The role of transmembrane segment 5 (TM5) in Na2 release and the conformational transition of neurotransmitter:sodium symporters toward the inward-open state

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Neurotransmitter:sodium symporters (NSSs) terminate neurotransmission by the reuptake of released neurotransmitters. This active accumulation of substrate against its concentration gradient is driven by the transmembrane Na+ gradient and requires that the transporter traverses several conformational states. LeuT, a prokaryotic NSS homolog, has been crystallized in outward-open, outward-occluded, and inward-open states. Two crystal structures of another prokaryotic NSS homolog, the multihydrophobic amino acid transporter (MhsT) from Bacillus halodurans, have been resolved in novel inward-occluded states, with the extracellular vestibule closed and the intracellular portion of transmembrane segment 5 (TM5i) in either an unwound or a helical conformation. We have investigated the potential involvement of TM5i in binding and unbinding of Na2; i.e., the Na+ bound in the Na2 site, by carrying out comparative molecular dynamics simulations of the models derived from the two MhsT structures. We find that the helical TM5i conformation is associated with a higher propensity for Na2 release, which leads to the repositioning of the N terminus and transition to an inward-open state. By using comparative interaction network analysis, we also identify allosteric pathways connecting TM5i and the Na2 binding site to the extracellular and intracellular regions. Based on our combined computational and mutagenesis studies of MhsT and LeuT, we propose that TM5i plays a key role in Na2 binding and release associated with the conformational transition toward the inward-open state, a role that is likely to be shared across the NSS family.

The neurotransmitter:sodium symporter (NSS)3 family is comprised of both prokaryotic and eukaryotic integral membrane proteins with 11 or 12 transmembrane segments (TMs). Mammalian NSSs, including transporters for dopamine (DAT), serotonin (SERT), and norepinephrine, modulate the temporal and spatial aspects of neurotransmission by transporting released neurotransmitters back into the presynaptic neuron where they can be repackaged and reused (1). Biogenic amine NSS are important therapeutic targets for psychostimulant and antidepressant drugs (2). Crystal structures of LeuT (3–7), a prokaryotic NSS homolog from Aquifex aeolicus, revealed a novel structural fold characterized by two five-TM pseudo repeats. This LeuT-fold has since been found in a number of other transporter families without obvious sequence similarity to LeuT (reviewed in Refs. 8 and 9). Recent crystal structures of Drosophila melanogaster DAT and human SERT show significant similarity to the LeuT structures (10–13), supporting the notion that the fundamental mechanistic elements of the transport process driven by the Na+ gradient across the membrane are shared between eukaryotic and prokaryotic homologs (14).

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The role of TM5 in Na2 release and inward opening

| MhsT  | LeuT  | hSERT |
|-------|-------|-------|
| 147 TPLPFWQALFMIATVAVGWGKESNGLLMTGLVPLLIALAYS | 186 LFAYIYVF1TMNFVINILIRGSGKERKAMTP7FL1AVFVLRV | 253 WQLGACLMLF1TVYLLFSPSWGK-5TSGSFF1W1ATMFPV1ALLIG |
| | | |
| 151 | 213 | 264 |
| 157 | 238 | 253 |
| 162 | 238 | 253 |
| 194 | 213 | 299 |

Figure 1. The sequence alignment of the TM4 to TM5 region of MhsT, LeuT, dDAT, and hSERT. The residues that face the Na2 site are underlined, and the residues in this region that are mutated for the mutagenesis studies (see text and Figs. 9 and 10) are shaded.

Two crystal structures of another prokaryotic NSS homolog, the multihydrophobic amino acid transporter (MhsT) from *Bacillus halodurans*, have also been resolved (15). One was obtained using a high lipid-detergent mix (HiLiDe) and another with lipid-cubic-phase crystallization (LCP) (MhsT<sup>HiLiDe</sup> and MhsT<sup>LCP</sup>; PDB codes 4US3 and 4US4, respectively). Both structures are bound with a substrate L-tryptophan in the central substrate binding (S1) site and two sodium ions (Na<sup>+</sup>) in the nearby Na1 and Na2 sites, similar to LeuT structures. However, in these two MhsT structures, the extracellular vestibule is in a closed state similar to the inward-open LeuT structure (PDB code 3TT3), whereas the intracellular portion of TM5 (TM5i; see “Experimental procedures” for the definition of intracellular, middle, and extracellular subsegments) is in either an unwound or α-helical (structured) configuration. These structures thus present transporter states that have not been identified in the multiple LeuT structures that have been solved to date.

In the outward-facing crystal structures of LeuT (PDB codes 3TT1 and 2A65), the Na2 site is formed by residues from TM1 and TM8; the Na2 (the Na<sup>+</sup> bound in the Na2 site) coordination is trigonal-bipyramidal; and bound Na2 does not have direct access to water (3–5). In contrast, in the crystal structures of MhsT, dDAT, and hSERT, the Na2 coordination is octahedral, with the sixth ligand being a water molecule in close vicinity to TM5i. Of note, in the MhsT<sup>HiLiDe</sup> structure with the unwound TM5i, there is a continuous water pathway from the Na2 site to the intracellular milieu, and we proposed that the unwinding and flexibility of TM5i facilitates the formation of such a solvation pathway in a step prior to full inward opening of the transporter (15). However, simulations of dDAT and a hDAT homology model (16) and of MhsT<sup>LCP</sup> (see “Results”) indicate that such a water pathway can readily form with a helical TM5i. In addition, the absence of the N terminus (NT) in MhsT<sup>LCP</sup> but not MhsT<sup>HiLiDe</sup> suggests that the dynamics of the NT may be coupled to that of TM5, which is in direct contact with the NT. A detailed analysis of the mechanism of Na2 release is therefore warranted, with a focus on the coordinated rearrangements of TM5 and the NT in such a mechanism.

In the structures of ApcT (17) from the amino acid-polyamine-organocarrier transporter family and in the structures of BetP (18) from the BCCT family, both of which share the LeuT-fold, TM5i also contributes to the aligned Na2 sites. In particular, the protonation and deprotonation of Lys-158 in TM5 of ApcT, in which the role of Na<sup>+</sup> is replaced by a proton, is believed to mimic Na<sup>+</sup> binding and dissociation, respectively (17). Previously, we showed how Na<sup>+</sup> or proton binding couples to the transport process by analyzing the results of comparative MD simulations of LeuT and ApcT: specifically, we identified a lead role of TM11 in coordination with TM5i in the transition to an inward-open conformation, when the Na2 site is neither occupied by Na<sup>+</sup> nor positively charged (19). Based on our homology modeling of DAT and SERT (19), we predicted that a conserved Thr from TM5i makes an H-bond interaction with a conserved Na2-coordinating Asp from TM8, and this interaction is likely involved in the reconfiguration of the Na2 site associated with binding and dissociation of Na<sup>+</sup>. This predicted H-bond was later confirmed by the dDAT and hSERT structures with Na2 bound (e.g. PDB codes 4M48 for dDAT and 517l for hSERT) (10, 13). Interestingly, in the MhsT<sup>LCP</sup> structure, Asn-176 from TM5i, which is one turn above Thr-269 in hDAT but is conserved in bacterial NSS except for LeuT (see the sequence alignment in Fig. 1), is in close vicinity to Ser-323 (the Na2 coordinating residue that aligns to Asp-421 in hDAT), whereas in the MhsT<sup>HiLiDe</sup> structure, Asn-176 faces outwards.

The potential involvement of TM5i in Na2 binding and exit suggests that it may play a role in coordinating intracellular conformational changes in both eukaryotic and prokaryotic NSSs. In this study, using molecular dynamics and energy calculations, we investigate the role of TM5i in these Na<sup>+</sup>-coupled conformational transitions.

**Results**

**The MhsT<sup>LCP</sup> structure is slightly more inward-facing than the MhsT<sup>HiLiDe</sup> structure**

Using the LeuT structures as references, we compared the degree of open versus occluded character between the MhsT and LeuT structures and between the two MhsT structures (MhsT<sup>HiLiDe</sup> and MhsT<sup>LCP</sup>). For each comparison, we evaluated the structural differences by measuring distances along the ends of TMs on either the extracellular or intracellular side (see “Experimental procedures”). Unlike our previous comparison, which relied upon a structural superposition to identify the relative rearrangements of TMs, the current analysis focused on direct measurements of openness.

On the extracellular side, the three LeuT structures in outward-open (PDB code 3TT1), outward-occluded (PDB code 2A65), and inward-open (PDB code 3TT3) states, which will be referred to as LeuTO<sup>OO</sup>, LeuTO<sup>OC</sup>, LeuTO<sup>HO</sup> below, clearly present three different levels of openness (Figs. 2 and 3). With respect to these structures, MhsT<sup>HiLiDe</sup> and MhsT<sup>LCP</sup> are similarly occluded on the extracellular side, can be considered as intermediates between LeuTO<sup>OC</sup> and LeuTO<sup>IC</sup> (Fig. 3). These intermediates are much more similar to LeuTO<sup>IC</sup> than to LeuTO<sup>OC</sup>, yet remain slightly more open than LeuTO<sup>HO</sup> near the extracellular portion of TM10 (TM10e).

On the intracellular side, MhsT<sup>HiLiDe</sup> is similarly occluded as LeuTO<sup>OC</sup> and LeuTO<sup>IC</sup>, with the exception of the unwinding of the TM5i region that opens a water tunnel to the Na2 site (15). The intracellular gating network surrounding Tyr-245 (which aligns to Tyr-268 of LeuT) is in the same configuration as in LeuTO<sup>OC</sup>/LeuTO<sup>IC</sup>, e.g. the backbone atoms of Tyr-245 and Trp-12 interact directly, just like Tyr-268 and Trp-8 in LeuTO<sup>OC</sup>/LeuTO<sup>IC</sup>. In contrast, MhsT<sup>LCP</sup>, in which TM11 is slightly more tilted toward the membrane, is more inward-facing than MhsT<sup>HiLiDe</sup> and opens at a region that has been implicated as the substrate permeation pathway (20) (circle in Fig. 2C). This difference between the two MhsT structures results from the
altered conformational coupling between TM5i-IL2 and TM1i-NT, which is propagated to the ends of the other TMs (Fig. 2C).

Taken together, the findings show that MhsTHiLiDe and MhsTLCP are similarly occluded on the extracellular side, whereas on the intracellular side MhsTLCP is more open than MhsTHiLiDe (Fig. 3A). Therefore, we propose that the MhsTHiLiDe represents an earlier intermediate and MhsTLCP represents a later intermediate in the transport cycle that leads to full inward opening (Fig. 3B, and see below).

**The role of TM5 in Na2 release and inward opening**

**The impact of the TM5i conformation on the Na2 site and the overall transporter conformation**

To investigate the different impacts that the unwound and structured conformations of TM5i have on the dynamics of the...
transporter, we carried out both conventional (cMD) and replica-exchange molecular dynamics (reMD) simulations in an explicit lipid bilayer environment. To differentiate the simulation results obtained by starting from the different crystal structures, we refer to the simulated conditions as MhsTU (where U indicates unwound TM5i) and MhsTS (where S indicates structured/helical TM5i), which start from the MhsT\textsuperscript{HiLiDe} and MhsT\textsuperscript{LCP} structures, respectively. Because the NT was not resolved in the MhsT\textsuperscript{LCP} structure, we started the MhsTS simulations from a model of the MhsT\textsuperscript{LCP} structure in which the NT was built according to the MhsT\textsuperscript{HiLiDe} structure (see “Experimental procedures” and Table 1).

In our prolonged cMD simulations (Table 1), we observed that the Na\textsuperscript{+} ion can gradually dissociate from the Na2 binding site in the MhsT\textsuperscript{S} state (3 of 5 simulations; Fig. 4), whereas in the MhsT\textsuperscript{U} state, it always remains stably bound. In the simulations in which Na2 dissociates from its binding site, TM1i consistently swings out toward the lipids, so that the MhsT transitions into an inward-facing conformational state (MhsT\textsuperscript{S}), which emerged from our hierarchical clustering analysis (see “Experimental procedures”). The dynamics of such a transition are revealed by the principal component analyses (PCA) we performed on the MhsT\textsuperscript{U}, MhsT\textsuperscript{S}, and MhsT\textsuperscript{S} simulation trajectories (Fig. 5): along the first dominant principal component of the MhsT\textsuperscript{S} simulations (shown as blue arrows in Fig. 5C), a helical TM5i rearranges so that TM1i can dissociate from the TM domain. Such remarkable conformational changes are not observed in any PCA eigenmode of the Na2-bound MhsT\textsuperscript{U} and MhsT\textsuperscript{S} simulations (Fig. 5, A and B), suggesting that the impact of the TM5i conformation on the overall transporter conformation is tightly associated with Na2 binding and unbinding. Interestingly, the helical or unwound TM5i conformations result in the polar Asn-176 of TM5i facing either toward the protein interior or the lipid membrane, respectively. We propose that Asn-176 plays an important role in Na2 dissociation (Figs. 4 and 7) by destabilizing Na2 binding when it faces the Na2 site: when Na2 is bound in the MhsT\textsuperscript{S} state, Asn-176 is in

Figure 3. The MhsT structures represent intermediate states between the outward-occluded and inward-open states of LeuT. In A, MhsT crystal structures (MhsT\textsuperscript{HiLiDe} and MhsT\textsuperscript{LCP}) are compared with the LeuT\textsuperscript{O0}, LeuT\textsuperscript{OC}, and LeuT\textsuperscript{O} structures with regard to their extent of opening at both the intracellular and extracellular sides using the same comparison as Fig. 2 and summarized by the average distances difference on the extra- and intracellular sides. B is a cartoon showing the relative position of the MhsT crystal structures (MhsT\textsuperscript{HiLiDe} and MhsT\textsuperscript{LCP}) compared with the LeuT\textsuperscript{O0}, LeuT\textsuperscript{OC}, and LeuT\textsuperscript{O} structures in the transport cycle, according to A. Each structure is distinguished by a different color for the NT/TM1i and TM5i/TM5m regions; the bound substrate and sodium ions are shown as colored and yellow van der Waals spheres, and the side chain of the conserved Pro in TM5m (Pro-181 for MhsT and Pro-200 for LeuT) is rendered as sticks.

Table 1

| Condition          | Number of replicas | Total simulation time (Ns) |
|--------------------|--------------------|---------------------------|
| MhsT\textsuperscript{U} | 2                  | 3000                      |
| MhsT\textsuperscript{S}\textsuperscript{S} | 6                  | 10,200                    |
| Total              | 8                  | 13,200                    |
close proximity and forms interactions with Na2 through one or two water molecules; it may also transiently form an H-bond with the side chain of the Na2-coordinating residue Ser-323 in TM8, which may facilitate its rotation away from Na2. Notably, when Na2 leaves its site, an H-bond between Ser-323 and Asn-176 is stably formed (see Fig. 7D). Similarly, in our reMD simulations (24 replicas each; Table 2), Na2 dissociates from its binding site in seven replicas in MhsTS (and significantly weakens its binding in many others), but Na2 is always stably bound in the MhsTU state (Fig. 4B). Furthermore, the reMD revealed a similar role of the dynamics of Asn-176 and its stable interaction with Ser-323 after Na2 dissociation, as observed in the cMD simulations.

These consistent results from our cMD and reMD simulations suggest that the Na2 binding affinity is higher in the MhsTU than in the MhsTS state, which prompted us to carry out free-energy perturbation (FEP) calculations to compare the Na2 binding free energy in the MhsTU and MhsTS states. To this end, we alchemically transformed the bound Na1/H11001 in the Na2 site into a water molecule. Indeed, the energy of this transformation is 106.2 ± 2.0 kcal/mol in the MhsTU state, ∼5 kcal/mol higher than that in the MhsTS state (101.7 ± 1.8 kcal/mol). In addition, we calculated the energy of the transformation in the MhsTIS state when Ser-323 rotates away from the Na2 site to

Table 2
List of reMD trajectories

| Condition | Number of replicas (41.28 ns each) | Total simulation time (ns) |
|-----------|-----------------------------------|---------------------------|
| MhsTU     | 24                                | 990.7                     |
| MhsTS and MhsTIS | 24                             | 990.7                     |
| Total     | 48                                | 1981.4                    |

Figure 4. Impact of the absence of Na2 on the conformations at the extracellular or intracellular sides. A and B show the dynamics of the distance between Na1 and Na2 along cMD and reMD simulations representing the “unwound,” “wound,” and “inward-facing wound” (MhsTU (orange), MhsTS (green), and MhsTIS (cyan)) states. The conformational changes near TM1i, TM5m, and TM5i in these states are illustrated in C–E by their representative frames from the cMD simulations.

Figure 5. Intracellular release of Na2 is associated with overall conformational changes of TM1i and TM5i. Representative conformations of the MhsTU (A), MhsTS (B), and MhsTS-to-MhsTIS (C) simulations are shown as gray ribbons and distinguished by different colors for the NT/TM1i and TM5i/TM5m regions; the Trp substrate is rendered as sticks, and the Na1 and Na2 ions are shown as yellow spheres; in the MhsTIS state (C) Na2 has left its binding site. These conformations are overlaid with blue arrows indicating the first principal components of each simulation set (see “Experimental procedures”). For a qualitative comparison between the three principal components, the lengths of these arrows (and thus of the first principle components) are scaled by the total dynamic content (∑λi, with eigenvalues λi) of the MhsTIS simulations.
transiently form an H-bond with Asn-176 and found the Na\textsubscript{2} affinity to be decreased by an additional $\sim 5$ kcal/mol ($94.9 \pm 2.6$ kcal/mol). Taken together, our results indicate that the TM5i conformation and intracellular Na\textsubscript{2} dissociation are coupled to a conformational transition of the transporter to an inward-facing state.

**The allosteric pathways from the intracellular gate to the extracellular vestibule**

We next pursued a systematic characterization of how molecular changes related to the different TM5i conformations can allosterically propagate throughout the entire transporter via an intramolecular interaction network. To this end, we applied the pairwise interaction analysis (PIA) approach adapted from our previous study on allosteric propagation in LeuT (21, 22). PIA detects pairwise interactions between molecular components, at either the atomic, residue, or subsegment level throughout the entire protein, which are significantly affected by a perturbation—in this case the unwound versus helical conformation of TM5i. The identities of the affected interactions are then used to assemble networks that form coherent pathways.

At the subsegment level, our analysis revealed coordinated rearrangements in the form of heterogeneous interaction changes as a consequence of the different TM5i conformations. As shown in Figs. 6 and 7, the significant changes in the interaction network originate from the TM5i and TM5m regions pivoting around Pro-181 and propagate to the NT, TM1i, IL2, TM6i, and IL3 regions on the intracellular side and to the TM1e, TM10e, and EL4b regions on the extracellular side. In the MhsT\textsubscript{HiLiDe} state, the absence of Na\textsubscript{2} leads to the dissociation of the NT from the TM domain and a drastic outward tilting of TM1i on the intracellular side, the impact of which propagates to TM1m and TM1e (Figs. 6B and 7, C and F); such changes result in a much more inward-facing conformation than the MhsT\textsubscript{HiLiDe} and MhsT\textsubscript{CP} structures, with TM1i tilted to a similar extent to that observed in the LeuT\textsuperscript{IO} state (Fig. 8).

At the residue level, by comparing the simulation results of the MhsT\textsuperscript{U} and MhsT\textsuperscript{S} states, we found on the intracellular side that the rearrangement of TM5i triggers reconfiguration of the packing between Trp-12 of the NT, Phe-18 of TM1i, and Tyr-242 and Tyr-245 of TM6i (Fig. 7, C and F). Trp-12, Phe-18, and Tyr-245 are highly conserved among NSSs, and the residues aligned to Tyr-245 in LeuT and DAT had been found to play a critical role in intracellular gating (21, 23). Compared with the stable interactions in this aromatic cluster seen in the MhsT\textsuperscript{U} state, a reconfiguration of this cluster in MhsT\textsuperscript{S} weakens the association between the NT and TM domain (Figs. 6 and 7). Thus, consistent with the MhsT\textsuperscript{TLC} structure in which the NT was not resolved, our simulation results revealed a highly flexible NT in the MhsT\textsuperscript{S} condition.

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**Figure 6. Allosteric interaction changes from the intra- to the extracellular side of MhsT are associated with the intracellular release of Na**\textsubscript{2}. Significant changes in the allosteric interaction network between the MhsT\textsuperscript{U} and MhsT\textsuperscript{S} states (A) and between the MhsT\textsuperscript{HiLiDe} and MhsT\textsuperscript{S} states (B) are represented in the form of a coarse-grained network of subsegments (see “Experimental procedures”): if any of the residue pairs in two subsegments exhibits significant differences in the interaction frequencies between two compared states, the subsegment pair is connected by an arrowed edge if the interactions are more frequent in one state or by an black edge if the subsegment pair involves more frequent interactions in both states. Functional sites are indicated by squares and are connected to the subsegments that form these sites with blue edges, with the indirect contribution of TM5m to the Na2 site indicated by dotted blue edges.
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Figure 7. Conformational changes associated with the intracellular release of Na2. The superposition between representative frames of MhsT
t and MhsT
t states (A–C) demonstrate the changes near the Na2 site and TM5i propagate to the extracellular and intracellular sides. For clarity, only selected segments are shown. B and C are zoomed-in views of indicated regions in A on the extracellular (B) and intracellular (C) sides. The scatter plots of selected measures show correlated changes near the Na2 binding site (D), between the extracellular and intracellular side (E), and between the NT and TM6i (F); these changes indicate the transition from MhsT
 to MhsT
t via the MhsT
 state. The Pearson correlation coefficients for each data set (combining all three states) shown in D, E, and F are 0.55, −0.59, and −0.63, respectively.

which in MhsT
 interacts with Trp-12, Tyr-242, and Tyr-245, faces in the opposite direction toward the lipid headgroups in MhsT
.

On the extracellular side, the packing of Trp-33 and Tyr-37 of TM1 and Pro-289 of EL4 in the extracellular vestibule is also affected by the binding or dissociation of Na2. Our PIA analysis indicates that this effect is propagated through an aromatic cluster formed by Phe-107 and Tyr-108 of TM3 and Phe-230 of TM6 (Fig. 7B). In the MhsT
 state, when Na2 is dissociated, the changes near Trp-33, Tyr-37, and Pro-289 evolve further, with Phe-107 and Phe-381 of TM10 rotating to directly pack against each other (Fig. 7B). In particular, we found Tyr-37 to adopt distinct rotamer distributions in the MhsT
 and MhsT
t states, resulting in the tip of EL4 tilting more toward the extracellular end in the MhsT
 state (Fig. 7B).

Taken together, our findings specifically reveal how the TM5m and TM5i configurations, which is associated with the exit of Na
 from the Na2 site, is allosterically connected both to the reconfiguration of residues in the extracellular vestibule and to the opening of the intracellular gate. As an indicator for correlated changes between the extracellular and intracellular sides, we found the χ
 rotamer changes of Tyr-37 to be correlated with the formation of an ionic interaction between Lys-169 and Glu-330 on the intracellular side (Fig. 7, B and E; Pearson correlation, −0.59).

Comparative experimental validation of the functional role of TM5i in LeuT versus MhsT

Curiously, in LeuT, the TM5i residue that aligns with Asn-176 in MhsT is Ala-195, which cannot form an H-bond with the Na2 binding residue Thr-354 but rather forms a hydrophobic interaction with the methyl group on its side chain. Thr-354 is unique to LeuT, because other bacterial NSSs all have Ser at that position, e.g., Ser-323 in MhsT. We hypothesized that this difference between a hydrophobic and polar environment near the Na2 site in LeuT and MhsT contributes to the much slower transport rate observed in LeuT compared with MhsT (15, 24).

To test this hypothesis and investigate whether TM5 plays a similar role in LeuT as in MhsT, we exchanged Ser-323 in MhsT and Thr-354 in LeuT, individually or in combination with their neighboring residues, Ser-175/Asn-176 in MhsT and Phe-194/Ala-195 in LeuT, and generated the following mutants in MhsT: S323T, N176A, S175F/N176A, and S175F/N176A/S323T; and conversely in LeuT: T354S, A195N, F194S/A195N, A195N/T354S, and F194S/A195N/T354S.

Measuring the uptake of 0.1 µM [3H]Trp by the MhsT mutants in intact Escherichia coli cells (Fig. 9A), we found that transport in MhsT-S175F/N176A and MhsT-S175F/N176A/S323T was dramatically impaired (with initial rates and steady-state levels of transport ≤ 5% of that observed for MhsT-WT). Whereas the N176A mutation yielded initial rates and steady-state levels of transport ≤ 13% of WT, the S323T mutation led to a lesser impairment (~44 and ~54%, respectively). In LeuT (Fig. 9B), substitution of Thr-354 with Ser caused a significant reduction in [3H]Ala transport activity regardless of whether the T354S mutation was introduced individually (~7%/~3%, initial rate and steady-state level of transport, respectively, when compared with LeuT-WT), in combination with A195N (~16%/~22%), or in combination with F194S and A195N (~12%/~13%). Mutating A195N alone or in combination with F194S also significantly reduced transport activity (42%/34 and 17%/22%, respectively). The decreased transport activity of the mutants is not due to reduced expression or membrane insertion of the individual transporter constructs. This is demonstrated by immunoblotting that yielded similar amounts of all MhsT and LeuT variants in the membrane of the E. coli cells.
used for the uptake studies (Fig. 9, C–F). Taken together, these data underscore the significant decrease of transport produced by T354S in LeuT and S323T in MhsT. However, because the triple mutation did not enhance the transport activity of LeuT, these findings suggest that the Na2 site and its neighborhood are robust and that “simple” swapping of key residues does not result in a gain of function of the complex overall transport reaction.
To assess the direct interaction of Na\(^+\) with the MhsT and LeuT mutants, avoiding the complexity of the coordinated binding steps of Na\(^+\) and substrate in the coupled transport mechanism, we measured \(^{22}\text{Na}\) equilibrium binding to the purified protein constructs using the scintillation-proximity assay. Fitting the data shown in Fig. 10A to the Hill equation revealed an EC\(_{50}\) for Na\(^+\) (the concentration of Na\(^+\) that caused half-maximal inhibition of \(^{22}\text{Na}\) binding) for LeuT-WT of 11.3 ± 0.87 mM with a Hill coefficient of 2.09 ± 0.29 (see also Ref. 24), consistent with the presence of two Na\(^+\) ions bound in the Na1 and Na2 sites (PDB code 2A65). The Na\(^+\) binding isotherm for LeuT-T354S yielded a Hill coefficient of 1.08 ± 0.1 with ~3-fold lower affinity (EC\(_{50}\) = 36.2 ± 4.0 mM; Fig. 10A). The smaller Hill coefficient can be interpreted as the loss of one Na\(^+\) binding site. Interestingly, combining the T354S mutation with F194S/A195N restored Na\(^+\) binding of this triple mutant to WT values (LeuT-F194S/A195N/T354S: EC\(_{50}\) = 11.4 ± 1.02 mM, and a Hill coefficient of 2.09 ± 0.34), consistent with the restoration of Na2 binding. The results for MhsT are very similar, because the S323T mutation reduced the Na\(^+\) affinity ~3-fold compared with MhsT-WT and changed the Hill coefficient dramatically (MhsT-WT: EC\(_{50}\) = 10.4 ± 0.84 mM with a Hill coefficient of 2.04 ± 0.33, compared with MhsT-S323T: EC\(_{50}\) = 35.53 ± 1.96 mM with a Hill coefficient of 1.12 ± 0.05; Fig. 10B). Na\(^+\) binding by MhsT-S175F/N176A/S323T positions this mutant construct between MhsT-WT and the MhsT-S323T mutant (with an EC\(_{50}\) of 18.55 ± 2.47 mM and a Hill coefficient of 1.63 ± 0.27) consistent with a partial restoration of Na2 binding in the triple mutant compared with S323T alone. Thus, the optimal “second layer” residues clearly ameliorate Na\(^+\) binding, although their mutations do not rescue substrate transport.

**Discussion**

The results from the combined computational and mutation analyses support a critical role of TM5 in Na2 release in MhsT and in the transition to the inward-open conformation. When TM5i adopts an \(\alpha\)-helical conformation, the transient (in MhsT\(^\text{S}\)) and eventually stable (in MhsT\(^\text{t186}\)) interaction between Asn-176 and the Na2-coordinating residue Ser-323 facilitates its rotation away from bound Na2, thereby weakening Na2 binding. In contrast, when TM5i is unwound (in MhsT\(^\text{F}\)), Asn-176 faces away toward the lipid headgroups, and Ser-323 can thus maintain its tight coordination with the Na\(^+\) in the Na2 site. Consistent with this interpretation, the Ser-323 \(\chi\)1 rotamer is significantly more stable in MhsT\(^\text{U}\) than in MhsT\(^\text{S}\) (Fig. 7D).

In all bacterial NSS transporters except for LeuT, the position of Asn-176 is conserved as a polar residue (and in many cases as Asn). We hypothesized that the hydrophobic interface in LeuT between Ala-195 of TM5 and the Na2-binding Thr-354 of TM8 contributes to its much slower transport rate compared with other NSSs, such as MhsT, in which the aligned residues are Asn-176 and Ser-323, respectively. Substitution of Thr-354 with Ser reduced the \[^3\text{H}\]Ala transport activity and disrupted Na\(^+\) binding, suggesting that Na\(^+\) binding is sensitive to even conserved mutations and likely subtle changes of the local configuration, e.g. the “compatibility” of residues at the TM5 and TM8 interface. Indeed, combining the T354S mutation with F194S/A195N restored Na\(^+\) binding to WT values. However, this triple mutant did not improve the transport rate, which argues that the rate is determined by elements beyond the immediate polar environment for Na2 release: either more extensive changes in the Na2 exit pathway are needed (see below) or other elements have to be considered.

In all our simulated states of MhsT (Fig. 11), and also of hDAT (16), we found that water was consistently present near Na2-TM5i, which forms a water tunnel to the intracellular side and was shown recently to be essential for the release of the sodium from the Na2 site (25). In contrast, the Na2-TM5i region in the simulated LeuT\(^{\text{DO}}\) and LeuT\(^{\text{DC}}\) is much less hydrated (Fig. 11). Such differences may alter the energy landscape in the Na2 exit pathway or even result in different pathways; indeed, analysis of our MhsT simulations in which Na2 escaped indicates that Asn-176, Ser-327, and Glu-330 of MhsT contribute to the Na2 exit pathway, whereas the aligned positions in LeuT are Ala-195, Ala-358, and Gln-361, which are less favored to interact with Na\(^+\). In the mammalian NSS, water accessibility is likely facilitated by a conserved polar Thr/Ser residue present not at the position that aligns to Asn-176 of MhsT, but at a position in TM5i that is one turn above. This is
In line with our previous prediction (19) that in DAT (or SERT), this conserved Thr-269 (or Thr-284) from TM5i would interact with Asp-421 (or Asp-437), which aligns with Ser-323 of MhsT, to facilitate the reconfiguration of the Asp and thereby the exit of Na2.

In MhsT5, TM5i is sufficiently flexible to allow Na2 to escape readily through the water tunnel toward the intracellular side. This flexibility likely results from the distancing of the NT from the TM domain, which is reflected in the higher B-factor values in the TM5i region in the MhsTLCP structure than in the MhsTHiLiDe structure (15). In MhsT5, when the NT is separated from the TM domain, TM5i is in a more mobile state, which is reflected in higher per-residue root-mean-square fluctuations for residues 168–176 than in both MhsTU and MhsT5. Consistently, in our previous simulations of LeuT (20, 24), when the NT is tightly packed with the TM domain in the outward-facing states (LeuT5O and LeuT5C), TM5i is significantly less mobile than when the NT is dissociated from the TM domain. In a recent study of LeuT, we characterized conformational coupling between NT-TM1i and TM5i (26); starting from the LeuT5C state in which a structured TM5i is tightly associated with the NT, we gradually pulled the NT away from the TM domain using targeted MD simulations and monitored in subsequent cMD simulations how TM5i accommodates this change (26). Interestingly, in response to the rearrangement of NT-TM1i, the TM5i-IL2 region evolved into a different helical conformation that reconfigured the interaction between Ile-138 in IL2 and the critical intracellular gating residue Tyr-268. Nevertheless, TM5i in the LeuT5O and LeuT5C states has certain similarity to that in the MhsTLCP structure. Partially for this reason we inferred previously (15) that this configuration likely represented a step prior to the unwound conformation, although our detailed analysis now suggests that the helical state revealed by the MhsTLCP structure facilitates Na2 release. This raises the question of why the NT does not dissociate from the TM bundle in LeuT5O and LeuT5C. We propose that the overall outward-facing conformation in these two states does not allow such a rearrangement, whereas the occluded conformation on the extracellular side observed in both MhsT structures and also in the LeuT5O state allows the transporters to transition to inward-open states, facilitated by unbinding of Na2 and separation of the NT from the TM domain. Such a coupling between the conformations on the extracellular and intracellular sides is consistent with an alternating access mechanism (27). Our results argue that a conformational state occluded at both the extracellular and intracellular sides would be in a relatively high-energy state, with a tendency to transition toward a more inward-facing state as observed in the later stages of the MhsT5 simulations.

The unwound TM5i would allow water to penetrate toward Na2 easily, but in our simulations this can also be achieved when TM5i is helical. Given that the MhsTHiLiDe structure has a smaller opening than the MhsTLCP structure on the intracellular side (Fig. 3) and that the NT in the MhsTHiLiDe structure is tightly associated with the TM domain, the MhsTHiLiDe structure with an unwound TM5i may have captured an earlier event in the transition toward the inward-open state, more closely associated with the initial simultaneous occlusion of both the extracellular and intracellular sides. Our simulation data show that Na2 readily dissociates from MhsT5 but never from MhsTU (Fig. 4), which is also consistent with the unwound structure being an earlier intermediate in the transport cycle. Interestingly, this observation is consistent with the structural changes traversed in the various pathways of Na2 release from the Markov model for DAT (25). Based on these observations, we propose a specific role for the MhsTU state in the transport cycle: during the transition from the MhsTU to the MhsT5 state, the unstructured TM5i leads to the weakening of the association of NT with the TM domain, because of the close association between NT-TM1i and TM5i. The weakening of this association is a pivotal step that eventually leads to the inward-open state (20). Although we have not observed spontaneous transition from the MhsTU to the MhsT5 state, in all of the MhsT5/5 simulations we find that the NT cannot form stable interactions with the TM domain.

Curiously, the transition between unwound and helical TM5i conformations is reminiscent of the accessibility studies of TM5i residues in SERT performed with the substituted-cysteine-accessibility method. In the absence of ligand, the residues of IL2 and TM5i were all water-accessible (from Val-271 to Thr-290), which suggested the possibility of an extended structure and made it difficult to assign the boundary between IL2 and TM5i (28). The rates of reaction, however, were consistent with an α-helical structure in TM5i from Lys-272 through Ala-285, with six highly reactive residues clustering on a single face. The overall water accessibility throughout this stretch of residues, together with the helical pattern of reactivity, might be consistent with a helical structure that transiently unwinds. The inhibitor cocaine, which would be expected to stabilize the outward-open state, protected all the highly reactive positions (29), consistent with a decrease in the accessibility of this region associated with inward closing. In contrast, serotonin, in the presence of its symported ions Na+ and Cl−, enhanced accessibility at these positions, consistent with this region contributing to the substrate permeation pathway. Notably, there is strong evidence for regulation of SERT function through phosphorylation of Thr-276 (30), which aligns with Gly-171 in MhsT, adjacent to unbound Arg-174–Met-180 in MhsTU. In the outward-facing SERT structure, this residue is located on the back side of the helical TM5i-IL2 region with its side chain forming an H-bond with the backbone of Ser-269 (13), in a position and orientation that would seem rather inaccessible to kinases. That this position is phosphorylated in a regulated manner is thus consistent with a transient unwinding of the SERT TM5i helix to allow kinases access to an extended region and with the possibility of a transient state similar to the unwound configuration in the MhsTHiLiDe structure. In the context of our findings from this study, we propose that in the absence of bound ligand, TM5i of SERT is likely in equilibrium between unwound and helical conformations, whereas binding of the substrate serotonin or the inhibitor cocaine differentially affects TM5i, promoting its unwound and helical conformations, respectively.

We find that in MhsT the conformational changes near TM5i and the Na2 binding site are allosterically coordinated with conformational rearrangements on the extracellular side;
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in particular, we observed that after Na2 escapes, the transporter transitions to a new conformation (MhsTiS), which is more inward-open, with TM11 swinging out, and a drastic rotameric reconfiguration of an aromatic residue cluster in the extracellular vestibule. Interestingly, in the occluded MhsT and the outward-open dDAT/hSERT structures, the conserved Trp-33 and Trp-51/Trp-103 near the center of the extracellular vestibule are in different χ̃ rotamer configurations (trans and gauche+, respectively). This finding supports our inference that the reconfiguration of this aromatic cluster is involved in the collapse of the extracellular vestibule in a manner that allosterically couples this region to conformational changes on the intracellular side of the transporter.

Experimental procedures

Comparing conformational states by intersegment distance differences

We defined a distance between the ends (either extracellular or intracellular) of two segments to be the average Ca-Ca distance of all intersegment pairs of the four most extracellular (or intracellular) residues of each segment and then calculated the distance differences for each segment pair between two compared structures. We defined each such distance difference to be significant, if its absolute value was larger than a threshold Δ = 1 Å; this threshold was chosen so that the minor structural differences, such as those between the outward-open and outward-occluded states of LeuT on their intracellular sides are not shown (Fig. 2).

The segment ends in LeuT are: TM1i (residues 13–16), TM1e (residues 34–37), TM2e (residues 41–44), TM2i (residues 66–69), TM3i (residues 88–91), TM3e (residues 121–124), TM4e (residues 166–169), TM4i (residues 180–183), TM5i (residues 191–194), TM5e (residues 210–213), TM6e (residues 241–244), TM6i (residues 264–267), TM7i (residues 276–279), TM7e (residues 303–306), TM8e (residues 337–340), TM8i (residues 366–369), TM9i (residues 375–378), TM9e (residues 392–395), TM10e (residues 399–402), TM10i (residues 421–424), TM11i (residues 447–450), TM11e (residues 474–477), TM12e (residues 483–486), and TM12i (residues 510–513). To compare the distances in LeuT and MhsT structures, we used the structure-based sequence alignment reported in Ref. 14 and mapped the following residue ranges in LeuT onto residue ranges in MhsT: LeuT: 13–70 (MhsT: residues 17–74), 75–128 (residues 75–128, 138–154 (residues 129–145), 165–218 (residues 146–199), 223–296 (residues 200–273), 299–308 (residues 275–284), 315–318 (residues 285–288), 320–397 (residues 289–366), 398–442 (residues 379–423), and 444–468 (residues 424–448). Distances were computed and evaluated with VMD (31) and R (32), and visualized with PyMOL (33).

MD simulations

All simulations were started from the substrate-bound crystal structures of MhsT with the TM5 in unwound (PDB code 4US3) and structured (PDB code 4US4) forms listed in Tables 1 and 2. Based on our established simulation protocols of NSS molecular systems (21, 24, 34), we carried out the cMD simulations of MhsT using NAMD (35) as described previously (21, 24). Briefly, all-atom simulations of MhsT models immersed in explicit 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipid bilayer were carried out with the CHARMM27-CMAP force field (36). In the isothermal-isobaric (NpT) ensemble, constant temperature (310 K) was maintained with Langevin dynamics, and 1 atm constant pressure was achieved with the hybrid Nose-Hoover Langevin piston method (37) applied to an anisotropic flexible periodic cell, with orthogonal pressure components computed independently. The particle mesh Ewald method was used to evaluate long-range electrostatic effects. A time step of 1 fs was used for the first 30 ns and was then increased to 2 fs for the rest of each simulation, which altogether resulted in an accumulated simulation data set of ~13 μs (Table 1). The equilibrated simulation segments of this set were then used for analysis at a time resolution (stride) of 240 ps.

The reMD simulations were performed using NAMD, with all other parameters set to the same values as in the cMD described above. We used 24 replicas in the temperature range of 310–340 K. The temperature increments, with smaller spacing at lower temperatures (from ΔT1,2 = 1.25 K to ΔT23,24 = 1.36 K), were chosen to ensure homogeneous acceptance ratio between all temperature pairs over the entire temperature range (38). The exchange between replicas was attempted every 4.8 ps. The acceptance ratio was ~0.26–0.31.

Principal component analyses

PCA were performed with the Gromacs program on each cMD trajectory set exclusively capturing either the MhsTiL, MhsTc, or MhsTiS state, considering the heavy atom positions of (i) backbone of residues 12–448, (ii) substrate Trp, and (iii) Na1. In the PCA results, the relative dynamic content (κj = λj/∑λi where λi is the ith eigenvalue) of the first principal component is κ1 = 37, 31, and 33% and κ2 = 12, 12, and 20% for other principal components of the MhsTciL, MhsTciS, and MhsTiSc states, respectively.

Interaction network analysis

We performed an interaction network analysis to characterize the MhsT conformations in different states, using the PIA protocol described in Refs. 21 and 22. Briefly, the workflow of this approach is as follows. (i) From the entire set of cMD trajectories (Table 1), identify conformational states with a hierarchical clustering protocol (21, 22) and extract the ensemble of frames for each conformational state. (ii) In each frame, we infer whether an interaction between a residue pair is formed or not based on geometric criteria, i.e. a distance criterion for van der Waals contacts and distance and angle criteria to determine polar contacts. (iii) For each residue pair in each ensemble of frames, compute the interaction frequency f with standard error,

$$\Delta f = \sqrt{\frac{f(1-f)}{t_{tot}/2\tau}}$$  

(Eq. 1)

where ttot is the total simulation time of a simulated condition, and τ = 20.4 ns is the maximum of autocorrelation times computed for a set of selected global observables of the molecule
water molecule by changing the FEP coupling parameter using the NAMD program (35). In particular, we applied an FEP Free-energy perturbations to IL5 (residues 406–426), and TM11i (residues 427–448). 380–387), TM10m (residues 388–392), TM10i (residues 393–405), EL5 (residues 365–379), TM10e (residues 182–194), EL3 (residues 195–217), TM1e (the extracellular section (e) of TM1, residues 24–31), TM1m (residues 218–226), TM1i (residues 227–238), TM1 (residues 239–245), IL3 (residues 246–252), TM7i (residues 253–262), TM7m (residues 263–269), TM7e (residues 270–282), EL4a (residues 283–288), EL4b (residues 289–305), EL4e (residues 306–319), TM8m (residues 320–328), TM8i (residues 329–338), IL4 (residues 339–343), TM9i (residues 344–353), TM9e (residues 354–364), EL5 (residues 365–379), TM10e (residues 380–387), TM10m (residues 388–392), TM10i (residues 393–405), IL5 (residues 406–426), and TM11i (residues 427–448).

### Free-energy perturbations

To compute the free energy of Na2 binding in the MhsT\textsuperscript{U}, MhsT\textsuperscript{S}, and MhsT\textsuperscript{F} states, we performed MD-based FEPs using the NAMD program (35). In particular, we applied an FEP wrapper script we had developed (34) to transform Na2 into a water molecule by changing the FEP coupling parameter \( A \) from 1 to 0 in steps of \( \Delta A \), which were automatically determined at each window of 25-ps simulation time, so that the absolute free energy changes in each window did not exceed \( 4k_B T \) = 2.4 kcal/mol. To stabilize the Na2 binding site during the annihilation of Na2, we harmonically restrained the distances between Na2 and the oxygen atoms of its coordinating residues (Gly-24, Val-27, Ala-320, Ser-323, and Ser-324) to their equilibrium distances with a force constant of 100 kcal/mol/Å\(^2\), whereas the oxygen atom of the appearing water is restrained to Na2 position using the same force constant, so that either molecule would not leave the binding site while appearing/being annihilated. The free energy contributions of these restraints (\( \Delta G_{\text{restraints}} \)) and of the alchemical difference in total charge (\( \Delta G_{\text{Ewald}}\)) (40) because of Na2 annihilation were considered for the total free energy difference of Na2-to-water transformation (\( \Delta G_{\text{Na}^+→\text{H}_2\text{O}} \)) as follows,

\[
\Delta G_{\text{Na}^+→\text{H}_2\text{O}} = \Delta G_{\text{restraints}} + \Delta G_{\text{FEP}} + \Delta G_{\text{Ewald}}
\]

where \( \Delta G_{\text{restraints}} \) is estimated as the difference in average harmonic potential energies between the Na\textsuperscript{+} and H\textsubscript{2}O states, and \( \Delta G_{\text{Ewald}} \) was computed as in Ref. 40. We used representative starting points (both coordinates and velocities) from the corresponding MD simulations and performed at least seven replicates for each set of FEP computations. For “Results,” we summarized the free-energy values from these replicates into averages and standard deviations for each simulated condition.

### Protein expression, purification, immunological detection, binding, and transport assays

Mutations in the leuT and mhsT genes were generated with the QuikChange method in pQO18 and pQO6 derivatives, respectively, using mutagenic primers and were confirmed by sequencing the resulting plasmid constructs (MacroGen, Inc.). [\textsuperscript{3}H]Ala (60 Ci/mmol) or [\textsuperscript{3}H]Trp (20 Ci/mmol) (both American Radiolabeled Chemicals, Inc.) were used to measure the uptake activity of LeuT and MhsT variants, respectively, expressed in E. coli MQ614 (41) following standard uptake protocols (15, 24). Immunological detection of the relative amounts of the LeuT and MhsT variants in the membrane fraction of the MQ614 cells used for the uptake studies was assessed with the monoclonal H-3 His-probe antibody (Santa Cruz Biotechnology, Inc.) in conjunction with a horseradish peroxidase-conjugated secondary antibody and the ECL method (Millipore Luminata Forte HRP, MilliporeSigma). The intensity of the immunoreactive bands was densitometrically analyzed with ImageJ (National Institutes of Health). For the purification of LeuT variants, the proteins were expressed from pQO18 derivatives in E. coli CD41(DE3) (24). mhsT gene variants were introduced into the Lactococcus lactis expression vector and expressed in L. lactis NZ9000 (42). Protein was purified and, when indicated, desalted (with Zeba\textsuperscript{TM} desalt spin columns; Pierce) as described previously (24, 42).

**Author contributions—**S. S., Z. L., M. Q., H. W., J. A. J., and L. S. designed the study. S. S., Z. L., and L. S. carried out the computations and analysis. M. Q. carried out the experiments and analysis. All the authors took part in interpreting the results. S. S., Z. L., H. W., M. Q., J. A. J., and L. S. wrote the initial draft, with L. M. and P. N. participating in revising the manuscript.

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