The Rocking Bundle: A Mechanism for Ion-Coupled Solute Flux by Symmetrical Transporters

Crystal structures of the bacterial amino acid transporter LeuT have provided the basis for understanding the conformational changes associated with substrate translocation by a multitude of transport proteins with the same fold. Biochemical and modeling studies led to a "rocking bundle" mechanism for LeuT that was validated by subsequent transporter structures. These advances suggest how coupled solute transport might be defined by the internal symmetry of proteins containing inverted structural repeats.

Alternating Access as a Mechanism for Transmembrane Solute Transport

For the last 40 years, our understanding of the mechanistic basis for solute transport across membranes has been shaped by the alternating access mechanism (22, 37, 56). In this model, transported solutes bind to a site in the transporter that can be exposed by conformational changes to one side of the membrane or the other. Early mechanistic interpretations of this model suggested that the transporter (carrier) bound its substrate on one side of the membrane and then migrated to the other side, where substrate was released (6). Although this moving carrier model is probably accurate for ionophores such as valinomycin, nigericin, and 2,4-dinitrophenol, our current understanding of solute membrane structure suggests that the energetic barrier for such movement is prohibitive.

A compelling feature of the alternating access model is that it can account for many types of transport. In the simplest of these, uniport (also facilitated or mediated diffusion), the transporter allows passage of a single solute across the membrane. An example of this process is the transport of glucose into mammalian cells catalyzed by the GLUT family of transporters (5). For alternating access to account for uniport, the substrate would bind to the transporter from one side of the membrane, and a conformational change would close access to the binding site from that side and open access to the opposite side. The solute would then dissociate, and another conformational change would bring the transporter back to its original conformation (FIGURE 1).

The alternating access mechanism also accounts for coupling the flux of two or more solutes. This process is sometimes referred to as secondary active transport to distinguish it from primary active transport, which is driven by metabolic energy such as ATP hydrolysis. In symport (co-transport) the solutes cross the membrane in the same direction, and in antiport (countertransport, exchange) they cross in opposite directions. For symport, two or more solutes bind and are released to the opposite side of the membrane in the same way as uniport, followed by reorientation of the transporter as with uniport (FIGURE 1). In antiport, one or more solutes is transported across the membrane (like uniport or symport), but then before reorientation of the transporter, a different solute binds and is subsequently transported in the opposite direction (FIGURE 1). In both symport and antiport, the energy of a solute transmembrane concentration gradient can thus be used to concentrate another solute on one side of the membrane.

If different transporters use this same general mechanism to catalyze uniport, symport, or antiport, there must be additional parameters that determine which kind of transport occurs in each case. Jencks (23) postulated that there must be "rules" followed by different coupled vectorial processes to account for different
modes of coupling. In alternating-access transport mechanisms, these rules must determine when the transporter may undergo the conformational change that switches binding site access from one side of the membrane to the other. For example, a transporter will catalyze symport if it undergoes the conformational change when either both solutes or none are bound, but not if only one is bound. Antiport, in contrast, requires that the conformational change occurs only when a substrate is bound but not when the binding site is empty (see FIGURE 1). The conformational change that transforms a transporter from outward- to inward-facing is likely to go through some intermediate state. One could imagine states in which the binding site is accessible simultaneously from both sides of the membrane and others in which it is accessible from neither side, as in FIGURE 1. A transporter that moves its substrate against a concentration gradient is less efficient if there is an intermediate from which the substrate could bind and dissociate to either side of the membrane. Such an intermediate would allow uncoupled flux of the bound substrate and would be equivalent to a channel through the membrane, albeit one selective for the substrate. A stark example is the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, which can be converted from an ATP-coupled transporter to a channel for Na\textsuperscript{+} and K\textsuperscript{+} by binding of the marine natural product palytoxin (4). In its channel mode, the protein dissipates cation gradients much faster than they can be generated by ATP hydrolysis. Consequently, we expect that transport by alternating access requires an occluded intermediate in which the substrate binding site is not accessible from either side of the membrane (FIGURE 1). Nevertheless, there are transporters with associated uncoupled ion fluxes, and these may occur through intermediate states in which the pathway is incompletely occluded (13, 31, 51).

A reasonable expectation of many proposed implementations of the alternating access mechanism is that the substrate binding site would be located near the center of the membrane so that the path from either side of the membrane would be of similar length. Indeed, structures with bound ligands have borne out the expectation of a binding site at the midpoint of the permeability barrier (1, 18, 38, 59, 60). These findings highlight the intrinsic symmetry of the transport process. Binding of substrate from one side of the membrane is the converse of dissociation of that substrate to the opposite side of the membrane. The process of substrate transport can thus be reduced to a set of binding and dissociation steps on either side of the membrane, in addition to the opening and closing of permeability barriers between a central binding site and the aqueous solution on either side.

**Mechanistic Implications of Transporter Structure: LeuT as an Example**

The neurotransmitter sodium symporter (NSS) family illustrates key elements in transporter structure and function. In 2005, Eric Gouaux and his colleagues presented the first atomic structure of a member of this family. It was a high-resolution X-ray diffraction structure of LeuT, a transporter from the thermophilic bacterium Aquifex aeolicus (58). This structure was remarkable in many ways, but perhaps the most significant is that it made sense of a wealth of biochemical data collected from neurotransmitter transporters. Although there is only 20-25% amino acid identity between LeuT and the mammalian NSS transporters, the regions of highest homology cluster around the positions where a leucine molecule and two Na\textsuperscript{+} ions were bound. LeuT is now understood to be an amino acid transporter, and leucine is a very poor substrate but one that binds tightly to the protein (49). Crystal structures are now available for LeuT with other substrates and non-substrate amino acids bound, and in complex with tricyclic antidepressants—compounds that inhibit the homologous transporters SERT and NET (which transport serotonin and norepinephrine, respectively) (48, 42).

In the original LeuT structure with leucine bound (59), an aqueous pathway is clearly visible from the extracellular membrane to the intracellular space. Further, the ion translocation pathway is directly connected to the substrate binding site, rather than being occluded by a central permeability barrier. In contrast, the sodium symporter NET (which transport serotonin and norepinephrine, respectively) (48, 42).

In contrast to LeuT, NET is a sodium-coupled transporter, with a natural product palytoxin (4). In its channel mode, the protein dissipates cation gradients much faster than they can be generated by ATP hydrolysis. Consequently, we expect that transport by alternating access requires an occluded intermediate in which the substrate binding site is not accessible from either side of the membrane. Nevertheless, there are transporters with associated uncoupled ion fluxes, and these may occur through intermediate states in which the pathway is incompletely occluded (13, 31, 51).

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In the channel mode, the rate of entry and the rate of hydrolysis.

When both amino acid and Na+ ions are present in the extracellular medium, almost reaching the binding site for amino acid and Na+ ions. However, between the bound amino acid and the cytoplasmic side of the membrane, there is no pathway for substrate diffusion. Rather, 20 Å of packed protein structure prevent dissociation of the amino acid to the cytoplasm. Indeed, diffusion of bound leucine to the extracellular side is also blocked (59), although only a few amino acid side chains constitute this steric barrier. Thus, although the overall structure resembles an extracellular-facing conformation, leucine is occluded within its binding site by barriers to both sides of the membrane, similar to the prediction from alternating access models of transport.

LeuT structures have since been reported with bound alanine, glycine, methionine, tyrosine, or tryptophan (49). In all but one of these, the protein structure is almost identical to that of the LeuT-leucine structure. However, the LeuT-tryptophan structure is different. Although tryptophan was found in the same binding pocket, its greater bulk results in a protein conformation that is more open, providing a continuous path from the extracellular medium to the binding site. Tryptophan is not a substrate for LeuT and acts as a competitive inhibitor of leucine and alanine transport. Its inhibitory action seems to be that of a classical competitive inhibitor: it competes with substrate for binding to LeuT but is not itself transported.

In contrast to competitive inhibition by tryptophan, tricyclic antidepressants inhibit LeuT non-competitively (48). In other words, although inhibition by tryptophan molecule was bound in the cytoplasmic pathway leading from the extracellular medium to the binding site. Antidepressant binding, by preventing closure of the extracellular pathway, could thus block transport if opening of the cytoplasmic pathway is coupled to closing the extracellular one. Because the antidepressant was bound to a site distinct from that for leucine, increasing the leucine concentration could not overcome inhibition, and the kinetics were classically non-competitive.

The cytoplasmic pathway

Through what pathway do substrates bound to LeuT and other NSS transporters diffuse to the cytoplasm? By measuring the reactivity of inserted cysteines, and this increase required Na+ and Cl−, both of which are also required for serotonin-dependent conformational changes in the overall structure resembles an extracellular-facing conformation, leucine is occluded within its binding site by barriers to both sides of the membrane, similar to the prediction from alternating access models of transport.

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The cytoplasmic pathway

Through what pathway do substrates bound to LeuT and other NSS transporters diffuse to the cytoplasm? By measuring the reactivity of cysteine residues placed in SERT, we found that many positions were accessible to reagents from the cytoplasmic side of the membrane despite the fact that these positions were predicted, by comparison with the structure of LeuT, to be buried in the protein interior (16). These reactive positions delineated a pathway from the substrate binding site to the cytoplasm composed of one face of each of TMs 1, 5, 6, and 8 (FIGURE 2A). Similar reactivity was subsequently found in the corresponding residues of TM8 in GAT-1 (6).

The location of the cytoplasmic permeation pathway was confirmed by changes in its accessibility in response to ligands and substrates. For several positions in this pathway, addition of serotonin increased the reactivity of inserted cysteines, and this increase required Na+ and Cl−, both of which are also required for serotonin-dependent conformational changes in

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FIGURE 2. Cytoplasmic pathway of SERT and repeat structure of LeuT.

A: Cytoplasmic pathway of serotonin transporter SERT is lined by amino acid residues (spheres) from transmembrane helices 7 (red), 5 (pale green), 6 (green), and 8 (cyan). The model of SERT was built by homology to LeuT and is viewed from the plane of the membrane with the backbone trace in ribbons, with bound serotonin (yellow), chloride (magenta), and sodium ions (dark blue) shown as spheres. B–D: the LeuT fold contains inverted topology repeats of five transmembrane helices. Repeat A contains transmembrane helices 1–5 (B), and repeat B consists of transmembrane helices 6–10 (C), according to the LeuT numbering. Superposition (D) of the last three transmembrane helices from each of those repeats (pale colors) indicates a relative difference in the orientation of the first two transmembrane helices (dark colors). These two conformations of the repeats may contain the essence of the two alternating-access states of the transporter.
transport (61). Almost all positions in this pathway were less accessible in the presence of cocaine, which binds from the cell exterior, holding the extracellular pathway open and the cytoplasmic pathway closed (61). The opposite was observed with ibogaine, an inhibitor that favors the SERT conformation in which the cytoplasmic pathway is open and the extracellular pathway is closed (16, 23).

"...the involvement of repeated structural elements is clearly an elegant and appealing solution to the problem of bringing the substrate into the binding site from one side of the membrane and allowing it to exit on the opposite side..."
The cytoplasmic pathway revealed in this model was lined by the four transmembrane domains identified in the cytoplasmic pathway by accessibility measurements in SERT, namely, TMs 1, 5, 6, and 8. Moreover, the calculated accessibility of residues in these helices agreed extremely well with the experimental measurements (FIGURE 3B). Thus the model generated by swapping the conformations of the two repeats of LeuT is highly consistent with a cytoplasm-facing state conformation of the whole protein.

Comparison of the LeuT X-ray structure with the cytoplasm-facing model highlights those regions that may change during transport. The greatest observed change between the LeuT crystal and the model is in the orientation of the bundle of TMs 1–2 and 6–7 with respect to the scaffold. The bundle orientation differs by a tilt of ~25° around an axis running through the binding site region (FIGURE 3A). In effect, the cytoplasmic ends of TMs 1 and 6, which are packed against the scaffold helices 5 and 8 in the structure of Yamashita et al. (59), are separated from these regions in the cytoplasm-facing conformation. In addition, in the model, the extracellular ends of TMs 1 and 6 pack closely against TMs 3 and 10 on the other side of the binding site. A simple molecular interpretation therefore would be that “rocking” of this bundle between two alternate orientations would suffice to open and close the pathway in a coordinated manner.

Other, more limited, differences were identified between the X-ray structure and the cytoplasm-facing model, for example, for the long, tilted helices of TMs 3 and 8, which curve slightly toward or away from the binding site, respectively, in the X-ray structure. In this model, the curvature of these helices is exchanged, as if each helix was flexing during the conformational change. Such a flexing motion may help to exaggerate the pathway opening on one side or the other but also requires subtle movements of the two V-shaped pairs of helices (TMs 4–5 straddling TM8 and TMs 9–10 straddling TM3). Although these movements may contribute to the accessibility changes in the binding site, they are probably not sufficient to expose the binding site to the cytoplasm, a transition that appears to require movement of the TM1, 2, 6, 7 bundle.

The LeuT structure and proposed mechanism for transport by LeuT, SERT, and other members of this family fulfill many requirements of an alternating access transport process. For example, a mechanism by which accessibility of the central binding site would be located at the interface between the moving bundle and a stationary scaffold, providing a way for the ligand to control the orientation of the bundle through direct interactions. A recent proposal suggests that, in LeuT, binding of a second molecule of leucine in the extracellular pathway facilitates the conformational change (47). Studies testing this hypothesis and others are critical to understanding the mechanisms that couple solute binding to conformational change in transport proteins.
Inverted Repeats in New LeuT-Like Structures

Four years after the publication of the LeuT structure, crystallographic studies of several other transporter proteins originally categorized in very different families from NSS have revealed that the 5-TM inverted-repeat fold is more common than expected. In 2008, Faham et al. reported a 2.7 Å-resolution X-ray diffraction structure of a bacterial homolog of the sodium-glucose symporter SGLT from the solute sodium symporter (NSS) family (12). This new structure to the NSS family had been suggested by comparisons of hydrophobicity involving alignments of so-called hydrophathy plots (28, 29) and from careful interpretation of biochemical data on the related sodium-iodide symporter (10). Nevertheless, the presence of a transmembrane helix that precedes the 5-TM repeats, of a total of 14 TM domains, as well as the extremely low sequence identity, were sufficient to confound any general expectation of a structural relationship. Thus it was a surprise that the Vibrio parahemolyticus vSGLT structure in fact has a close relationship to that of LeuT. Given that it probably represents a cytoplasm-facing conformation, it is useful to compare the structure of vSGLT with the cytoplasm-facing model of LeuT described above. Most remarkably, the cytoplasmic pathway in vSGLT consists of the very TM domains (2, 6, 7, and 9) that correspond to those forming the pathway in the cytoplasm-facing model of LeuT (1, 5, 6, and 8) (FIGURE 4). This observation suggests that LeuT and vSGLT use the same rocking bundle mechanism and that the two crystal structures represent the two main conformational states. The vSGLT structure also confirms predictions that the two pathways in these proteins are symmetrical and are formed by the corresponding regions of the structural repeats, on opposite sides of a pseudo-symmetric binding site (16, 59). According to the suggestion of Abramson and Wright (2), we will subsequently refer to TM helices in vSGLT and other proteins that share the LeuT fold according to the corresponding helix in LeuT so that repeat A contains TMs 1-5 and repeat B contains TMs 6-10 in each case.

Subsequently, the structure of a sodium-benzyl- hydradoinum symporter Mhp1 from Microbacterium flavifaciens and belonging to the nucleobase:cation symporter (NCS) family revealed it to be even more similar to LeuT, and also in an extracellular-facing orientation at 3.35 Å resolution (42). This structure also contains a LeuT-like fold but has two TM helices preceding the first repeat, which contains TMs 3-7, whereas the second repeat contains TMs 8-12. The BetP structure is apparently in an occluded, substrate-bound state: no pathway is apparent on the periplasmic side, although a narrow pathway on the cytoplasmic side is formed by helices corresponding to those lining the pathway in vSGLT and in the cytoplasm-facing model of LeuT. Interestingly, the two repeats in the BetP structure are more similar to one another than in the other structures, consistent with a more intermediate conformation. Under resting conditions with low osmolality, as found in the crystalization conditions, BetP is inactive. Thus it is likely that this occluded state reflects a down-regulated, inactive state of the protein. However, it is also possible that this intermediate conformation occurs as part of the normal transport cycle of BetP.

Recently, consistent with bioinformatic analyses (30), three structures representing another major transporter family, the amino acid/polypeptide/organocation or APC superfamily, have been published. The two proteins, ArC and ApcT, both adopt the LeuT fold (14, 17, 46). The APC (or SLCT) family is itself related by sequence homology to the SLC32, SLC36, and SLC38 families that catalyze cation-chloride symport, amino acid/H+ antiport, amino acid/H+ symport, and amino acid:Na+ symport/H+ antiport, respectively. The variety of substrates and coupling modes suggests that this basic protein fold has evolved to allow wide mechanistic flexibility within the same structural framework. The finding that this transporter fold accommodates both symport and antiport mechanisms indicates that the key step in substrate transport may be catalyzed by a rocking bundle and that motional freedom in the structural repeats and extracellular loops is critical.
Scaffold and Bundle Movements in Proteins with the LeuT Fold

The scaffold of the STM-inverted-repeat structures appears to be relatively well conserved, both between repeats and across proteins. Between any two proteins of the same fold family, the helix packing and TM topology are essentially identical. However, it is reasonable to expect that there could be differences in the helix inclination and TM rotation, which is apparent in the SERT and GAT-1 (15, 54, 64), which is formed by residues in TMs 1, 2, 6, and 7 (12, 42), would also be maintained. Furthermore, moving the bundle as a unit provides a means for coupling the opening and closing of the two pathways, through the positioning of the long, unbroken TM helices 2 and 7. That is, helices 2 and 7 may serve as semi-rigid splints behind the broken TMs 1 and 6. Thus, if one pathway is opened by the swinging out of 1 and 6, then the splints would help ensure that the other pathway is simultaneously closed. However, this mechanism does not require the four-helix bundle unit to move as a perfectly rigid body, and some internal rearrangements between the helices are no doubt possible, as illustrated by the LeuT-Trp structure, in which the extracellular ends of the helices are swung further outward. Furthermore, accessibility and cross-linking measurements on the related GABA transporter GAT-1 and the amino acid transporter KAAT1 indicate some rearrangement of the bundle helices during transport (7, 43). In any case, this mechanism does not rule out the possibility of additional intermediate and/or occluded states.

In addition to the cytoplasmic and extracellular loops, the four-helix bundle is responsible for the leak. The gates therefore would fulfill the prediction of the alternating access model by preventing the formation of a continuous open pathway that would function as an uncoupled substrate channel. In transporters with uncoupled ion conductances (13, 31, 51), it may be irregularities in the closing of these thin gates or the motion of the bundle that are responsible for the leak.

FIGURE 5
Rocking of the bundle within the LeuT transporter fold structure results in opening and closing of pathways on either side of the membrane.

The surface of the scaffold region from LeuT is shown as a blue cut-away surface, viewed from the plane of the membrane. The axis of the four-helix bundle consisting of TMs 1, 2, 6, and 7 is shown as a line, colored from red for extracellular-facing through green for cytoplasm-facing conformations. Axes were defined as the line joining the average coordinates of all helical residues in the upper and lower leaflets of the membrane, after superposition of the residues in the scaffold onto those of LeuT. Four-helix bundles are shown as cartoons for the X-ray structures of tryptophan-bound LeuT (dark red) and for vSGLT (dark green), which are in the fully open extracellular-facing and cytoplasm-facing conformations, respectively.

Antipporter mechanisms portend that unipporter also may be catalyzed by some members of this structural family and that movement of the four-helix bundle may be the key step in transport for all of these proteins.
Inverted Repeats, Evolution, and Mechanism in Other Membrane Proteins

Repeated structural elements are a very common motif in membrane proteins, observed in proteins with diverse functions, including primary active transporters (27, 55), secondary transporters (11) and channels (35). For example, the SecY translocon, responsible for primary transport of newly translated proteins, contains a pseudo-symmetric repeat of five TM helices with a different topology of one TM helix (35). In many cases, these relationships were unexpected, due to a lack of obvious sequence homology, and revealed only by resolution of their atomic structures. Nevertheless, bioinformatic analysis suggests that such repeats originate from internal gene duplications of smaller membrane proteins and thus reflect a common evolutionary pathway for membrane proteins (44). For example, the last 3-TM segment of some major-facilitator superfamily (MFS) transporters is related in sequence to a voltage-gated sodium channel of a similar size (20). In the specific case of repeats with inverted topologies, their evolution appears to have occurred via half-sized intermediate proteins that were able to adopt both of the two transmembrane topologies, followed by internal gene duplication (40, 41, 45, 57). In any case, the commonality of such topologies suggests that additional novel structures containing inverted repeats are likely to be discovered.

For some proteins, such as LeuT, the use of inverted topology repeats may have had the added advantage, beyond the mechanism of evolution, of creating a symmetric pathway leading from either side of the membrane toward a central substrate binding site. That is, each of the structural elements that make up the extracellular permeation path has a counterpart in the other repeat that contributes to the cytoplasmic pathway. Aside from LeuT, this is potentially also the case for the sodium-proton antiporter, GltPh (9, 42a). This protein belongs to the DAACS/EAAT families, whose structures are very different from that of LeuT (60). The repeats in GltPh were also used to construct a model for the cytoplasm-facing form of the aspartate transporter, which is symmetrical relative to the extracellular-facing structure (42a). In contrast to the rocking bundle mechanism of LeuT, GltPh is predicted to function by a shuffling movement of a substrate-carrying domain through the protein—locking the gate to one side of the membrane while allowing the other gate to open. A similar exchange between two alternate conformations of inverted repeats may also occur in MFS transporters, typified by the lactose permease LacY. These proteins contain two 6-TM domains, each containing a 3-TM inverted-topology repeat (1). RTM segments 1–3 and 10–12 are considered to constitute one non-contiguous repeat, whereas TM 4–6 and 7–9 constitute a second repeat, then exchanging their conformations should lead to a similar exposure of pseudo-symmetrical pathways. It will be interesting to see whether such analysis of structural repeats can indeed prove to be a generally useful approach to deduce transport mechanisms from yet-to-be-discovered transporter structures. Fundamentally, however, these observations suggest that inverted-topology repeats not only contribute to formation of symmetric pathways in channels and transporters but provide an elegant mechanism by which nature can create a transporter protein capable of adopting two symmetry-related conformational states.
levels in which to a pseudo-parallel family, the adjacent beta strands form short helices in the channel, whereas the alpha helix is inserted. This is not only to form the C-terminal (Ricci et al., 2002). A comprehensive review of the latter two families is available in a recent comprehensive, structural organization is critical for transport activity. The mechanism of the intestinal absorption of sugars. Curr Opin Cell Biol 2009.


