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Alternating access mechanisms of LeuT-Fold transporters: trailblazing towards the promised energy landscapes

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Abstract

Secondary active transporters couple the uphill translocation of substrates to electrochemical ion gradients. Transporter conformational motion, generically referred to as alternating access, enables a central ligand binding site to change its orientation relative to the membrane. Here we review themes of alternating access and the transduction of ion gradient energy to power this process in the LeuT-fold class of transporters where crystallographic, computational and spectroscopic approaches have converged to yield detailed models of transport cycles. Specifically, we compare findings for the Na⁺-coupled amino acid transporter LeuT and the Na⁺-coupled hydantoin transporter Mhp1. Although these studies have illuminated multiple aspects of transporter structures and dynamics, a number of questions remain unresolved that so far hinder understanding transport mechanisms in an energy landscape perspective.

Graphical Abstract

Introduction

Building and maintaining solute and ion concentration gradients across cell membranes is fundamental to the physiology of organisms across kingdoms of life. Dedicated membrane proteins mediate transport processes that include the acquisition of essential nutrients, the

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extrusion of cytotoxic drugs and the uptake of signaling molecules, often against their concentration gradients. To drive energetically uphill movements of substrates, secondary active transporters harness input energy from electrochemical ion gradients. The general dogma in the field posits that transporters operate by “alternating access” wherein centrally located binding sites for ligands are exposed to extracellular and intracellular solutions through conformational rearrangements of the transporter structure [1–4]. Transport proceeds through a series of intermediates separated by energy barriers and may involve the population of transition states. Thus, a mechanistic understanding of transport entails i) the elucidation of the structural motifs of alternating access, and ii) how the underlying conformational changes are powered by the potential energy of electrochemical gradients. Addressing these two critical aspects of transport awaited the dawn of the structural biology age. With atomic resolution structural contexts at hand, the integration of biophysical, biochemical and computational methods has made it possible to formulate and test models of transport with increasing sophistication and detail.

An example of the confluence of biophysical methods is the evolving understanding of complex relationships between sequence, structure, and dynamics in the LeuT-fold class of transporters. The leucine transporter (LeuT), a Na⁺-coupled symporter from *Aquifex aeolicus* belonging to the neurotransmitter:Na⁺ symporter superfamily (NSS) became the founding member of the LeuT-fold structural class following the publication of its first crystal structure in 2005 [5]. Subsequently, ten additional transporters from different functional superfamilies were found to have the LeuT-fold architecture [6–17]. Below, we outline the common structural features that define this fold, review the current understanding of the structural orchestration of alternating access, and expound on the emerging insight into the energetics and mechanistic divergence of transport cycles for two LeuT-fold transporters.

The inverted repeat as the conserved motif of LeuT-fold transporters

Comparative analysis of LeuT-fold structures identified common structural motifs that define the architecture of the fold and are intimately associated with ligand recognition and binding. Central among them is a conserved five-helix inverted repeat motif [18] where the structure of the first five core transmembrane helices (TM) is related to the next five helices by a two-fold symmetry around an axis nearly parallel to the membrane (Fig. 1). Structural similarity between the inverted repeats within individual LeuT-fold transporters is consistently high with RMSD values between 2 and 6 Å, despite the lack of significant sequence similarity between the repeats.

The second common structural motif pertains to ligand recognition and binding. TM helices equivalent to TM1 in LeuT (henceforth TM1') show a centrally-located discontinuous region in all LeuT-fold transporters crystallized so far. The structure of TM6' is discontinuous in LeuT [5] and most LeuT-fold members, though TM6' is bent in CaiT [11,14] and fully continuous in BetP [9]. The discontinuous nature of TMs 1' and 6' provides hydrogen bonding partners and dipole moments that participate in ion and substrate coordination [19]. Substrates bound at the primary binding site (sometimes referred to as S1)

are stabilized by proximally-bound ions or, in the case of ion-independent members, interactions with side chains of optimally positioned residues [14].

Ion dependence and stoichiometry vary widely among LeuT-fold transporters. The LeuT structure [5] identified two Na^+ binding sites: Na1 located proximal to the substrate binding site and coordinated by discontinuous regions of TMs 1' and 6' and Na2 at the interface of TMs 1' and 8' (Fig. 1). Na1 directly coordinates the substrate, while Na2 indirectly interacts through intervening residues. The Na2 site appears to be conserved in Na^+ -dependent transporters with evidence of bound Na^+ derived from electron density difference maps or inferred from functional studies of predicted Na2 site mutants [8]. By analogy, the structure of the H^+ -dependent symporter ApcT revealed that the amine group of a lysine side chain occupies the Na2 site [10]. When protonated, this residue is presumed to provide a positive charge that performs a similar function to a Na^+ . For the ion-independent CaiT, the Na2 site is occupied by a positively charged Arg residue providing similar interactions with TMs 1' and 8' [14]. In contrast with Na2, occupation of the Na1 site is rare within the current library of LeuT-fold transporters with only NSS members showing crystallographic evidence of Na^+ binding at this site [5,15–17]. Although Na1 was modeled originally at a structurally equivalent site in BetP, an alternative location for Na1 was subsequently proposed [20]. Whereas Cl^- dependence is an important feature of eukaryotic NSS, LeuT was shown to be Cl^- -independent as a consequence of the substitution of a highly conserved serine in NSS by an acidic residue (E290) in close proximity to Na1 [17,21–23]. This glutamate has been proposed to be involved in H^+ counter transport during the rate limiting return step from the inward-facing to the outward-facing conformation [24].

Structural mechanisms of alternating access

The Rocking Bundle model

The recurrence of the inverted repeat in LeuT-fold transporters stimulated conjectures of a common structural mechanism of alternating access. This hypothesis was initially formalized by Forrest et al. [18] in the context of a computational model of LeuT's inward-facing conformation which was generated by threading the sequences of each inverted repeat onto the alternate repeat. A comparison of the outward-facing LeuT structure and the inward-facing computational model identified a 4-helix bundle (TMs 1, 2, 6, and 7) which undergoes a rotation around an axis that passes through the substrate binding site. Referred to as the Rocking Bundle, this model was distinguished by its prediction of rigid-body, collective motions of the bundle [25] relative to a scaffold motif consisting of TMs 3–5 and 8–10. In recent analysis, inverted symmetry was uncovered in secondary transporters of different folds thus extending the Rocking Bundle as a general model of alternating access [26].

Mhp1 crystal structures: Experimental support for the Rocking Bundle Model

Mhp1 was the first member of the LeuT-fold class to be represented by a complement of states including a Na^+ -bound, substrate-free outward-facing conformation [7], a Na^+ - and substrate-bound outward-facing occluded conformation [7], and a Na^+ - and substrate-free inward-facing conformation [27] (Fig. 2). The alternating access model inferred from these

structures [27] was generally consistent with the Rocking Bundle predictions [25]. Both the bundle and scaffold motifs were found to be rigid units, with 0.7Å and 0.9Å RMSD for the motifs, respectively, among the three structures [27]. The only deviation was the inference that symmetrically-related TM helices 5 and 10 participate in occlusion of the permeation pathway from the intracellular and extracellular sides respectively, acting as thin gates. MD simulations revealed that the gating motions of TMs 5 and 10 were likely stochastic and not directly coupled to the relative motion of the bundle and scaffold motifs [27].

Model of LeuT alternating access inferred from crystal structures

The determination of outward-facing and inward-facing structures of LeuT led to an atomic resolution model of the conformational cycle underlying LeuT transport [28]. Relative to the substrate-occluded structure [5], the substrate-free, Na⁺-bound conformation showed opening of the extracellular permeation pathway as a result of outward shifts in TMs 1b, 2a, and 6a (Fig. 2) as well as extracellular loop (EL) 3 and TM11 while the intracellular vestibule was completely occluded in a similar configuration to the outward-facing occluded structure. In contrast, the inward-facing structure was characterized by the prominent displacement of intracellular TM1a away from the protein core, tilting 45° into the membrane. The repositioning of TM1a was coupled to a large-scale outward translation of TM5 and a 17° tilt of TM6b away from the central binding site. These rearrangements lead to opening of the intracellular vestibule providing a plausible path for substrate release. On the extracellular side, TMs 1b and 6a and EL4 shift into a more closed conformation than was evident in any of the preceding crystal structures. Notably, the inferred alternating access model of LeuT is dissimilar from the predicted Rocking Bundle [25]. The core of the bundle motif aligns only to 1.6Å C_α RMSD between the outward-facing and inward-facing structure, with the deviation predominantly the result of shift in position of TM1a (Fig 2).

Hybrid Models of alternating access: BetP and vSGLT

The largest complement of crystal structures for a LeuT-fold transporter in different conformations was obtained for BetP [29], a trimeric Na⁺-coupled symporter of betaine. Strikingly, the BetP structures demonstrated an asymmetric trimer organization with each protomer manifesting a distinct orientation of the bundle motif. These orientations were interpreted based on solvent access of the betaine binding site and included multiple apo and substrate-bound conformations. A comparison of BetP crystal structures inferred elements of conformational changes identified individually in the LeuT and Mhp1 alternating access models. A relatively modest rotation of the bundle relative to the scaffold of 13° was identified as was a small-scale translation of TM1a' of 5Å with an 18° tilt relative to the protein core (Fig. 2). Subtle rearrangements of TM5' and 10' were predicted to support uncoupled gating of the intracellular and extracellular sides, respectively [29], as in Mhp1 [27].

Although only inward-facing [30] and inward-facing occluded [8] conformations have been captured for the bacterial galactose transporter vSGLT, the conformational transition implied by these structures is quite similar to that described for BetP involving rather small-scale rotation of the bundle relative to the scaffold of 6° and an independent 13° tilt of TM1a'.

All in the NSS family: LeuT, DAT, SERT and MhsT

Perhaps it was not surprising that conservation of the LeuT fold appears to be the rule among evolutionarily-related transporters across kingdoms of life. Crystal structures of the drosophila dopamine transporter (DAT [17,31,32]) and the human serotonin transporter (SERT [16]) revealed a core LeuT-fold motif which supports similar coordination of substrate and ions to that of LeuT. While DAT and SERT have been captured in inhibited conformations similar to the outward-facing state adopted by LeuT, the bacterial NSS homolog tryptophan transporter MhsT [15] showed a novel inward-facing conformation with distinct structural features. When compared to the outward-facing conformation of LeuT, the inward-facing MhsT structure displays essentially no rotation of the bundle motif relative to the rest of the structure. Furthermore, MhsT forms a solvent accessible intracellular cavity exclusively through the unwinding and subtle translation of TM5. Further experiments are necessary to determine if the differences in the LeuT and MhsT structures represent snapshots of conformational sampling along the mechanistic cycle.

Whereas these structures have been interpreted in the context of a ligand-driven outward-facing to inward-facing conformation, recent crystallographic analysis of LeuT in the putative rate-limiting return step has identified residues involved in stabilizing an empty transporter in absence of mutations or an antibody [33]. A LeuT structure, determined at acidic pH and devoid of Na^+ or substrate, revealed structural changes in the polypeptide backbone of the unwound region of TM1 that enabled occupancy of the primary substrate binding site with a strictly conserved Leu side chain (Leu25). In this structure, two residues that participate in coordination of Na1 (Asn27 and Thr254) in the outward-facing conformation were found to directly interact with a presumably protonated E290. Based on these observations, the authors proposed an intricate model of the inward- to outward-facing transition in which Leu25 functions as a gate to control Na^+ access to Na1 and Na2 from the extracellular side and is coupled to protonation and deprotonation events of E290.

The next frontier: Analysis of conformational dynamics using spectroscopic approaches

While crystal structures provide atomic resolution of putative intermediates and detailed descriptions of ligand coordination, defining the mechanistic identity of these structures along the transport pathway can be challenging. Many factors conspire to cloud the interpretation including but not limited to conformational selection in the confines of the lattice, solubilization in harsh detergents which could alter the relative energies of states, and the use of mutations and antibodies. Advanced biophysical approaches such as electron paramagnetic resonance (EPR) spectroscopy and single-molecule fluorescence resonance energy transfer (SM-FRET) allow framing crystal structures in mechanistic contexts and the investigation of equilibrium-based conformational transitions that power the transport cycle [34,35]. Below, we describe the application of these approaches to LeuT-fold transporters and highlight their contribution to understanding transport mechanisms.

Equilibrium-based Model of Mhp1 transport

Site-directed spin labeling (SDSL) and double electron-electron resonance (DEER) spectroscopy were employed to uncover the contribution of ion and substrate binding to Mhp1 conformational dynamics [36]. Distance distributions monitoring helices predicted to undergo relative movements demonstrated that the crystallographically-captured outward-facing and inward-facing conformations are sampled in solution, supporting the Rocking Bundle model of Mhp1 alternating access mechanics (Fig. 3). Importantly, these conformations were shown to exist in a dynamic equilibrium under all ligand conditions, with ligand-binding shifting the equilibrium between conformations. In the ligand-free apo state, Mhp1 predominantly adopts the inward-facing conformation but low probability fluctuations enable sampling of the outward-facing conformation. Concurrent Na⁺ and substrate binding stabilizes the outward-facing state as the major population, yet the transporter still samples the inward-facing state infrequently as manifested by its low steady-state population under these conditions. These findings, specifically the stabilization of the outward-facing conformation by the substrate, ran counter to prevailing models that assumed substrate binding would trigger transition to the inward-facing conformation [37]. The insight into the structural dynamics of Mhp1 framed the crystal structures into a novel model of Mhp1 transport that emphasized equilibria between conformations [36], and highlighted the synergy between atomic resolution models and spectroscopic approaches.

Ligand-dependent conformational transitions in LeuT

Initial EPR analysis of LeuT uncovered local and global structural changes that modulate access to the extracellular vestibule as a consequence of Na⁺ and Leu binding [38]. However, interpretation of the overall mechanism of alternating access was impaired by the limited set of EPR constraints. This limitation was overcome in a subsequent extensive investigation [39] of LeuT conformational dynamics with the goal of identifying structural motifs that contribute to transporter isomerization between inward- and outward-facing intermediates. Similar to Mhp1, multicomponent distance distributions underscored the presence of equilibria between states. However, in contrast to Mhp1, exhaustive DEER analysis revealed substantial deviations from the crystallographic model [28] both at the level of the mechanistic identity of the structures as well as the conformational changes underlying the transition between states. Analysis of distance distributions in conjunction with restrained-ensemble MD simulations indicated that the crystal structure assigned as substrate-occluded is in fact an outward-facing conformation (Fig. 4). Occlusion on the extracellular side necessitates a larger displacement of TMs 1b, 6a and 7, and EL4 than what was implied by the crystal structures.

Moreover, the DEER analysis identified distinct structural motifs underlying transition to the inward-facing conformation. The EPR-derived model for the inward-facing conformation implicated TMs 6 and 7 rather than TMs 1 and 5 as the motif associated with opening of the intracellular vestibule (Fig. 4). The distance distributions also revealed that TM1a is located in a position between the two extremes captured by the outward-facing and the inward-facing crystal structures. While the distributions monitoring the position of TM1a were broad, suggestive of a dynamic element, they did not change under different ligand conditions in contrast to the inferences from the crystal structures.

Importantly, capturing the inward-facing crystal structure necessitated a number of conserved residue mutations and stabilization with an antibody [28], which may explain the disparate results. A fundamentally unresolved question is whether the crystal structure captured a transition-like state that is fleetingly populated under equilibrium conditions of the DEER analysis.

Kinetics of LeuT alternating access

Single molecule techniques are ideal to resolve such questions and to determine the kinetics of transporter isomerization. In two elegant papers, Zhao et al. observed fluctuations in the efficiency of energy transfer between pairs of fluorescent probes at the extracellular and intracellular sides of LeuT [40,41]. This was the first report of movement of the N-terminal region preceding TM1, referred to as the intracellular gate, which was predicted by MD simulations [41] and later verified by DEER analysis [39]. Dwelling times in the inward-open and inward-closed states corresponded well with overall transport rates thereby establishing the functional relevance of the observed movements [41]. Moreover, the substrate Ala, which is transported at higher rates than Leu, enhanced rates of opening and closing of the intracellular gate [40]. While these studies were pivotal in revealing fundamental aspects of LeuT dynamics, the explicit role of TM1 was not conclusively answered owing to the placement of the fluorescent probe at residue 7 which precedes TM1a [41].

Together with functional analysis and MD simulations, these studies advanced an allosteric model of LeuT transport that relies on the functional significance of a secondary substrate binding site in the extracellular vestibule of LeuT, known as the S2 site, hypothesized to control intracellular gating [42]. Notably, the existence of this site in LeuT continues to be controversial [43] due to the conspicuous absence of crystallographic evidence for binding of transporter substrates. Interestingly, two (*S*)-citalopram molecules, a widely prescribed reuptake inhibitor, were shown to simultaneously bind human SERT [16]. One molecule occupied the primary binding site, and the other an allosteric site situated in the extracellular vestibule reminiscent of an inhibitor binding site in LeuT [44]. Indeed, inhibitor occupation of this allosteric site in SERT significantly reduced the dissociation rate of (*S*)-citalopram bound in the primary site [16].

Perspective: Energy landscapes and transport mechanisms

In contrast to the conservation of the core fold and the determinants of ligand binding, the conformational dynamics of LeuT-fold transporters appears to be divergent and mirrors the remarkable diversity of their substrates, driving ions, and stoichiometries. While the relative rotation of the bundle and scaffold motifs provide an elegant explanation for the formation of intracellular and extracellular vestibules, rigid body movement between these domains appears to be the exception so far. Not unexpectedly, elements of ligand-dependent conformational changes appear to be recycled but no *a priori* predictable pattern has emerged. High resolution structures of new LeuT-fold transporters in different conformational states as well as applications of spectroscopic and computational tools may further uncover similarities and differences in the structural basis of alternating access.

An important aspect that emerges from the spectroscopic analysis is the role of conformational equilibria in the transport mechanism. Under virtually all conditions, LeuT and Mhp1 are in equilibrium between at least two conformations (Figs. 3 and 4). Substrate and ion binding shift the equilibria through selective stabilization of conformations. We submit that this ensemble perspective is central to understanding transport mechanisms. Although not yet explicitly tested, lipids are also likely to modulate this conformational equilibrium. Recent spectroscopic analysis of the proton-dependent multidrug transporter LmrP uncovered shifts in the free energy landscape between inward- and outward-facing conformations that were dependent on lipid headgroup interaction with a conserved charge-relay network [45]. The observation of bound cholesterol in DAT crystal structures [17] to stabilize an outward-facing state that promotes inhibitor binding [46] supports the notion of potential lipid effects on the conformation of LeuT-fold transporters.

The ultimate goal is to construct a reaction coordinate for transport, or an energy landscape. This endeavor entails an atomic resolution description of intermediates, determining their relative energies and the rate constants of the transitions, and defining the pathway(s) that connect them. The combinations of high resolution structure determination and spectroscopic approaches can now be utilized to obtain the first three descriptions as has been demonstrated elegantly in the case of the bacterial glutamate transporter [35]. Characterization of transition pathways remain an experimentally challenging task. Recently, computational methods have begun to elucidate this aspect of energy landscapes [47–49] and specific models can be experimentally tested. The lesson from protein folding, where a similar undertaking has been attempted, is that the road to energy landscapes is likely to be an arduous one, best navigated through the integration of multiple techniques and approaches.

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by DEER spectroscopy uncovers a Na⁺- and Leu-dependent conformational equilibrium and identifies unique motifs involved in forming the inward-facing state. The study also reports the role of the crystallization detergent toward stabilizing an outward-facing conformation and the impact of mutations on the intracellular gate to create a distinct conformation that was not observed in the wild type background.

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Highlights

- Alternating access in the LeuT-fold class of transporters is discussed
- Structural analysis suggests mechanistic divergence among class members
- Ligand-dependent conformational equilibria is a defining feature of transport
- Descriptions of transport energy landscapes require a multifaceted approach

Figure 1. Inverted repeat and motifs of ligand recognition and binding in representative LeuT-fold transporters

Ligand-bound crystal structures of LeuT (PDB 2A65), Mhp1 (PDB 4D1B), BetP (PDB 4AIN chain b) and CaiT (PDB 2WSX chain a) are shown with helices colored similarly to illustrate conservation of overall topology as well as substrate (cyan) and ion (magenta) binding sites. Helices and Na⁺ are designated numerically using LeuT nomenclature. Ion binding sites are substituted by residue side chains in CaiT. Superimposition of helices composing the inverted repeat (IR, bottom left of each panel) can be achieved by an approximate 180° rotation of one repeat (IR2) in the membrane plane.

Figure 2. Alternating access models derived from crystal structures

(a) Inward-facing (IF, left) and outward-facing (OF, right) conformations of LeuT (PDB 3TT3 and 3TT1), Mhp1 (PDB 2X79 and 2JLN), and BetP (PDB 4LLH chain c and b) are shown. Scaffold helices 3, 4, 8, and 9 are shown as a grey surface within the outline of the complete protein structure. Bundle helices 2 and 7 and EL4 are shown as colored surface to demonstrate degree of bundle rotation. Bundle helices 1 and 6 are depicted as colored cartoons. The extracellular and intracellular vestibules are shown in cyan. (b) The core motif of the bundle for outward-facing and inward-facing conformations is shown after alignment

along TM helices 2 and 7 to display deviations from rigid body movements. RMSD calculations are shown for each alignment.

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Figure 3. Spectroscopic analysis of Mhp1 alternating access supports the Rocking Bundle model Outward-facing (OF, PDB 2JLN) and inward-facing (IF, PDB 2X79) crystal structures are shown with relevant helices depicted in cartoon representation. Distances measured by DEER between sites of interest are indicated by a line connecting black spheres that identify spin label locations. The corresponding distance distributions are shown in two middle panels for TM7 (extracellular) and TM1 (intracellular) under apo (black), Na⁺-bound (blue), and Na⁺/substrate-bound (red) conditions, emphasizing shifts in conformational equilibria between closed and open states (grey). As seen in the four right panels, these EPR-derived states are correlated to distributions calculated from each of the three available crystal structure conformations: outward-facing occluded (OFO, PDB 2JLO), outward-facing (OF), and inward-facing (IF). Predicted distance distributions from crystal structures were calculated by the Molecular Dynamics of Dummy Spin Labels (MDDS) approach [50,51].

Figure 4. Ligand-dependent changes in distance distributions deviate from the crystallographic model of alternating access

Outward-facing (OF, PDB 3TT1) and inward-facing (IF, PDB 3TT3) crystal structures are shown with relevant helices depicted in cartoon representation. Distances measured by DEER between sites of interest are indicated by a line connecting black spheres that identify spin label locations. Colors correspond to those in Figure 3. Distance distributions shown in the top panels for TM6 on either side of the membrane underscore ligand-dependent conformational equilibria. The bottom six panels compare the EPR-derived closed/open states to distributions calculated from each of the three crystal structure conformations of

LeuT: outward-facing occluded (OFO, PDB 2A65), outward-facing (OF), and inward-facing (IF). Predicted distance distributions from crystal structures were calculated by the Multiscale Modeling of Macromolecular systems (MMM) approach [52].

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