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Minireview

Camilo Perez and Christine Ziegler*

Mechanistic aspects of sodium-binding sites in LeuT-like fold symporters

Abstract: Secondary active transporters are of paramount biological impact in all living cells, facilitating the movement of many different substrates across the membrane against a concentration gradient. The uphill transport of one substrate is coupled to the downhill transport of another and driven by the electrochemical gradient. In the last decade, an increasing number of atomic structures of secondary transporters have been reported, confirming a very fundamental mechanistic concept known as the alternating-access cycle. The wealth of structures of transporters sharing the so-called LeuT-like fold that is characterized by two five-transmembrane-helix repeats sharing a 2-fold inverted pseudo symmetry has raised big hopes to finally describe alternating access on a molecular level. Although comparing the individual transporter states of different LeuT-like fold transporters revealed striking similarities, the coupling process, which represents the heart of secondary transport, is far from being understood. Here, we review the structural, functional, and biophysical validation of sodium-binding sites in four different LeuT-like fold transporters. The conservation of sodium sites is discussed in light of their role as key elements connecting symmetry-related structural domains, which are involved in substrate translocation. Moreover, we highlight their crucial roles in conformational changes of LeuT-like fold transporters and their implication on a unifying mechanism in secondary transport.

Keywords: alternating-access cycle; ion coupling; LeuT-like fold; secondary transporter; sodium binding; structure.

***Corresponding author: Christine Ziegler**, Max Planck Institute of Biophysics, Structural Biology Department, Max-von-Laue-Strasse 3, D-60438 Frankfurt (Main), Germany; and Faculty of Biology and Preclinical Medicine, University of Regensburg, Universitätstraße 31, D-93053 Regensburg, Germany,
e-mail: christine.ziegler@biophys.mpg.de
Camilo Perez: Max Planck Institute of Biophysics, Structural Biology Department, Max-von-Laue-Strasse 3, D-60438 Frankfurt (Main), Germany

Introduction

Secondary transporters are widely spread throughout all kingdoms of life and are found in every biological cell. In general, they facilitate the movement of essential nutrients across the membrane and regulate metabolite concentrations by catalyzing the excretion of end products of metabolic pathways. Moreover, they mediate the active extrusion of drugs and toxic substances (Saier, 2000). Transporters bind their substrates with high stereospecificity and catalyze transport at rates well above the limits of free diffusion across membranes. Similar to the activity of enzymes, secondary transport is saturable. The substrates are driven across the membrane against a concentration gradient. Thereby, the uphill transport of one substrate is coupled to the downhill transport of another (Figure 1A) (Widdas, 1952). Coupling of substrates can occur either as a co-transport (symporters) or a counter-transport (antiporters). In symport, the transport of H^+ or Na^+ used as a motive force is favored by the electrochemical gradient.

Since 2003, an increasing number of atomic structures of secondary transporters have been reported and were discussed in numerous reviews (Krishnamurthy et al., 2009; Forrest et al., 2011; Zdravkovic et al., 2012), providing solid support for the long-standing alternating-access hypothesis (Jardetzky, 1966). In a nutshell, it is assumed that the catalytic cycle of a transporter involves dynamic transition through several distinct transporter states. Sequential conformational changes assure the exposure of a central substrate-binding site only to one side of the membrane at once, but never to both at the same time (Figure 1A) (Jardetzky, 1966).

Surprisingly, several transporters previously assigned to different families on the basis of sequence homology share common folds. One conserved secondary transporter fold is the LeuT-like fold named after LeuT, a bacterial homologue of the neurotransmitter:sodium symporter (NSS) family (Yamashita et al., 2005), which was the first identified member of this structural superfamily. The LeuT-like fold is characterized by an internal 2-fold pseudosymmetry

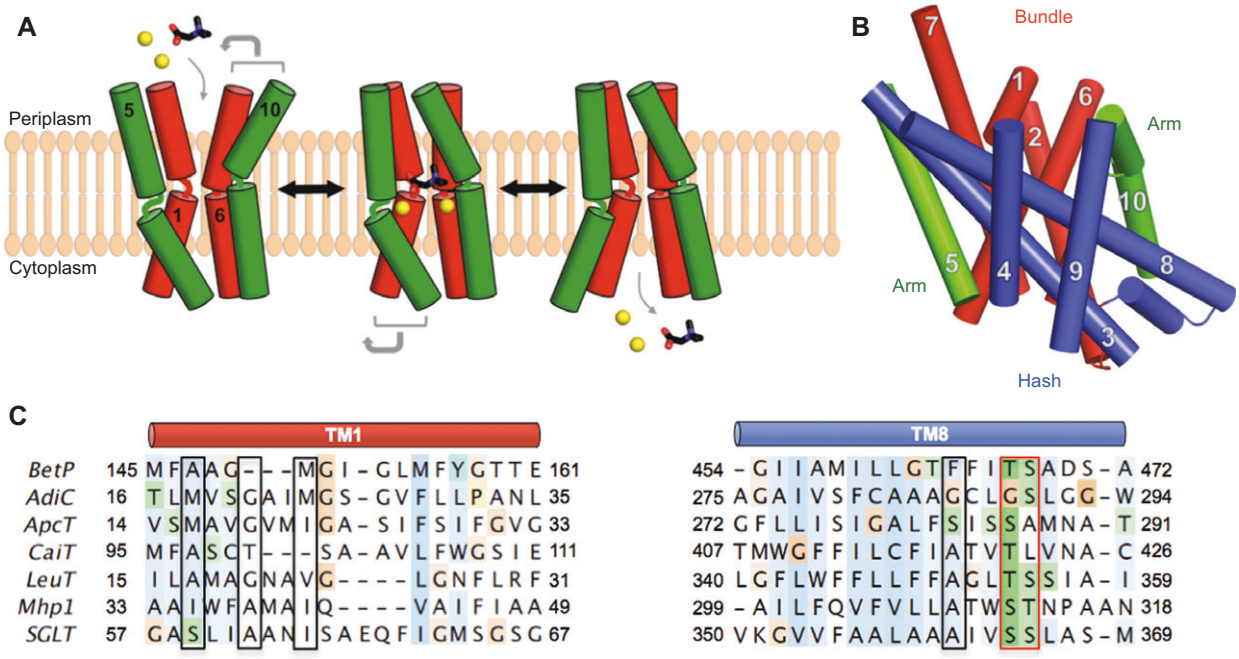

Figure 1 (A) Alternating-access mechanism of a sodium-coupled LeuT-like fold symporter. Exposure of the binding sites to the periplasm allows sodium and substrate to enter, most likely in an order such that the Na2 sodium ion site is occupied first. Subsequently, a series of conformational changes triggered by binding and dissociation of ions and substrate result in their release into the cytoplasm. Sodium ions are shown in yellow, and substrate in black (carbon), red (oxygen), and blue (nitrogen). (B) Characteristic structural elements of LeuT-like fold transporters. (C) Sequence alignment of TM1 and TM8 in LeuT-like fold transporters. Boxes indicate residues involved in the formation of the Na2 site. The red box highlights the two highly conserved residues providing the hydroxyl side chains for coordination.

relating the five N-terminal transmembrane (TM) helices of the first repeat to the following five TM of the second repeat along an axis running parallel to the membrane plane through the center of the transporter, such that their TM topologies are inverted with respect to one another (Yamashita et al., 2005; Khafizov et al., 2012). Three distinct structural elements are characteristic for this conserved transporter architecture: a four-TM helix bundle consisting of the first two helices of each repeat, a hash domain consisting of the third and fourth helices of each repeat, and two peripheral arms corresponding to the fifth helix of each repeat (Figure 1B). For simplicity of comparison, we use the LeuT repeats TM numbering for all transporters discussed here, as some of these contain additional TM helices either preceding or following the five-TM repeats.

To date, seven different transporters originally assigned to five sequence-unrelated transporter families share the LeuT-like fold, namely, LeuT from the neurotransmitter:sodium symporter (NSS) family; BetP and CaiT from the betaine/carnitine/choline transporter (BCCT) family (Schulze et al., 2010; Tang et al., 2010; Perez et al., 2012); AdiC, ApcT, and GadC from the amino acid/polyamine/organocation (APC) transporter family (Fang et al., 2009; Shaffer et al., 2009; Ma et al., 2012); Mhp1 from the nucleobase:cation symporter-1 (NCS1) family (Weyand et al., 2008); and vSGLT from the solute:sodium symporter (SSS) family (Faham et al., 2008). A full description of the alternating-access mechanism has been possible only for the sodium-coupled symporters LeuT, Mhp1, and BetP, which have been crystallized all in outward- and inward-facing states, although at very different resolutions ranging from 1.65 to 4 Å (Weyand et al., 2011; Krishnamurthy and Gouaux, 2012; Perez et al., 2012). The sodium-binding sites in these transporters have been identified by a combination of structural, computational, biochemical, and biophysical approaches (Yamashita et al., 2005; Weyand et al., 2008; Khafizov et al., 2012). Comparison of the distinct conformations of different transporters revealed striking similarities in the conformational changes, even between symporters and antiporters, which were discussed in great detail in a number of recent reviews (Forrest et al., 2011; Zdravkovic et al., 2012).

However, the molecular mechanism of coupling is far from understood. Therefore, in this mini-review, we will focus on a thorough description of conserved and non-conserved sodium ion sites and their distinct roles in conformational changes in LeuT-like fold transporters.

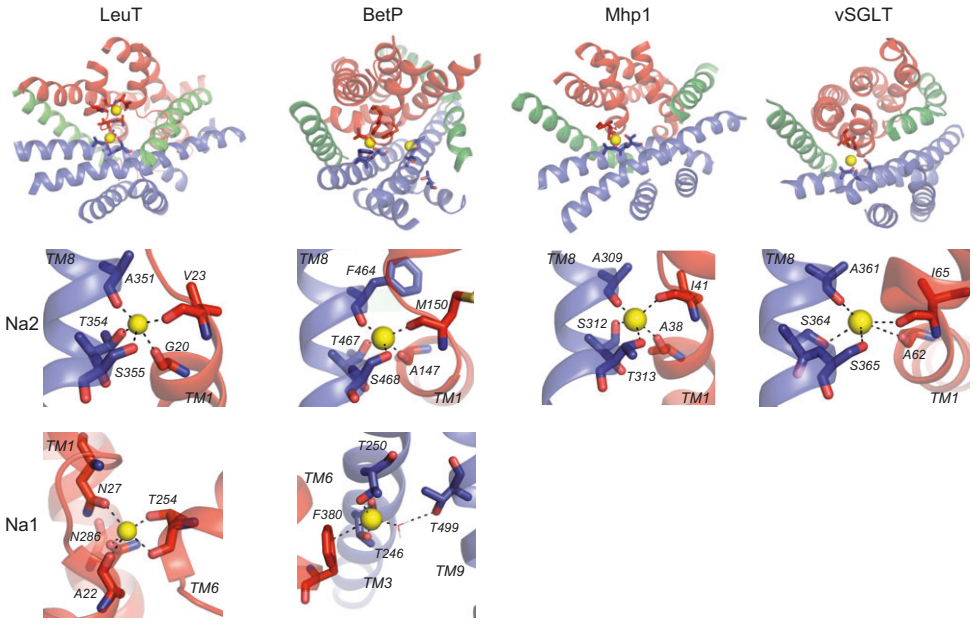

Figure 2 Periplasmic view of the sodium-binding sites in LeuT-like fold symporters.
Sodium ions are in yellow. Bundle, hash, and arms are in red, blue, and green, respectively. The highly conserved Na2 site for all four LeuT-like fold symporters is formed between TM1 and TM8, whereas the position of the Na1 site differs in LeuT and BetP.

Sodium-binding sites in LeuT

The crystal structure of LeuT from *Aquifex aeolicus*, a bacterial homologue of Na^+/Cl^- dependent neurotransmitter transporters, was obtained to a 1.65 Å resolution by Yamashita et al. (2005). This transporter shows an overall sequence identity to the eukaryotic counterpart of 20–25%. In the LeuT structure, two sodium-binding sites could be identified, named Na1 and Na2 (Figure 2). The sodium in the Na1 site is coordinated by two main-chain carbonyl oxygens from A22 (TM1) and T254 (TM6), and three side-chain oxygens from N27 (TM1), N286 (TM7), and T254 (TM6) (Figure 2). N27 is fully conserved in the NSS family, whereas N286 is conserved in most family members and, in some others, it is replaced by aspartate. T254 is replaced in most NSS transporters by serine. Sodium in the Na2 site is coordinated by three main-chain carbonyl oxygens, from G20 and V23 (TM1) and from A351 (TM8), as well as by two side-chain hydroxyl oxygens from T354 and S355, which are highly conserved in all LeuT-like fold transporters (Figures 1C and 2). The conservation of T354 is less than that of residues coordinating Na1; however, S355 is almost fully conserved throughout the NSS family.

It is very challenging to distinguish between a sodium ion and a water molecule even at high resolution, and assignment of density peaks to sodium requires some precautions. For LeuT, two putative sodium ion peaks were modeled as oxygen atoms of water molecules, resulting in negative peaks in the Fo-Fc difference density map at sigma levels >3 σ, which disappeared when these densities were modeled as sodium ions. Moreover, valence calculations yielded a sodium-specific valence (v_{Na+}) of 1.32 for Na1 and 1.34 for Na2, in agreement with the values expected for bound sodium ions ($v_{Na+} \geq 1.0$) and in agreement with the distances between the sodium ions and coordinating atoms (2.5–2.8 Å) (Yamashita et al., 2005). In contrast, Li^+, K^+, and Mg^{2+} valence values and distances would have been far off. This thorough validation of sodium sites in transporters should be considered a gold standard to give a measure to judge the meaningfulness of structural ion coordination sites.

The LeuT architecture of inverted repeats suggested that binding of sodium to at least the Na2 site is required to stabilize the architecture of the substrate-binding site. Indeed, molecular dynamic (MD) simulations implementing free energy perturbations confirmed that the presence of sodium at the Na2 site is required for structural stability of the binding pocket. Interestingly, Na2 occupation by sodium causes also an amplification of the selectivity toward sodium at the Na1 site in the case of double ion occupancy (Caplan et al., 2008). Furthermore, these sites are strongly selective for Na^+ over K^+ (for single and double ion occupancy) but weakly selective for Na^+ over Li^+. Besides,

for both the single- and double-occupancy models, the Na1 site is less selective for Na^+ than the Na2 site. It can be concluded that the sodium ion at the Na2 position stabilize both the binding pocket for sodium at the Na1 site and the central substrate-binding site, implying that in the sequential binding events of substrate and coupled substrates, this Na2 sodium might bind first. In agreement with these observations, sodium binding to LeuT displays positive cooperativity between Na1 and Na2, with a Hill coefficient of 1.9±0.3 (Shi et al., 2008). MD simulations of constructs in which the substrate was removed in the presence or absence of the two sodium ions (Shi et al., 2008) revealed that in the absence of sodium, the substrate binding site is shielded from the extracellular milieu, whereas the presence of sodium facilitates an opening to the extracellular milieu and the entry of water molecules into the site. These observations were confirmed experimentally by single-molecule Förster resonance energy transfer methods (Zhao et al., 2010). The relative population of outward-open conformation increased significantly depending on the sodium concentration. In the future, experiments with LeuT reconstituted into proteoliposomes will be required to account for the impact of the electrochemical sodium potential on the transporter dynamics. Site-directed spin labeling and electron paramagnetic resonance analysis were applied to capture the dynamics of LeuT (Claxton et al., 2010), and revealed that the exposure of the central substrate-binding site in a dynamic outward-facing intermediate is sodium dependent.

Comparison of outward- and inward-facing states (Krishnamurthy and Gouaux, 2012) in LeuT highlights that conformational changes of TM1 and TM6 have profound consequences on the conformation of the sodium ion sites (Figure 3). During the transport cycle, TM1a moves away from TM6 and TM8, resulting in the disruption of the sodium ion sites, implying that the movement of the cytoplasmic half of TM1 and the opening of the transporter are coupled (Figure 3).

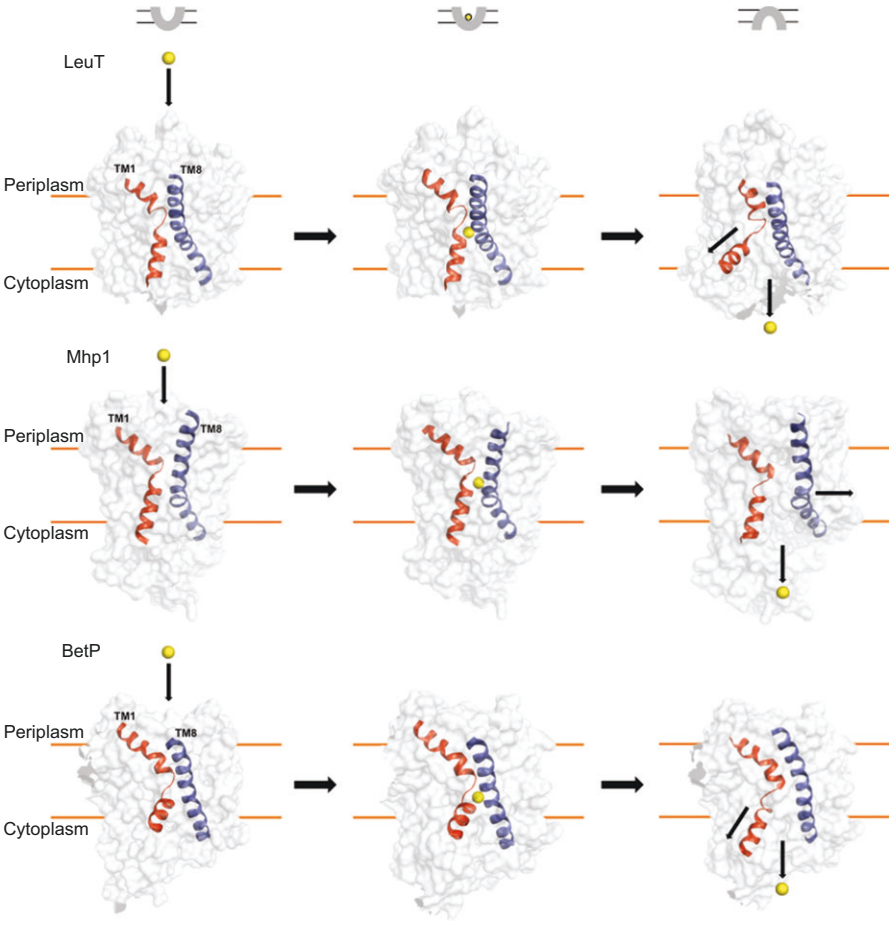

Figure 3 Schematic of sodium-induced conformational changes of TM1 and TM8 in sodium-coupled LeuT-like fold symporters.
The vectorial movement of sodium through the Na2 site induces a spring-like movement of the cytoplasmic part of TM1 in LeuT and BetP or the displacement of TM8 in Mhp1. These conformational changes render the cytoplasmic pathway accessible. The substrate and additional sodium ions are not shown for clarity.

Sodium-binding sites in Mhp1

Crystal structures of the hydantoin transporter Mhp1 from *Microbacterium liquefaciens* have been obtained in outward and inward-facing states (Shimamura et al., 2010). Mhp1 was first reported to be a proton-coupled transporter according to whole-cell uptake experiments (Suzuki and Henderson, 2006); however, tryptophan fluorescence measurements showed a clear benzyl-hydantoin-sodium-dependent binding (Weyand et al., 2008). The affinity of benzyl-hydantoin to the protein increased >10-fold in the presence of sodium. Similarly, the presence of benzyl-hydantoin increases the affinity for sodium; these results indicated that binding of sodium and benzyl-hydantoin were tightly coupled in Mhp1 (Weyand et al., 2008). Presumably, the sodium dependence in whole-cell transport assays was obscured by the presence of a separate compensating sodium transport system.

The electron density map at 2.85 Å resolution of an outward-facing occluded state showed a single positive peak in the Fo-Fc difference density map, at a position structurally equivalent to the Na2 site of LeuT. A sodium ion modeled at this site is surrounded by carbonyl oxygen atoms from A38 and I41 from TM1 and A309 from TM8, plus side chain hydroxyl atoms from S312 and T313 from TM8 (Figure 2) (Weyand et al., 2008). Neither the 1:1 stoichiometry of sodium/benzyl-hydantoin transport nor the coordination of this site was biochemically and genetically confirmed, e.g., by mutagenesis. A link between the sodium-binding site and the substrate-binding site through residues N318 on TM8 and Q42 on TM1 was reported, which suggests that cation binding to Na2 is required for substrate translocation.

The crystal structure of an inward-facing conformation of Mhp1 at 3.8 Å (Shimamura et al., 2010) allowed describing overall conformational changes during alternating access. Compared with the outward-facing state, in the inward-facing structure TM8 was displaced ~4.5 Å away from TM1 so that the Na2 site was no longer intact (Figure 3). Furthermore, a sodium ion was stably bound in equilibrium MD simulations of the outward-occluded state (Shimamura et al., 2010), similar to the behavior of Na2 in simulations of LeuT (Caplan et al., 2008; Noskov and Roux, 2008), whereas in a structure of the inward-facing state, the displacement of TM8 leads to the disruption of the sodium-binding site (Shimamura et al., 2010). Furthermore, in multiple MD simulations of the inward-facing state, sodium did not remain stably bound to this site (Shimamura et al., 2010), consistent with the observations discussed above for LeuT.

Sodium-binding sites in vSGLT

The Na^+/galactose symporter vSGLT from *Vibrio parahaemolyticus* is a homologue of the mammalian Na^+/glucose symporter SGLT1, which is directly involved in the human disorder glucose/galactose malabsorption (Turk et al., 1991). vSGLT is a member of the SSS family (Turk et al., 2000). When reconstituted into proteoliposomes, vSGLT displayed sodium-dependent galactose uptake with initial rates increasing 10- to 25-fold in the presence of 100 mM sodium (Turk et al., 2000). Galactose and sodium are transported with an apparent 1:1 stoichiometry, which was assessed from Hill coefficient determination, $n_{Hill(Na+)}$~0.9; $n_{Hill(galactose)}$~1.0, suggesting mechanistic differences in the coupling mechanism compared with LeuT, which exhibits a stoichiometry of 1:2. Two crystal structures of vSGLT have been obtained in inward-facing conformations, which preclude from a description of the alternating-access cycle for this symporter. The position of the sodium-binding site was identified on the basis of a comparison with the Na2 site in LeuT (Faham et al., 2008) and mutational analysis. The proposed coordination of sodium at this site involves the hydroxylic side chains of S365 and S364 from TM8 and backbone oxygen atoms from residues A361 from TM8, and A62 and I65 from TM1 (Figure 2). Binding to this site is affected by mutagenesis of S365 to alanine and of analogous residues in other members of the SSS family (Faham et al., 2008). Consistent with the observations for other LeuT-like fold symporters, several equilibrium simulations of a membrane-embedded model of vSGLT in inward-facing conformations are characterized by instability of the ion in the proposed binding site (Li and Tajkhorshid, 2009; Watanabe et al., 2010). This indicated that the crystal structure of vSGLT was actually representing an ion-releasing state of the transporter. Furthermore, Li and Tajkhorshid (2009) observed that the dynamics of the sodium ion suggested that the cytoplasmic release of this ion precedes that of the substrate, thus shedding light on a key step in the transport cycle of this transporter. Watanabe et al. (2010) also showed that sodium exit causes a reorientation of TM1, opening the cytoplasmic pathway for substrate exit.

The validated 1:1 stoichiometry in vSLGT also strengthened the aforementioned discussion on the sodium stoichiometry in Mhp1, i.e., the differences in mechanistic impact between the Na2 (non-optional) and the Na1 (optional) sites. In fact, the presence and position of the Na1 site in LeuT-like fold transporters are not conserved, which was demonstrated after localization of the sodium-binding site in the betaine transporter BetP.

Sodium-binding sites in BetP

Recently, crystal structures of the Na^+/betaine symporter BetP from *Corynebacterium glutamicum* obtained at a 3.1–3.5 Å resolution described, besides inward- and outward-facing states, a unique closed state (Ressl et al., 2009; Perez et al., 2011, 2012). Actually, BetP is the secondary transporter with the highest number of different conformational states crystallized so far and therefore with the most complete molecular description of the alternating-access cycle. However, only one out of six different conformations showed putative density for sodium. In the closed state observed in a 3.1 Å resolution structure, a positive peak in the Fo- Fc difference density map was identified in a position similar to the Na2 site in LeuT (Figure 2) (Perez et al., 2012). The putative sodium ion at the Na2 site was coordinated by two carbonyl oxygen atoms from residues in TM1 (A147 and M150) and three oxygen atoms from helix TM8, namely the backbone carbonyl of F464 and the side-chain hydroxyl oxygen atoms of T467 and S468 (Figure 2). However, at a 3.1 Å resolution, it was not possible to unambiguously assign the density to sodium, although the conserved pentacoordinate oxygen coordination was strongly confirmative. To cross-validate the coordination of a sodium ion at the Na2 site in the closed structure, MD simulations of this structure embedded in a hydrated POPG lipid bilayer were performed (Khafizov et al., 2012). As a result, the sodium coordination network in the Na2 site was stable on the simulation timescale, both in terms of the coordination number and oxygen-ion distances. Moreover, mutations of the coordinating residues abolished or reduced the binding and transport of sodium (Khafizov et al., 2012). Experimental evidence supporting the location of the Na2 site in BetP has been presented on the basis of [^{14}C]betaine uptake assays in cells and proteoliposomes, as well as sodium binding measured by tryptophan fluorescence and $^{22}Na^+$ binding measured by scintillation proximity assays (SPA). Moreover, transient currents derived from sodium charge displacement have been measured in proteoliposomes using solid-supported membrane-based electrophysiology (Khafizov et al., 2012).

The co-transport stoichiometry of betaine and sodium was determined to be 1:2, derived from steady-state accumulation ratios of betaine and sodium in thermodynamic equilibrium (Farwick et al., 1995), indicating the presence of a second sodium-binding site, which could not yet be deduced from structural data. Interestingly, in BetP, the positive trimethylammonium group of betaine is located within the four-TM bundle, which is similar to the location of Na1 in LeuT. Consequently, a sodium-binding site at the position of Na1 in LeuT was not conserved in BetP, which was also confirmed by MD simulations and mutagenesis studies (Khafizov et al., 2012). The position of the second sodium site (Na1′ to distinguish it from Na1 in LeuT) was proposed on the basis of the structural symmetry between repeats, which is extraordinarily pronounced in BetP (Khafizov et al., 2012). In all LeuT-like fold sodium-coupled symporters, the Na2 site is mainly formed by residues from the first helix of the first repeat and the third helix of the second repeat. A simple assumption for the highly symmetric BetP was to propose a coordination for the Na1′ site residues from the first helix of the second repeat and from the third helix of the first repeat. Surprisingly, at this position, a positively charged residue (K110) is located in Mhp1, which does not require a second sodium ion for transport. The proposed coordination of the Na1′ site involved the oxygens from the side chains of T246 and T250 and the backbone carbonyl of T246 from TM3, a cation-π interaction with F380 from TM6, and a single water molecule coordinated between the sodium ion and T499 from TM9 (Figure 2). Furthermore, mutations of the coordinating residues increased the apparent Km and Kd. A mutant of BetP, in which an aspartate was inserted in the unfolded stretch of TM1, increased the sodium affinity 6-fold and changed substrate specificity in a way that in addition to the zwitterionic betaine, a positively charged choline was transported (Perez et al., 2011, 2012). In a structure of this mutant, the trimethylammonium group of choline was observed in the Na2 site, suggesting that in BetP the Na2 site serves as a cation-binding site.

In the APC, NCS1, and SSS transporter families, the corresponding residues forming the Na1′ site in BetP do not contain a significant population of hydroxylic side chains, suggesting that this binding site is not conserved in those families. Nevertheless, the serine and threonine residues involved in the formation of Na1′ are found in a substantial fraction of all sequenced BCCT transporters, implying that the Na1′ binding site may be found in other members of this family as well (Khafizov et al., 2012).

Similarly to what was shown for LeuT, tryptophan fluorescence and SPA sodium-binding measurements provided clear evidence for a positive cooperativity between the two sodium-binding sites in BetP with a Hill coefficient of 2.0±0.2 (Khafizov et al., 2012). A possible origin of the cooperativity is that similarly to LeuT, the Na2 site might be occupied first in order to increase the affinity of the Na1 site for sodium.

In the outward and closed states, the Na2 site is formed, providing reasonable coordination for sodium.

By contrast, in the inward state, the coordinating residues are too distant to coordinate a sodium ion, which is a consequence of the main-chain conformational changes of TM1a (Figure 3), similarly to what was observed for the Na2 site in LeuT. However, a proper coordination sphere of sodium in the Na1′ site is only established in the closed state; in the outward and inward states, residues in TM3 and TM6 are too distant to coordinate a sodium ion.

From symporter to antiporter

LeuT-like fold structures of sodium-independent transporters suggest some similarities between ion-coupled symport and substrate/product-coupled antiport. For example, in the outward-facing structure of AdiC that co-transports arginine and agmatine independent of sodium or protons, the side chain of S289 in TM8 forms a hydrogen bond directly with the backbone carbonyl of G21 in TM1 (Kowalczyk et al., 2011), resembling the Na2-mediated interaction between these two TM helices in LeuT-like fold symporters (Shaffer et al., 2009; Khafizov et al., 2012). Interestingly, in other antiporters, the sodium ions at Na2 have been replaced by basic side chains. For example, in the carnitine/γ-butyrobetaine antiporter CaiT, an arginine residue (R262) located at a position equivalent to Na2 bridges T100 in TM1′ and T421 in TM8′ (Schulze et al., 2010). In the proton-coupled amino acid transporter ApcT, K158, whose amino group is located at the position structurally equivalent to Na2, interacts with the backbone carbonyl of G19 and the side-chain hydroxyl oxygen of S283, from helices TM1 and TM8, respectively (Shaffer et al., 2009). Protonation and deprotonation of K158 were proposed to play the same role as the binding of Na2 in LeuT-like fold symporters (Shaffer et al., 2009), suggesting a common mechanistic principle, which was supported by subsequent MD simulations (Shi and Weinstein, 2010).

Conclusion

The molecular understanding of the coupling mechanism between the downhill transport of an ion and the uphill transport of a substrate can be considered as the holy grail of the investigation of secondary transporters. Atomic structures of transporters in different conformations have recently provided a glimpse on coupling, owing to combination with computational methods and powerful biochemical tools. Interestingly, the symmetry relation between repeats is less conserved than previously anticipated and reveals very distinct properties for individual transporters with respect to coupling. Localization and characterization of coupling-ion binding sites in different LeuT-like transporters set the stage for in-depth investigations into the mechanisms of coupling. In the same way, major breakthroughs have been obtained in the understanding of the sodium-coupling mechanism of secondary transporters adopting other folds than the LeuT-like fold, e.g., owing to the comparison of sodium/proton exchangers such as NhaA (Hunte et al., 2005) and sodium-dependent bile transporters such as ASBT (Hu et al., 2011). Their fold, although quite different from the LeuT-like fold, also contains internal symmetries. Unwound stretches in TM helices participate in cation-binding sites, quite similar to those found in LeuT-like fold transporters. However, more high-resolution structures and single-molecule techniques together with subsequent long-time scale simulations on the kinetics of coupling are required to push these results to a more complete picture of coupling. Moreover, it remains a challenging task to extrapolate these results to a common, fold-independent concept of ion coupling in secondary transporters.

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