**TITLE:** Principles of Alternating Access in LeuT-fold Transporters: Commonalities and Divergences

**AUTHORS:** Diego del Alamo (1, 2), Jens Meiler (2, 3), Hassane S. Mchaourab* (1)

**AUTHOR AFFILIATIONS:**
1. Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, USA
2. Department of Chemistry, Vanderbilt University, Nashville, TN, USA
3. Institute for Drug Discovery, Leipzig University, Leipzig, DE

**CORRESPONDING AUTHOR INFORMATION:**
*Hassane S. Mchaourab: hassane.mchaourab@vanderbilt.edu

**GRAPHICAL ABSTRACT:**

**ABSTRACT**

Found in all domains of life, transporters belonging to the LeuT-fold class mediate the import and exchange of hydrophilic and charged compounds such as amino acids, metals, and sugar molecules. Nearly two decades of investigations on the eponymous bacterial transporter
LeuT have yielded a library of high-resolution snapshots of its conformational cycle linked by solution-state experimental data obtained from multiple techniques. In parallel, its topology has been observed in symporters and antiporters characterized by a spectrum of substrate specificities and coupled to gradients of distinct ions. Here we review and compare mechanistic models of transport for LeuT, its well-studied homologs as well as functionally distant members of the fold, emphasizing the commonalities and divergences in alternating access and the corresponding energy landscapes. Our integrated summary illustrates how fold conservation, a hallmark of the LeuT-fold, coincides with divergent choreographies of alternating access that nevertheless capitalize on recurrent structural motifs.
INTRODUCTION
Secondary active transporters tap the potential energy stored in electrochemical gradients to traffic nutrients and cytotoxic drugs across cell membranes[1]. Despite extensive divergence in topologies and ligands[2,3], current dogma hypothesizes that these transporters operate via alternating access, a generic term referring to the exposure of the substrate-binding site to no more than one side of the membrane at a time[4,5]. This mechanism allows transporters to couple conformational changes critical to substrate binding and/or release while minimizing the uncoupled flux or leak of ions down their electrochemical gradients. Although this basic working model of transport was devised nearly sixty years ago[4,5], describing its precise structural choreography enabling coupled ion/substrate translocation in individual transporters presents a formidable scientific challenge (Figure 1A). Until recently, capturing high-resolution snapshots of intermediates along the transport cycle was a daunting task[6,7]. To complement the static snapshots, solution-state measurements, carried out in the absence or presence of various substrates under conditions conducive to interconversion between conformations, directly report on the transporter's energy landscape and allow high-resolution snapshots to be assigned to specific intermediate states observed in the functional cycle[8–11]. However, few transporters have been exhaustively characterized to enable integration of structure, dynamics, and function[12].
Figure 1. Transport cycle, reaction coordinate, and architecture of the bacterial amino acid transporter LeuT. (A) Cartoon depiction of a generic symporter's transport cycle as it isomerizes between outward-facing, occluded, and inward-facing conformations. Sodium ions and substrate shown as orange and purple circles, respectively. Mock reaction coordinate shown below. (B) Panel of experimentally resolved structures of LeuT.

The bacterial Neurotransmitter-Sodium Symporter (NSS) homolog LeuT found in the thermophilic archaea *Aquifex aeolicus* presents a rare example of a transporter that has been studied to this extent[8] (Figure 1B). LeuT couples the uptake of small aliphatic amino acids such as leucine and alanine to an inward sodium gradient and an outward proton gradient[9]. An extensive record of atomic-resolution crystal structures, complemented by solution-state experimental data, enables exquisite insight into its transport cycle although a consensus mechanism remains elusive[8,10,11,13–22]. Here, we review the LeuT body of knowledge and
explore the extent to which its structural homologs, including many transporters found in humans and whose dysfunction contributes to a range of diseases, undergo similar ligand-dependent structural rearrangements[18]. These homologs include mammalian NSSs, such as the serotonin transporter SERT and the dopamine transporter DAT[23–25], as well as a multitude of more distant transporters that have been identified across nearly a dozen families (Tables 1 and 2) belonging to the LeuT-fold. As many of these transporters have been the subject of extensive study over the course of many decades and have been the subject of numerous recent reviews[26–34], here we focus on comparing their structural dynamics and energy landscapes. Necessarily, emphasis is placed on NSSs which are the most extensively studied family of transporters with this topology.
Table 1. Curated library of LeuT-fold protein structures. Conformations are assigned to either outward-facing open (OFOp), outward-facing occluded (OFOc), fully occluded (OO), inward-facing occluded (IFOc), and inward-facing open (IFOp). Structures are marked if they were mutated (†) or bound to inhibitors (‡) or antibodies (§). Chain IDs indicated in lower-case where relevant. Redundant structures have been omitted.

<table>
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<tr>
<th>Protein (Organism)</th>
<th>Conf.</th>
<th>PDB</th>
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<th>Resn.</th>
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Table 2. Curated library of LeuT-fold protein structures (continued).

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<td>6CSF[82]</td>
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STRUCTURAL AND FUNCTIONAL DIVERSITY WITHIN THE FOLD

The ten transmembrane helix topology

Members of the LeuT-fold class belong to the Amino Acid-Polyamine-Organocation (APC) superfamily of transporters[83] and share a core ten-helix structure consisting of two consecutive pairs of five transmembrane helices (TMHs) related by pseudo-twowfold symmetry around an axis parallel to the membrane surface[84,85]. The fold encompasses symporters and antiporters engaged in ion-coupled transport as well as substrate exchange. Analysis of internal symmetry and structural similarity led to assignment of these ten helices into three domains: the bundle domain (TMHs 1, 2, 6, and 7), the hash domain (TMHs 3, 4, 8, and 9), and gating helices (TMHs 5 and 10; we use this canonical numbering scheme and ignore transmembrane helices preceding this ten-helix core in sequence). Additional N- and/or C-terminal helices flank the ten-helix core and vary across individual transporter families comprising the LeuT fold. Almost universal to members of the LeuT fold, TMH 1 and often TMH 6 contain conserved unwound regions, observed near the geometric center of the ten-helix core, and their involvement in ligand coordination is a hallmark of the fold[86]. Importantly, fold recurrence is not accompanied by sequence similarity. Few structural motifs are identifiable at the sequence level[14,18,87], which prevented these transporter families from being co-categorized into a fold prior to the determination of their structures[23,88] (Figure 2).
Figure 2. Functional and structural diversity within the LeuT fold. The LeuT fold is adopted by proteins found in a range of transporter families.

In addition to the NSS family[27], members of the Sodium-Solute Symporter[30] (SSS), Cation-Chloride Cotransporter[28] (CCC), Amino Acid-Polyamine-Organocation Transporter[29] (APC), Natural resistance-associated macrophage protein[26] (Nramp), Nucleobase-Cation Symporter[32] (NCS-1), and Amino Acid/Auxin Permease (AAAP) families adopt this fold. The same topology also describes transporters in protein families unique to prokaryotes, such as those in the Betaine-Carnitine-Choline Transporter[89] (BCCT), Alanine-Glycine-Cysteine Symporter (AGCS), and Potassium Uptake Permease (KUP) families. Finally, transporters in the Branched-
Chain Amino Acid-Cation Symporter (LIVCS) and the Carbon Starvation Family (CstA) families, which have no representatives in the PDB, are also predicted to adopt this fold[83].

**Structural diversity outside the ten-helix core**

Variations in the structural features decorating the ten-helix core further contribute to functional specialization. A remarkable example is SLC38A9, which both exports amino acids from the lysosome and activates the regulatory complex mTORC1 under nutrient-rich conditions[90,91]. An N-terminal domain elegantly couples these two functions by binding to the cytoplasmic cavity and, following displacement by the transported substrate arginine, recruiting GTPases involved in downstream signaling[78,92]. In a potential case of convergent evolution, the C-terminal domain of the pH-dependent Glu/GABA exchanger GadC arrests transport by binding to the intracellular cavity at neutral pH in a nearly identical conformation[48]. Autoinhibition by disordered terminal domains has also been visualized directly in several potassium-chloride symporters [57,58]. In other transporters, such as eukaryotic NSSs, disordered termini instead regulate transport by interacting with a range of cytoplasmic proteins and lipids[93,94]. In BetP, a cytoplasmic C-terminal helical domain regulates transport in response to osmotic stress[95,96]. These domains often go unobserved in structural studies due to truncation and/or intrinsic disorder[24,25,54,56], prompting speculation regarding their role in transport.

Further diversity is found in the range of higher-order assemblies of certain LeuT-fold transporters[97,98]. Whereas oligomeric interfaces in LeuT-fold transporters visualized by crystallography are highly compact, those of structures recently determined by cryo-EM appear weaker and more flexible. For example, several eukaryotic APC and NSS transporters associate with proteins proposed to facilitate trafficking and localization (the experimental structure of b\(^0\)AT1/ACE2 was determined as part of a larger complex that included the receptor-binding domain of the SARS-CoV-2 spike protein[35]). Additionally, protomers of the bacterial potassium transporter KimA and eukaryotic CCCs appeared to dimerize using similar domain swapping mechanisms, which may be indicative of a recurring quaternary assembly mechanism[57,59,61,76]. Notably, experimental structures and MD simulations indicate that these assemblies are highly flexible.

**Conserved elements of ion/substrate binding**
Retention of the ten-helix core, which encodes conserved motifs of substrate binding and ion coordination, is all the more remarkable considering the extent to which the transported ligands differ in size, structure, and polarity. The centrally located substrate-binding site, shared by symporters and antiporters, accommodates ligands ranging in size and charge from halogen ions and divalent metals to sugars and aromatic amino acids. Beyond plasticity of the substrate-binding site, coordination of the coupled ions provides rich examples of conservation and evolutionary tuning across distantly related transporters. While the identity and number of these ions tend to be family specific, the location and geometry of coordination are often recycled. A widely discussed example is the conserved sodium site, termed Na2, found in most sodium-coupled symporters[8,61,62,79,99]. The exception, the sodium-coupled amino acid symporter AgcS, coordinates its only sodium at a position equivalent to LeuT's Na1 site and leaves the Na2 site unoccupied[82]. This is despite its alanine binding site overlapping nearly perfectly with the substrate-binding sites of unrelated amino acid transporters from the NSS and APC families (Figure 3C). To our knowledge, no cations besides sodium ions have been observed in the Na2 site, and no comparable degree of structural conservation has been observed at other ligand-binding sites, such as those involved in binding potassium (transported by SERT, as well as the KUP and CCC families) or chloride (transported by NSSs and CCCs). Moreover, no such conservation is observed in the position of the other sodium binding site in symporters that bind two sodium ions such as the NSSs, SSSs, and BetP[100]. In proton-coupled symporters and exchangers, such as CaiT and members of the APC family, positively charged residues occupy the Na2 position[45,47,49,65], highlighting the malleability of ion coupling throughout the fold.
Figure 3. Examples of conserved ligand coordination. (A) Sodium ions (orange) and leucine (pink) in LeuT. (B) Conservation of the Na2 site. Sodium binding in NKCC1 was inferred from sequence and not directly observed. (C) Partial recurrence of amino acid binding modes. Native substrates are shown in purple, while substrates shown in white are shared in all four panels.

STRUCTURAL BASIS OF ALTERNATING ACCESS
Inferences from crystal and cryo-EM structures

Coupled transport of ions and substrates, i.e. alternating access, entails the interconversion of the transporter between conformational states. In contrast to conservation of motifs of substrate binding and ion coordination, comparison of structural elements of alternating access between LeuT-fold transporters suggest divergence is the rule, not the exception. As high-resolution snapshots of transporters in different states such as Mhp1 were determined in conjunction with comparative spectroscopic studies, this fact became apparent nearly a decade ago with the publication of structures of Mhp1[79,81], BetP[7,62], and LeuT[8,13,85]. Examination of their structural changes revealed a striking lack of consensus over the molecular details of alternating access that has since been reinforced by similar studies in SERT[24,40], DraNramp[66,67], and LAT1[50,51] (Figure 4). Additional OF-open and OF-occluded structures of AdiC[44,101], as well as IF-open and IF-occluded structures of vSGLT[75,99], further expand how these transporters grant access to the substrate-binding site. Overall, comparison of pairs of structures
from different transporters reveals fundamental differences in which helices move and which stay fixed. Below we delve into the specific elements of alternating access, with an emphasis on commonalities and divergences between different families in the fold.

**Figure 4. Variations in inferred conformational dynamics within the LeuT-fold.** LeuT-fold transporters show striking differences in how alternating access between IF and OF conformations is facilitated. Dynamic and static helices are depicted as ribbons and cylinders, respectively. Charts show observed distance between alpha carbons in the indicated superimposed structures (dashed lines indicate extensive stretches of unobserved density).

The gating helix TMH5
Along with the intracellular loop preceding it, TMH5 ranks among the most consistently mobile and dynamic regions in the transporters studied so far[18,87]. In OF conformations, TMH5 nestles against the bundle domain helices TMH1a and TMH6b, forming the highly ordered intracellular "thick gate". In the IF state, by contrast, opening of the intracellular vestibule is often driven by rearrangements that vary across families and even individual transporters within families.

The contribution of TMH5 to alternating access has been most extensively studied in NSSs[18,87]. A GX_{5,6}P sequence, strictly conserved within the family and partially conserved throughout the fold, putatively mediates both bending and unfolding motions instrumental to the initiation of substrate release (Figure 5). Mutagenesis of glycine or proline with this sequence severely abrogates transport in the bacterial NSS MhsT[14], highlighting the importance of the dynamic processes facilitated by this motif. Partial unwinding of TMH5, first observed in a substrate-bound IF-occluded conformation of MhsT[14], has been corroborated in several other NSSs by hydrogen-deuterium exchange/mass spectrometry (HDXMS) studies under conditions promoting the IF conformation of each transporter[11,16,102–104]. However, although IF-open structures of LeuT, SERT, and GlyT1 show this helix protruding out from the rest of the transporter[13,22,37,40], orthogonal measurements in LeuT suggest that substrate and ion binding promote the adoption of conformations that occlude, rather than open, the intracellular cavity[20,21,105,106]. As elaborated below, however, these results in LeuT are qualified by the frequent use of leucine, which has a low transport rate and nanomolar binding affinity[15,102]. Subsequent solution-state experiments bound to different amino acids found that quenching of fluorescent probes attached to the intracellular half of TMH5 was inversely correlated with transport rate[18], suggesting that this IF-occluded conformation may be less stable, relative to IF-open, when transporting substrates with higher turnover rates such as alanine. Nevertheless, in conjunction with other findings discussed below, this points to a mechanism in which TMH5 preferentially adopts the partially unwound occluded conformation when bound to its substrates but transiently bends to facilitate their release.
Figure 5. Bending of TMH5 and TMH10 is observed in a subset of LeuT-fold transporters. **Top left:** LeuT with TMH4/5 and TMH9/10 highlighted. **Top right:** Movement of TM10 in Mhp1. **Bottom:** Movements of TMH5 observed in NSSs, ApcT, and DraNramp in the IF state. Conserved proline residues are highlighted in LeuT, MhsT, and Mhp1.

It is notable that TMH5 adopts a similar, but not identical, conformation in IF-open Mhp1 which shares this GX₃P sequence[81]. Despite this agreement, electron paramagnetic resonance (EPR) spectroscopy measurements revealed disorder in TMH5 that is altogether absent from similar measurements carried out on LeuT in the presence of leucine[20,107]. Interestingly, ApcT also shares a LeuT-like bend despite lacking a proline in TMH5 at the equivalent position[45]. Since its structure has only been determined in a single conformation, and since its homologs such as GadC and BasC maintain a straight conformation of this helix[48,49], the extent to which the aforementioned NSS movements occur in ApcT and its homologs remains unclear[108]. Finally, although TMH5 is also involved in opening the intracellular cavity in DraNramp66, which also lacks the conserved mid-helical proline, it undergoes a rigid-body up-and-out translation rather than bending and unfolding. Many other IF structures, such as those observed in vSGLT and GkApcT, lack a fully resolved stretch of residues corresponding to IL2, located between TMHs 4 and 5, indicating a high degree of heterogeneity in the crystal lattice or the cryo-EM ensemble[49,75]. In summary, the crystallographic data indicate substantial conformational
variation observed across the fold in TMH5 and the loop preceding it that is corroborated by solution-state data indicative of local disorder. Ultimately, these data suggest that the protruded conformation observed in some transporters, though perhaps physiologically relevant and likely fundamental to the transport cycle, may not represent a well-defined low-energy state.

**The gating helix TMH10**

Movement in TMH10, despite its pseudosymmetry relation to TMH5, is less frequently observed (Figure 5). In NSSs, for example, no evidence has been advanced to show involvement in either ligand-dependent conformational dynamics or partial unwinding[11,20,104,109]. Mhp1 shows partial symmetry of TMH10 to TMH5 in both sequence and structure, with comparable increases in conformational heterogeneity detected by EPR under OF-stabilizing experimental conditions[79,81,107]. However, the movement of TMH10 inferred from crystal structures is less dramatic than that of TMH5. In both BetP and DraNramp, differences between their OF-closed and OF-open conformations in this region, though less drastic than in Mhp1, are nonetheless unmistakable; indeed, the corresponding proline in TMH10 facilitating this bend is strictly conserved in the BCCT family and partially conserved among Nramps[26,64]. Although the APC transporter AdiC both shares this specific residue and shows evidence of this structural movement, its structural similarity to the eukaryotic homolog LAT1, which instead has a cysteine at the equivalent position, indicates that this proline positioned halfway across TMH10 may be coincidental[44,50].

**The bundle domain helix TMH1a**

LeuT's twofold pseudosymmetry initially appeared to imply that a rigid-body rotation of the bundle domain relative to the rest of the structure mediates alternating access[84]. This elegant mechanism, elaborated under the name rocking bundle, failed to predict subsequent structural evidence in two key respects. First, the contribution of this domain to alternating access, although prominent in some transporters, is far from universal. Movement in ancillary helices and loop regions has been observed in every transporter studied thus far. Second, the bundle domain virtually never moves as a rigid body. The exception, Mhp1, locks the bundle domain into place and instead pivots the hash domain and gating helices around this scaffold[79].
Figure 6. Movement of TMH1 and TMH7 across the LeuT fold. **Left:** Four representative LeuT-fold transporters with experimental structures determined in both OF and IF conformations. **Right:** Comparison of the LeuT's intracellular side in an outward-facing occluded conformation (top, PDB: 2A65) and an inward-open conformation (bottom, PDB: 3TT3). Stabilization of the IF conformation required disruption of an intracellular hydrogen bond network by mutagenesis of a conserved tyrosine (Y268) and the introduction of a high-affinity antibody, shown in teal. Electron density corresponding to the N-terminus, which contains R5, was not resolved in the inward-facing conformation.

Movements of TMH1a relative to the rest of the intracellular vestibule, in the context of alternating access, appear to be the most variable across this superfamily. Considered critical for substrate release, these movements were observed in X-ray and cryo-EM structures of NSSs and Nramps[13,40,66] and were corroborated in solution (both families, it should be noted, lack N-terminal helices and oligomeric interfaces capable of restricting the dynamics of TMH1a). Particular controversy surrounds the relevance of the signature 45° pivot observed in LeuT, which has been attributed to the use of short-chain detergents commonly used in membrane protein crystallography[103,110], alanine mutagenesis of a conserved tyrosine residue essential for function[13,111], and a conformationally selective high-affinity antibody (Figure 6). Molecular
dynamics simulations of LeuT's IF-open crystal structure in a lipid bilayer later revealed the steep energetic cost of this movement in a more physiological membrane environment[110]. Although this brought attention to the contribution of the membrane mimetic in stabilizing such an extreme conformer, these findings, alongside experimental measurements obtained using both luminescence resonance energy transfer[110] and HDXMS[16] in lipid environments, nonetheless corroborated the more general hypothesis that TMH1a becomes conformationally disordered in the IF-open state. Because these experiments were executed on similar tyrosine-to-alanine mutants, they do not address the extent to which this IF-open conformation is sampled by the wildtype protein in solution. For example, EPR measurements on equivalent tyrosine-to-alanine mutants indicate comparable disorder in TMH1a; by contrast, no such movement was detected without this mutation[11,20]. Experiments in SERT painted a similar picture to LeuT of large amplitude movements of TMH1a[40,104], and recent cryo-EM structures reinforce the distribution of conformations adopted by this helix[39].

This movement is also a hallmark of alternating access in Nramps[66]. In fact, deletion of residues in THM1a was necessary to determine the first atomic-resolution structure of an Nramp, suggestive of disorder comparable to LeuT and SERT[70]. By contrast, transporters in the BCCT, SSS, and other families show marginal movement, which may be in part due to steric hindrance by N- and C-terminal helices[7,72,73,75,99] (Figure 6). Instead, opening of the cytoplasmic pathway is mediated by structural changes elsewhere. Overall, the data suggest that transient detachment of TMH1a from the rest of the bundle domain exposes the substrate-binding site to the cytoplasm in a subset of LeuT-fold transporters.

**The rest of the bundle domain**

A similar pattern of increased disorder under conditions promoting opening of the cytoplasmic cavity has also been observed on the intracellular side of TMH7. EPR and HDX/MS experiments in LeuT suggest that TMH7 partially unfolds and/or translates under such conditions[11,20], contrasting with its lack of movement inferred from the crystal structures. Similarly, in DraNramp's IF-open conformation, but not its IF-occluded conformation, the eight N-terminal residues of TMH7 could not be assigned to electron density, suggesting increases in disorder correlated with substrate release[66]. The most pronounced motion of TMH7 is likely found in LAT1, which swings over 15 Å to close its intracellular cavity[50]. Notably, this
movement does not coincide with comparable movements on the extracellular side, which contrasts with observations in its bacterial homologs\[44,47,48,101\].

TMH1b and TMH6a, located on the extracellular sides of the LeuT topology, consistently undergo smaller scale but nonetheless significant movements \[15,19\]. These helices appear to open the extracellular vestibule by moving in concert with the conserved helix extracellular loop 4 (EL4), located between TMH7 and TMH8, in NSSs\[8,13,24,40\], APC transporters\[50,51\], and Nramps\[66–68,70\]. However, EPR spectroscopy of LeuT indicates that the crystal structures may understate the helices’ movement\[19,20\], a finding attributed to detergent molecules wedged in the extracellular vestibule\[112\]. Interestingly, on the intracellular side, no equivalent coupling between TMH1a, TMH6b, and intracellular loop 1 (IL1) has been detected to our knowledge. Indeed, unlike EL4, IL1 appears to be firmly stapled to the hash domain. Finally, TM2 was not shown to undergo any significant movement among the panel of transporters shown in Figure 4.

**The hash domain**

Relative to movements outlined above, independent helical movements within the hash domain are relatively rare. Mhp1 stands out in rigidly “rocking” this domain, alongside bending in TMH5 and TMH10, to mediate alternating access\[79,81,107\]. Similar movements were reported in vSGLT using EPR\[113\], although these coincided with additional movements distributed throughout the rest of the structure. In contrast, inferred hash domain movements in other transporters are limited to bending of TMH4 on the intracellular side and TMH9 on the extracellular side to complement bending of TMH5 and TMH10, respectively. The contribution of ancillary helices and/or oligomeric interfaces, which are frequently found adjacent to the hash domain, to this phenomenon is unclear. In an interesting twist, a preprint publication describing the IF-to-OF transition in KCC1 proposes that alternating access is purely mediated by movement of TMH3 and TMH8, while TMH4 and TMH9 remain fixed\[56,114\].

**Experimental conditions, mutations, and antibodies confound interpretation of alternating access**

Many LeuT-fold transporters, with Mhp1 and BetP being noteworthy exceptions, could only be coaxed into specific conformations using drastic mutations, conformationally selective antibodies, or high-affinity transport inhibitors (see Tables 1 and 2). In addition to the controversial
use of a transport-abrogating tyrosine-to-alanine mutation in LeuT discussed above[13], stabilization of a purported IF-occluded state was also achieved by mutating a conserved tryptophan similarly found to be essential for function[22]. Crystallographic snapshots of DraNramp in OF and IF conformations required glycine-to-arginine mutations in TMH1a and glycine-to-tryptophan mutations in TMH6a, respectively, that prevented isomerization by obstructing closure of the appropriate vestibule[66,67]. As noted in the release of these structures, the equivalent missense mutation in TMH1a in human Nramps is correlated with severely reduced iron uptake in vivo[115], highlighting the extent to which transport function is impaired. Similarly, crystallization of IF-open vSGLT required a lysine-to-alanine mutation that prevented ligand binding and showed no transport activity[75]. Capture of the OF-occluded conformation of AdiC, achieved using an aspartate-to-alanine mutation, may have played a role in stabilizing a ligand pose distinct from those observed in subsequent ligand-bound crystal structures of the wildtype protein[116]. Equivalent studies of the eukaryotic transporters SERT and LAT1 have employed potent inhibitors that preferentially bind to specific conformations[40,50,114]. Whereas apo SERT readily crystallized in OF conformations, capture of IF state required the small molecule ibogaine. Similarly, LAT1 was structurally characterized in an IF conformation in the absence of ligands[51,52] but could only be described in OF conformations using inhibitors[50]. The introduction of small molecules and/or inactivating mutations arrested both transporters by stabilizing conformers that may be off-path with respect to the protein's functional cycle.

**CONNECTING THE DOTS: FROM STRUCTURES TO LANDSCAPES**

Because secondary active transporters lack a molecular motor such as an ATPase, they must rely on the energy input of ion and ligand gradients to navigate their energy landscapes[117]. Mechanistic models of transport must, therefore, also map the conditions under which specific conformations are populated. The outstanding structural record of LeuT-fold transporters underrepresents conformations that are not amenable to structural characterization[118,119]. Although the experimentally determined structures can be foundational to mechanistic models of transport, such as for instance the glide-symmetry symport mechanisms in NSSs[120] and CCCs[121], these structures do not represent a direct test to these models. An example that will not be discussed further is the possible existence of an allosteric binding site in LeuT and other...
prokaryotic NSSs, which remains controversial despite nearly two decades of investigations[9,122,123].

Rigorous evaluation of energy landscapes requires solution-state techniques reporting on protein kinetics and thermodynamics during the transport cycle[85]. Although several LeuT-fold transporters have been studied using these techniques, it remains difficult to ascertain the extent to which these properties are conserved both within and between families. Below, we summarize the current data on NSSs and SSSs, the only LeuT-fold transporter families with data that permit these fundamental questions to be tackled.

Conformational dynamics of NSSs

The energy landscapes of NSSs have been extensively characterized by ensemble and single molecule biophysical studies carried out in solution. Among the conserved themes emerging from these studies: sodium binding stabilizes OF conformations, substrates tend to stabilize occluded conformations, and absence of either promotes interconversion between IF-open, occluded, and OF-open[11,20,124–126] (Figure 7). These data provide additional context for the experimental structures by reporting on how ligand-binding events bias the conformational ensemble. Concomitantly, the data can reveal steps unanticipated by canonical symport and/or antiport mechanisms. For example, recent data suggesting that potassium stabilizes LeuT into an IF-open conformation indicate a step in which intracellular potassium ions indirectly participate in the transport cycle by competing with sodium and accelerating its release from the central binding site[11,17] Facilitation of substrate release by allosterically bound ions has since been reported in several CCCs[56,59,127].
Despite their structural similarity, LeuT, SERT, and DAT were observed to have distinct steps underlying their conformational dynamics. Whereas LeuT adopts an occluded conformation when bound to leucine[11,20], serotonin-bound SERT fluctuates between IF-occluded and IF-open[104]. More intriguingly, the helical unwinding in IL2 and TMH5 initially proposed by the IF-occluded structure of MhsT[14], although plausible in LeuT and SERT, was altogether inconsistent with HDXMS data collected in DAT suggesting a lack of cooperative movement in this region[109]. In contrast, the results pointed to movement in IL4, a nearby region previously found to be critical to IF-opening in other eukaryotic NSSs but static in LeuT[20,128] (lack of coverage in HDXMS studies of SERT prevented this region from being studied[104]). The presence of lipids and cholesterol in DAT samples presents a confounding factor when attempting to directly compare these results to those collected in SERT, which was probed in detergent micelles. Previous studies reported modulation of conformational dynamics in both eukaryotic NSSs and other active transporters by detergent and lipids[40,104,129–134]. Therefore, while these results highlight the extent to which conformational steps are conserved, they also reveal key deviations in how evolution has fine-tuned these transporters to traffic substrates.

Single-molecule visualization of conformational changes using fluorescent resonance energy transfer (FRET) has added a layer of detail entirely missed by ensemble-level measurements[135,136]. A recent study employing this technique showed how LeuT appears to undergo uncoupled movements on the intracellular and extracellular sides of the membrane in the absence of substrate, including sampling a channel-like conformation simultaneously open to both sides[124]. Although canonical symport mechanisms of alternating access forbid population of such intermediates[137], LeuT appears to avoid uncoupled sodium flux by sealing the intracellular cavity in response to sodium binding. Additionally, this study corroborated previous experimental and computational studies on LeuT and other NSSs suggesting that substrate dissociation, and specifically substrate-dependent sodium dissociation from the Na2 site, is the rate-limiting step in
transport[18,138,139]. However, not all NSSs appear to follow this pattern. A study on wildtype MhsT, in which soluble amino acid-binding proteins labeled with pairs of complementary fluorescent probes were introduced to the interior of MhsT-containing proteoliposomes, determined that the substrate-free IF-to-OF transition was instead rate-limiting[123]. Electrophysiology studies in human NSSs led to similar conclusions[140], suggesting that divergences outlined above in the conformational dynamics of NSSs extend to their landscapes as well.

**Conformational dynamics in SSSs**

The differences in conformational dynamics among NSSs, although not trivial, are dwarfed by those distinguishing them from SSSs such as the eukaryotic sodium/glucose symporter SGLT1, the prokaryotic sodium/galactose symporter vSGLT, and the prokaryotic sodium/proline symporter PutP (Figure 7). While not as extensively studied, these proteins traverse an energy landscape that is fundamentally distinct from those of NSSs. Moreover, the outstanding data highlight the challenges inherent to the interpretation of solution-state dynamics data. EPR measurements of vSGLT[113] and PutP[141] as well as fluorescent labeling and cysteine accessibility measurements in SGLT1[142–144] suggest that ligand-dependent conformational dynamics are effectively inverted relative to NSSs, with apo and/or sodium-rich conditions favoring IF conformations and substrate binding stabilizing the OF conformation. As with NSSs discussed above, key differences exist between these SSS transporters: whereas sodium was not observed to engender large structural changes in vSGLT[113], sodium binding to PutP led to closing of EL4 and increased accessibility of residues lining the intracellular vestibule[141,145,146]. Importantly, similar sodium-invariant conformational dynamics were independently reported in the unrelated bacterial transporter Mhp1 using both EPR and cysteine accessibility measurements[107,147,148], and sodium-driven stabilization of an IF state was also suggested by EPR data in BetP[149].

These data prompted the conclusion that symport in SSSs may be driven by ion electrochemical gradients and/or membrane potentials absent in these *in vitro* studies[113]. Consistent with this hypothesis, accessibility measurements and fluorescent labeling data collected in human SGLT1 in cells that actively maintain a sodium gradient showed a conformational landscape nearly identical to NSSs: unrestricted isomerization between OF and IF in the apo
state[144] and stabilization of the OF conformation in the presence of sodium and absence of glucose[142,150]. Critically, the OF-promoting effect of sodium diminished when the electrochemical gradient was decreased[142]. However, while these data support the hypothesis that the gradient may play a similar role in prokaryotic SSSs, a critical difference is that SGLT1 is electrogenic, with a 2:1 sodium-to-glucose stoichiometry that contrasts with the 1:1 stoichiometry of the prokaryotic model systems discussed above.

CONCLUDING REMARKS: DETERMINANTS OF SUBSTRATE TRANSLOCATION

Single particle cryo-EM has resolved dozens of structures of LeuT-fold transporters that have reinforced questions about the extent to which distinct conformational steps are conserved across transporters with this fold. Some facets of this question can be answered with confidence, such as the evolutionary persistence of the Na2 site or the ligand-binding modes of transporters within the same family. For example, point mutations in residues involved in substrate binding in GAT-1[151], dDAT[152], and BetP[153] were sufficient to modulate cargo preferences. This indicates that, even without access to structures in multiple conformational states, initial crystallographic data could answer key questions about how evolution fine-tunes the substrate-binding site within individual families.

In sharp contrast, the recent surge of structural data has failed to unmask deeper relationships surrounding the sequence and structural determinants of alternating access. The source of variations in the alternating access mechanisms and the corresponding energy landscapes between NSSs and SSSs remains to be unlocked. Indeed, it is unclear why this variation evolved at all, or if they are relevant to other understudied families such as APC and/or Nramp transporters. In part, this knowledge gap stems from the recent prioritization of the acquisition of high-resolution structures, rather than a deeper understanding of dynamics. Recent HDX-MS studies in the NSSs LeuT, SERT, and DAT have shed light on how the kinetics and structural movements of even closely related transporters can differ[11,104,109]. Moreover, spectroscopic and cysteine accessibility in studies in the SSSs SGLT1, vSGLT, and PutP highlight variations in their response to sodium binding, suggesting that these intra-family differences extend to energy landscapes as well. Thus, the apparent variation between LeuT-fold transporters in different families may reflect accumulated changes that occur at the family level which are only now coming to light.
Although formidable, the task of determining alternating access mechanisms of LeuT-fold transporters will almost certainly benefit from the recent development of high accuracy *de novo* structure prediction tools, particularly for families lacking representatives in the Protein Data Bank[83,154,155]. Further advancements and modifications that facilitate prediction of conformational dynamics may reveal the extent to which mechanisms of alternating access are conserved both within and across the LeuT-fold[156]. However, despite these advancements, only experiments can provide a rich understanding of transport dynamics of individual proteins, such as their energy minima and the rate-limiting steps of transport.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Derek P. Claxton and Ms. Alexandra Shwartz for critical reading of the manuscript. Figures were designed using GNU Image Manipulation Program, Inkscape, ggplot2, and PyMol. All structural alignments shown were performed using TM-Align[157]. This study was funded by the National Institutes of Health (HSM: GM 128087) and the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) through CRC 1423, project number 421152132, subproject Z04 (JM).

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