involved in the cation-coupled symport in \( \text{PanF} \) (31) and \( \text{hSGLT1} \) (36). The association between

bond between Gly-7 and Ile-11 (Fig. 1D). Thus, according to our computational simulations, the residue assignment RA0 does not reflect an energetically favorable positioning of TM1 amino acid side chains with regard to their interactions with other regions in the transporter and the lipid bilayer. Similarly large scale distortion in the middle of TM1 was also observed in the second trajectory (supplemental Fig. S2).

In our sequence alignment (Fig. 1A), the N terminus of \( \text{vSGLT} \) extends those of other functionally characterized prokaryotic SSSs by five to seven residues. For example, in this alignment, the N terminus of Pan\( \text{F} \), the Na\(^{+}\)/panthothenate transporter (35), is seven residues shorter than that of \( \text{vSGLT} \). Together with the accessibility data for \( \text{PutP} \) (31), our sequence alignment (Fig. 1A) suggests that TM1 in \( \text{vSGLT} \) is likely to start from a more C-terminal position than was modeled with RA0. To test this hypothesis, we built a series of \( \text{vSGLT} \) models based on the crystallographic data but shifted the residue assignments in TM1 along the sequence (Fig. 2A). Each model in this series was constructed and simulated following the same protocol to identify the optimal residue assignment of TM1.

The Role of Tyr-19 in the Interaction Network Associated with Na\(^{+}\) Binding—In the \( \text{vSGLT} \) crystal structure (Protein Data Bank code 3DH4), the side chain of highly conserved Gln-192 faces TM1, but without forming a distinct H-bond with any of the originally assigned amino acid residues in TM1. When TM1 was entirely excluded from the simulations, in the previous studies (13–15), Gln-192 would be exposed to lipid tails, without making any favorable interaction. These unfavorable situations are resolved in our models 6 and 10, where Gln-192 finds a feasible stabilizing anchor in its water-mediated interaction with Tyr-19. Fig. 2 (C and D) shows the distances between the hydroxyl group of Tyr-19 and both the side chain of Gln-192 and the backbone of Ile-191 during the simulations. Tyr-19 in models 6 and 10 is seen to converge gradually toward similar positions and consequently faces the TM6–TM9 interface, which is in close vicinity to the Na\(^{+}\)-binding site. In addition, Asp-12, which was not in a favorable orientation in the simulation based on the residue assignment RA0 (see above), is now located in a positively charged environment (Fig. 2E).

In two independent simulations of model 10, which has the fewest rearrangements from its starting configuration, Na\(^{+}\) leaves the original binding site identified in the \( \text{vSGLT} \) crystal structure and slowly moves toward the intracellular side of the transporter after \( \sim 60 \) ns of simulation (supplemental Fig. S3). Consistent with previous observations (14, 15), when Na\(^{+}\) leaves the original binding site, it immediately forms a direct interaction with Asp-189, a conserved residue found to be involved in the cation-coupled symport in \( \text{PutP} \) (30) and \( \text{hSGLT1} \) (36). The association between

FIGURE 2. Alternative residue assignment of TM1. A, residue assignments of TM1 in a series of \( \text{vSGLT} \) models. The models are named according to the residue numbers that align to residue 3 of the crystal structure. For example, in model 6, His-6 is aligned to Asn-3. B, the models are evaluated by measuring the backbone RMSD (residues 3–19) between the simulated TM1 in our models and modeled TM1 in the crystal structure of \( \text{vSGLT} \) (Protein Data Bank code 3DH4). C and D, the evolutions of the distances between Tyr-19 and residues Ile-191 (C) and Gln-192 (D) near the Na\(^{+}\)-binding site indicate Tyr-19 in models 6 and 10 converged toward similar locations. The color codes of the models in B–D are the same as in A. E, the converged TM1 conformation in models 6 (green) and 10 (orange). TMs 2–14 are represented by their solvent-accessible surface and are colored according to the electrostatic potentials calculated by the Poisson-Boltzmann approach implemented in APBS (37) through its plug-in in PyMOL. In both models, the TM1 structure converged to have Tyr-19 facing the TM6–TM9 interface that is close to the Na\(^{+}\)-binding site (blue sphere).
Na⁺ and Asp-189 destabilizes the water-mediated interaction between Asp-189 and nearby Gln-192. Consequently, Gln-192 rotates to interact with Tyr-19 of TM1, and the nearby interaction network rearranges from the configuration depicted in Fig. 3A to that in Fig. 3B, which is stable in the absence of Na⁺.

To confirm the configuration of the interaction network in the presence of Na⁺, starting from a snapshot of model 10 taken at the end of 45 ns, a third MD simulation of model 10 was carried out with the bound Na⁺ restrained to maintain its distances to the carbonyl oxygen atoms of residues Ala-62, Ile-65, and Ala-361, whereas the rest of the network depicted in Fig. 3 is free to move. In this trajectory of 42 ns, the interaction network retains the configuration of model 10 before the Na⁺ left the binding site (Fig. 3A).

A similar behavior of Tyr-19, in response to the presence and absence of Na⁺, is observed in the trajectory of model 6, in which Na⁺ exits to the intracellular milieu within 3 ns because of the disruption induced by the upward shift of TM1. Thus, we propose that in the absence of Na⁺, Tyr-19 forms a water-mediated interaction with Gln-192, whereas in the presence of Na⁺ it interacts with the backbone of Ile-191, because Gln-192 rotates inwards and forms a water-mediated interaction with Asp-189.

To evaluate the impact of the absence of TM1 on the mechanistic conclusions that are relevant to the Na⁺-binding site in previous modeling studies of vSGLT (13–15), we built a vSGLT model without TM1 and carried out a MD simulation following the same protocol used for our other models with TM1. Fig. 3C shows the configuration of the interaction network in the equilibrated stage of this model (after 36 ns). Note that in this model, the initially embedded Na⁺ leaves the binding site quickly (supplementary Fig. S4), as was observed in the previous studies (13–15) (also see “Discussion”).

The rearrangements of the interaction network in response to the presence of Na⁺ and/or TM1 affect the shape and size of the Na⁺-binding site, represented by Cβ-Cβ distances among Na⁺-coordinating residues Ala-62, Ile-65, and Ser-364 (Fig. 3). Thus, in the presence of Na⁺, the Na⁺-binding site is narrower than that in the absence of Na⁺ and also that in the crystal structure, consistent with the conclusion that the binding site in the crystal structure is not in an optimal configuration to bind Na⁺ (see “Discussion” and Refs. 14 and 15). In the absence of both TM1 and Na⁺ (Fig. 3C), the Na⁺-binding pocket becomes significantly more inward-open than those in the models with TM1, suggesting a strong role of TM1 in maintaining the stability of the interaction network.

Involvement of the Conserved Tyr in Na⁺-coupled Substrate Transport and Binding in PutP—To probe the role of the conserved Tyr of TM1 in Na⁺-coupled symport in the broader context of SSS proteins, we tested the functional effect of mutating the corresponding Tyr (Tyr-14) in PutP (Fig. 4A), a well-established system for the study of Na⁺-coupled substrate transport and binding (1, 18, 20, 21, 30).

First, to assess the role of Tyr-14 as H-bonding partner, we built models of PutP-WT, -Y14E, and -Y14F, each with two configurations, in the presence and absence of bound Na⁺. Whereas the Tyr to Phe mutation only eliminates the H-bond capability, the Glu mutation drastically changes both the side chain size and the interaction profile, e.g. it can only serve as an H-bond acceptor. To obtain detailed information on the role of Tyr, Glu, or Phe at position 14, these models were immersed in a lipid bilayer and then relaxed with the same MD simulation protocol as that used for vSGLT. To reduce the impact of other divergent regions, however, only the portions of TMs 1, 2, 6, and 9 that encompass the Na⁺-binding site were allowed to equilibrate freely (totally 59 residues), with the rest of protein restrained on backbone (Fig. 4B). In addition, in the simulations with embedded Na⁺, the distances between oxygen atoms of Na⁺-binding residues Ala-53, Met-56, and Ala-337 and the Na⁺ were restrained (<3.2 Å) to maintain the Na⁺ in its binding site. For each configuration, we collected a 42-ns MD trajectory.

The simulations of PutP-WT in the presence or absence of Na⁺ show that the configurational changes of the interaction...
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FIGURE 4. The involvement of Tyr-14 in the interaction network associated with Na\(^+\) binding in PutP. A, the Na\(^+\)-binding site in PutP-WT model is lined by residues from TM5, 6, and 9 (in green sticks), with Tyr-14 from TM1 (in purple sticks) in close vicinity. B, in our MD simulations, the colored regions in TM5, 6, and 9 encompassing the Na\(^+\)-binding site are allowed to equilibrate freely, whereas the rest of protein is restrained on the backbone. C–E represent the resulting configurations of the interaction network in MD simulations of PutP-WT (C), PutP-Y14F (D), and PutP-Y14E (E), in the presence (left panels) or absence (right panels) of Na\(^+\). In the PutP-WT model (B), the configurational changes of the interaction network in the presence and absence of Na\(^+\) are similar to those observed for vSGLT (Fig. 3). In the mutants (C and D), this network is highly disrupted and loses the capability to make alternating configurational changes. TMs are in the same color scheme as in Fig. 1. The dashed lines represent direct interactions, whereas red crosses indicate no interaction.

network in PutP-WT are similar to those in vSGLT (Fig. 3, A and B). Thus, Tyr-14 interacts with the Val-189 backbone in the presence of Na\(^+\) and with the side chain of Gln-190 in absence of Na\(^+\) (Fig. 4C) in the same way as Tyr-19 of vSGLT interacts with the Ile-191 backbone or Gln-192 side chain. In contrast, the interaction networks are disrupted in the PutP-Y14F and PutP-Y14E mutants (Fig. 4, D and E), and importantly, there is no alternating pattern of the conformations observed in the vSGLT and PutP-WT simulations. The observed disruptions in each mutant seem to correspond to the nature of the mutation: in PutP-Y14F, the aromatic ring alone is not capable of making polar interactions as seen in PutP-WT, whereas in PutP-Y14E, the Glu cannot H-bond to the backbone Val-189, and it is difficult for its shorter side chain to interact with the side chain of Gln-190. It is also interesting to note that in the mutants, but not in the WT, the portion of TM1 that is free to relax has a tendency to deviate from the original backbone conformation. Such disruptions would be expected to further alter the dynamics of the interaction network.

To validate our in silico mutagenesis results, we replaced Tyr-14 in PutP with Glu and Phe. Fig. 5A shows the time course of 2 μM [\(^3\)H]Pro uptake by PutP-WT, -Y14E, and -Y14F in the presence of saturating NaCl. PutP-WT mediated active transport that reached a steady-state level of Pro accumulation of 19.5 ± 0.5 nmol-mg protein\(^{-1}\) after 30 s (19). The steady-state level of Pro accumulation catalyzed by PutP-Y14E or -Y14F was comparable with that of WT; however, it was reached only after 5 min. In striking contrast to the transport features in the presence of Na\(^+\), PutP-WT exhibited no significant transport activity when the uptake was performed in the virtual absence of Na\(^+\) (determined to be 3 μM by atomic absorption spectroscopy; Fig. 5B). Remarkably, replacement of Tyr-14 with Glu or Phe resulted in similar uptake characteristics in Na\(^+\)-free medium compared with those observed for each PutP-mutant in the presence of saturating Na\(^+\) (Fig. 5B). Note that changing the pH from 6 in the standardized PutP uptake protocol (20) to 7.5 did not significantly alter the uptake properties of all PutP variants.

To test the effect of the mutations on the facilitated influx (uniport) of Pro, a transport mode in which the driving force for proline transport comes exclusively from the chemical concentration gradient of Pro across the plasma membrane, we measured uptake of 20 μM [\(^3\)H]Pro in E. coli WG170 harboring PutP-WT, -Y14E, or -Y14F that were completely de-energized by incubation in the presence of m-chlorophenylhydrazine and monensin (19). All three PutP variants mediated facilitated Pro transport with virtually identical initial rates and steady-state levels of accumulation (~2 nmol min\(^{-1}\)-mg of protein\(^{-1}\) and ~2.3 nmol-mg of protein\(^{-1}\), respectively) (Fig. 5C), pointing out that mutation of Tyr-14 did not affect the interaction of Pro with the transporter per se but rather the coupling between Na\(^+\) and Pro symport.

Furthermore, analysis of the initial rates of transport revealed that the \(K_m\)\(^{Pro}\) and \(V_{max}\)\(^{Pro}\) for PutP-Y14E and -Y14F were not significantly different when the assay was performed in the presence or absence of Na\(^+\) (Fig. 5, D and E), paralleling the transport characteristics observed for the time course of Pro transport (Fig. 5, A and B). In contrast, reliable kinetic data for PutP-WT were only obtained in the presence of Na\(^+\), highlighting the strict Na\(^+\) dependence of this SSS family.
Na\(^{+}\)-dependent transporters with LeuT-like fold (10), suggests that the dynamic changes at the Na2 site may be a common element in their conformational transitions. Indeed, TM1 and TM8 (LeuT numbering), from which the residues form the Na2 site, can be well aligned in LeuT and Mhp1 (in outward-open conformations), or in BetP and vSGLT (in inward-open conformations), with a RMSD of ∼3 Å between the LeuT/Mhp1 and BetP/vSGLT groups (11). In addition to TM1 and TM8, TM5 (LeuT numbering) also contributes to the Na2 site in a few transporters with LeuT-like fold, including BetP (38) and ApcT (39, 40), and likely in vSGLT.

Interestingly, near the Na2 binding site of vSGLT, TM6 and TM9, which can be structurally aligned to TM5 and TM8 of LeuT, respectively, are in contact with TM1, a TM found exclusively in members of the SSS family. In the initial crystal structure of the inward-occluded vSGLT (Protein Data Bank code 3DH4), TM1 was modeled as a 17-residue \(\alpha\)-helical poly-Ala, with the closest atom only at 12.5 Å away from the bound Na\(^{+}\) and in close proximity of the Na\(^{+}\) binding interaction network composed of a set of polar or charged residues. In the absence of TM1, Gln-192 of this network would face the lipid bilayer directly and suggests a polar side chain from TM1 may be involved in maintaining the structural integrity of vSGLT in the lipid environment. Indeed, in our simulations with the properly modeled TM1 (model 10), the bound Na\(^{+}\) stays stably in its binding site for a significantly longer amount of time (supplemental Fig. S3) than those in

FIGURE 5. Effect of the replacement of conserved Tyr-14 in Na\(^{+}\)-coupled substrate transport by PutP. A and B, time course of 2 \(\mu\)M [\(^3\)H]Pro (1 Ci/mmol) uptake by intact E. coli WG170 harboring given PutP variants (WT, Y14E, Y14F, or a control plasmid) in the presence of 10 mM NaCl (A) or Na\(^{-}\)-free conditions (B, open symbols; symbols were used consistently throughout Figs. 5 and 6). C, facilitated diffusion of 20 \(\mu\)M [\(^3\)H]Pro in E. coli WG170 harboring PutP-WT, Y14E, or Y14F after incubating the cells in the presence of 5 \(\mu\)M m-chlorophenylhydrazone and 5 \(\mu\)M monensin. D, transport kinetics of [\(^3\)H]Pro by PutP-WT, Y14E, and Y14F in the presence of 10 mM NaCl, revealing \(K_D\) values of 1.2 ± 0.1, 1.2 ± 0.2, and 1.6 ± 0.2 \(\mu\)M, respectively, with \(V_{\text{max}}\) values of 26.8 ± 0.7, 21.3 ± 0.9, and 27 ± 0.7 nmol·mg of cellular protein\(^{-1}\)·min\(^{-1}\). E, uptake of [\(^3\)H]Pro in the virtual absence of NaCl revealed a \(K_D\) of 3.8 ± 0.8 and 2.6 ± 0.1 \(\mu\)M for PutP-Y14E and Y14F, and a \(V_{\text{max}}\) of 26.8 ± 1.6 and 25.9 ± 0.1 nmol·mg of cellular protein\(^{-1}\)·min\(^{-1}\), respectively. Initial rates of [\(^3\)H]Pro transport were determined from the linear portion of uptake between 0 and 10 s measured at Pro concentrations ranging from 0.1 to 50 \(\mu\)M. F, Immunological detection of given PutP variants using monoclonal anti-FLAG IgG against the C-terminal FLAG epitope in recombinant PutP. Protein originating from membrane vesicles of WG170 harboring the indicated PutP construct (or from cells transformed with the control plasmid lacking the putP gene) was subjected to 11% SDS-PAGE and electroblotted onto a polyvinylidene fluoride transfer membrane. Immunoreactions were visualized with the enhanced chemiluminescence method. Positions of the protein standards are indicated (kDa).
previous vSGLT modeling studies (13–15), all of which excluded TM1 in their simulations. In those studies, Na⁺ was seen to enter the intracellular space within 9 ns (13) or 6 ns (14) or to leave the original binding site immediately (15) after the release of the constraints applied in the equilibration phase. In our simulations of a vSGLT model in the absence of TM1, the Na⁺ also experiences high mobility and completely exits the transporter to the intracellular milieu in less than 18 ns (supplemental Fig. S4). Although Na⁺ does not leave the transporter as quickly as in the other studies, it already experiences a 8.2 Å deviation from its initial position after 2.4 ns of simulation, in stark contrast to the situations in the presence of TM1 (supplemental Fig. S3). This suggests strongly that our findings described here reflect a functional role of the conserved Tyr in TM1 in providing stability and integrity of the Na⁺-binding site (Fig. 3, B and C). Therefore, the quick release of Na⁺ observed previously, resulted most likely from the disruption of the Na⁺-binding site by the absence of TM1 (13–15), though we agree that the vSGLT structure is in an inward-facing conformation and that the identified Na⁺-binding site is not in an optimal configuration for Na⁺ binding.

In the absence of TM1, the bound galactose experienced significant mobility and dissociated from the original binding mode in less than 25 ns during the simulation (15). In contrast, our study shows that the bound galactose and its associated interaction network can remain in the position and configuration revealed by the crystal structure, but only in the presence of TM1. Thus, in addition to the nearby Na⁺-binding site, TM1 may contribute as well to the integrity and stability of the substrate binding site (supplemental text and Fig. S5).

The inferences from the computational studies were supported by the results of our functional studies using the cognate PutP, which is a well characterized model system for the SSS proteins as detailed kinetic and structural dynamic data are available. Replacing Tyr-14 in PutP (which corresponds to Tyr-19 in vSGLT) with Glu and Phe enabled us to validate the results of our comparative simulations, according to which in the presence and absence of bound Na⁺, the conserved Tyr makes alternating interactions with either the backbone of Val-189 or the side chain of Gln-190 (Fig. 4C).

Interestingly, our simulations of vSGLT also reveal that Asp-187 interacts with Gln-192 (which corresponds to Gln-190 in PutP) via a water molecule (Fig. 3A). Indeed, the mutation of Asp-187 in PutP (aligned to Asp-189 in vSGLT) to Cys has a phenotype close to the Tyr-14 mutations, i.e. proline binding is only slightly affected but becomes Na⁺ “independent” (30). Note, however, that a potential reduction of the EC⁵₀ Na⁺ to submicromolar values would preclude experimentally assessing the Na⁺ dependence of PutP-mediated Pro transport or binding because of the contaminating traces of this element from the environment in all buffer solutions.

Such phenotypes suggest that the transporter is shifted toward a conformation that readily binds the substrate; however, it loses the capability to undergo conformational transitions induced by Na⁺. We thus propose that Tyr-14 and Asp-187 play important roles in the Na⁺-coupled co-transport mechanism by being able to engage in alternating interactions in response to the presence or absence of Na⁺. Notably, removal of the charge of Asp-204 in hSGLT, which corresponds to Asp-189 in vSGLT, converts this archetype of Na⁺-coupled symporters into a substrate-gated H⁺ channel (36). It is tempting to speculate that the interactions occurring in the absence of Na⁺ might take advantage of a change in local electrostatic properties and allow an H⁺ to cause local changes mimicking those produced by binding and dissociation of Na⁺. Although we did not detect differences in the Pro uptake activity of the mutants in the absence of Na⁺ when the pH of the assay was varied between 6.0 and 7.5, we cannot rule out the possibility that the pKₐ of both mutants is high and that under our experimental conditions the proteins are fully protonated. A similar scenario has been described for the H⁺-dependent lactose permease (LacY), which exhibits a pKₐ of 10.5 (41).

In summary, our findings indicate that a conserved residue located in TM1, a unique TM that is solely found in members of the SSS family among transporters with a LeuT-like structure fold, contributes to Na⁺ coupling and protein integrity. The structural arrangement in SSSs may present a prototype for the transition of the energy stored in the electrochemical Na⁺ gradient into transport work of solutes. Our new residue assignment of the vSGLT structure also suggests that the missing loop region between TM1 and TM2, which is now only 16 residues long (Ser-31 to Leu-46), is more functionally relevant than previously appreciated. Thus, a number of conserved charged and aromatic residues in this loop (Fig. 1A) may be involved in the conformational transition during the galactose transport, as
those in the NSS family (32, 42), considering the involvement of both TM1 and TM2 in Na\(^{+}\) binding.

While this manuscript was being prepared, the crystal structure of the vSGLT mutant K294A (Protein Data Bank code 2XQ2) was released (9). This vSGLT structure was determined in the absence of both galactose and Na\(^{+}\), however, with the side chains of TM1 solved in monomer A. To our delight, the RMSD between TM1 of our model 10 and that of the new structure is only 0.55 Å, especially with Tyr-19 pointing similarly toward the Na\(^{+}\)-binding site (supplemental Fig. S6).

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Addendum—During preparation of this manuscript, we assumed that the sequence of TM1 in Protein Data Bank code 3DH4 was originally assigned as vSGLT residues 3–19; however, it was emphasized in recent communications with the Abramson and Wright labs that these residues were labeled UNK (unknown) in the Protein Data Bank, and the sequence should not be considered as assigned.

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