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Review

Atomistic models of ion and solute transport by the sodium-dependent secondary active transporters☆,☆☆

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ABSTRACT

The recent determination of high-resolution crystal structures of several transporters offers unprecedented insights into the structural mechanisms behind secondary transport. These proteins utilize the facilitated diffusion of the ions down their electrochemical gradients to transport the substrate against its concentration gradient. The structural studies revealed striking similarities in the structural organization of ion and solute binding sites and a well-conserved inverted-repeat topology between proteins from several gene families. In this paper we will overview recent atomistic simulations applied to study the mechanisms of selective binding of ion and substrate in LeuT, Glt, vSGLT and hSERT as well as its consequences for the transporter conformational dynamics. This article is part of a Special Issue entitled: Membrane protein structure and function.

Contents

1. Introduction

Membrane proteins represent an essential component of life. Lipid membranes allow for the compartmentalization of cells, but prevent the uptake of nutrients and expulsion of toxins. Membrane proteins circumvent this problem by providing a path for uptake and expulsion with well-controlled specificity and permeability. Membrane proteins also play a crucial role in neuron function and signal propagation [1]. One particularly important class of membrane proteins is

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transporters, which translocated solutes across cell membranes. Transporters are a very large family of integral membrane proteins classified as utilizing either primary (ATP driven) or secondary (electrochemically driven) transport. While both forms of active transport translocate the main substrate across the membrane against its concentration gradient [1,2], our focus will be on the secondary transporters. Secondary transporters can be found in every tissue type and are driven by the chemical gradients that are often created by ion pumps. The facilitated diffusion of ions down their electrochemical gradients is coupled to the active transport of molecules against their concentration gradient. Secondary transporters are further broken down into symporters and antiporters. Symporters transport one or more molecules and/or ions in the same direction, while antiporters transport their substrates in opposing directions [3,4].

With the recent availability of X-ray crystal structures, numerous advances in understanding these systems have been made. These systems include the sodium-coupled leucine transporter LeuT of the NSS family [5], the sodium/galactose symporter vSGLT of the SSS family [6], the sodium/hydantoin symporter Mhp1 of the NCS1 family [7,8], the sodium/betaine symporter BetP [9] and the L-carnitine/γ-butyrobetaine antiporter CaiT [10] of the BCCT family, and the proton-coupled amino acid transporters ApcT [11] and arginine–agmatine antiporter AdiC [12,13] of the APC family. These unrelated transport systems have several common structural features [3,4,14], including a two-fold symmetry with inverted repeats that involve 5 + 5 essential helices and a break in the TM helices that forms the substrate-binding pocket(s).

The currently accepted mechanism of transport for most of the systems with this structure is the alternating access model proposed in the mid-sixties [15]. Computational studies at the atomic level allow for the accurate understanding of how this mechanism can be achieved at the atomistic level of resolution. We will use different transporters to illustrate the basic principles of ion selectivity, transport coupling, and water pathways in order to elucidate how these crucial steps lead to the transport of molecules. In particular, we will focus on LeuT as a prototype for the large family of LeuT-fold proteins and its eukaryotic homologue, the sodium/neurotransmitter symporter serotonin transporter (hSERT). We will also discuss the sodium-dependent glucose transporter (SGLT), whose human homologue plays essential roles in the small intestines and nephrons, and the glutamate transporter (GLT), whose human homologue plays an important role in the central nervous system. Among these transporters, LeuT, hSERT, and SGLT are located in different cell types and have diverse functions with high-specificity and low sequence homology, yet evolution has maintained its conserved topological features and one of its sodium-binding sites (see Fig. 1). It is these similarities, which have been conserved as far back as bacterial organisms that allow us to study multiple systems and hopefully gain insight into a single unifying theory of transport. An excellent example of this is the use of the bacterial LeuT crystallized from *Aquifex aeolicus* [5] for multiple studies involving hSERT and other monoamine transporters [16–18].

Real-time simulations of the full transport cycle are beyond the capabilities of equilibrium molecular dynamics at this time. Therefore, we will focus on work on various conformational states to study these transporters. Currently, some of the most challenging questions in the field directly accessible by modern atomistic simulations are the following: how is a great level of selectivity for the substrates/

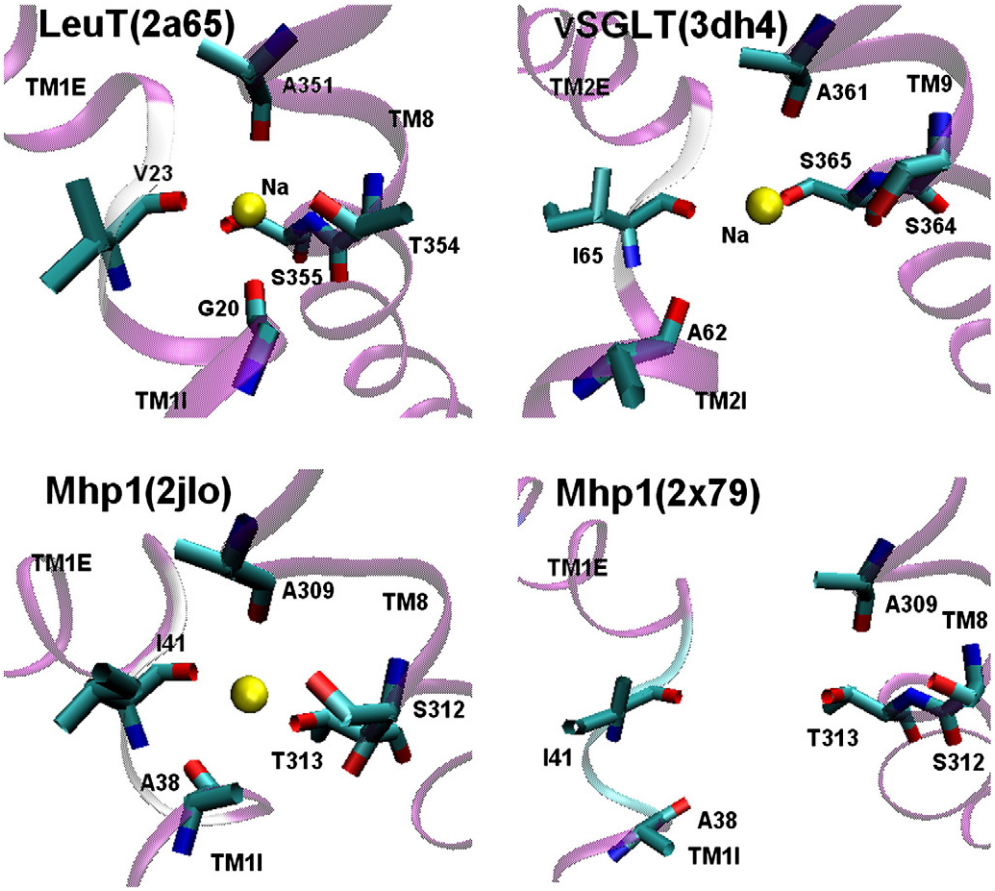

Fig. 1. The conserved Na^+ binding site for the LeuT-fold Na^+-coupled secondary transporters LeuT, vSGLT and Mhp1. Pdb entry names are shown in brackets. LeuT (2A65) [5] and Mhp1 (2JLO) [7] are in the occluded state with the Na^+ site intact, while vSGLT (3DH4) [4] and Mhp1 (2X79) [7] are in inward-facing conformations and the Na^+ site is (partially) disgruntled. The proteins are shown in ribbon representation and colored by the secondary structure: magenta for α-helices and white/blue for the breaks in the helices. The residues that form the Na^+ binding site are shown in stick representation and the Na^+ is shown as a yellow ball.

ions achieved and how is the energetics of ion transport coupled to the energetically unfavorable substrate transport? By focusing our efforts on addressing these problems, we hope to have reliable data that lead to a better understanding of the underlying mechanism without making rash conclusions or grand assumptions. We will start our mini-review with the atomic mechanisms responsible for the high-affinity/high-specificity binding of the transported substrates, and then proceed to the roles of small molecules/ions in the transport cycle: Cl^-, Na^+, and water molecules.

2. Molecular principles of high-affinity/high-specificity substrate binding to secondary transporters

2.1. Binding of Substrates and Inhibitors to LeuT-fold secondary transporters

The understanding of the molecular mechanism behind the specificity of substrate/inhibitor binding to secondary membrane transporters is of great medical importance. Particularly, homologues of the LeuT transporter, including hSERT and the GABA transporter, are targets for clinical antidepressants as well as addictive drugs. Therefore, the LeuT structures, solved with bound tricyclic antidepressants (TCAs) [19,20] and selective serotonin reuptake inhibitors (SSRIs) [21], have been excellent models for computational studies of the mechanisms of drug binding to these neurotransmitter transporters. Computational studies on LeuT and hSERT have led to the development of hSERT models that may be suitable for antidepressant drug design [22], have explored the mechanisms of antidepressant binding to hSERT models [23,24], and have demonstrated the applicability of free-energy simulations in determining the binding affinity of drugs to membrane transporters [25,26].

Two substrate/inhibitor binding sites, namely S1 and S2, have been suggested for the LeuT-fold secondary transporters [5,27]. The S1 site is located roughly halfway across the cell membrane. In the crystal structures [5–9], the substrates for LeuT, vSGLT, Mhp1, and BetP bind to this S1 site. The S2 site is located above the S1 site in the extracellular vestibule [27]. The presence of this putative binding site on LeuT was indicated in both experimental evidence and steered molecular dynamics (MD) simulations [27]. It was shown that the non-native tryptophan "substrate" could occupy both the S1 and S2 sites of LeuT simultaneously [28]. Antidepressants and detergent molecules were also found bound to S2, while S1 was occupied by the native substrate leucine (LEU) [19,20,29], indicating non-competitive inhibition of the transport function in LeuT. However, a structure with a native substrate bound to S2 is yet to be reported.

Based on the crystal structures of LeuT bound with TCAs [20], we recently computed the standard binding free energy of three TCAs, including clomipramine, imipramine, and desipramine, binding to S2 of LeuT [25] using MD/free-energy perturbation (FEP) with restraining potentials [30]. The computed binding affinity sequence and the free-energy differences between these three TCAs, shown in Table 1, are comparable to experimental results. Based on these computations, the main contribution to the favorable binding of the three TCAs is the van der Waals interactions between the drugs and LeuT. However, the electrostatic interaction between D401 and the protonated nitrogen atom in the TCA side chain also plays an important role in stabilizing the TCAs. In an earlier study of substrate specificity in the LeuT transporter [26], a similar MD/FEP technique was applied to elucidate the mechanisms of selective substrate binding to the S1 site of the protein. In addition, previous FEP simulations have shown that the leucine substrate adopts the zwitterion form (Table 2), rather than the neutral form. Therefore, the high-specificity/high-affinity binding of leucine to LeuT is achieved via both types of interactions with direct coordinating residues, such as F253, and some relatively long-range effects involving the collective dynamics of the Q250–R30–D404 residues. In addition to the scientific findings, these studies [25,26] demonstrated the applicability of the computational technique of FEP/MD [30,31] in exploring binding mechanism and potentially drug optimizing [32].

Table 2
Absolute free energy of binding (ΔG_{tot} highlighted) for leucine and aspartate to the reduced (GSBP) Na1–Na2 LeuT and Na1–Na2, Na1–Na2–Na3 Glt structures [36,39].

Substrate[a]	G_{rep}	G_{disp}	G_{elec}	G_{pos}	G_{conf}	G_{total}
N-Leu	4.7	−4.7	−0.9	13.7	13.2	**26.0**
ZW-Leu	−1.8	−11.9	−21.9	7.3	14.2	**−13.7**
Na1Na2-ASP	−3.3	−11.3	9.1	3.6	11.3	**9.4**
Na1Na2Na3-ASP	7.2	−9.3	−29.3	6.4	11.4	**−13.4**

[a] N-Leu corresponds to neutral leucine, ZW-Leu is a zwitterionic form. All units are shown in kcal/mol.

Monoamine transporters, such as serotonin and dopamine transporters, represent an important target for stimulants and drugs. LeuT has been used as a template to build various homology models for these transporters. Using crystal structures of LeuT [5,20], several homology models for the human serotonin transporter were created [23,24]. With these models, computational studies have identified the binding sites and modes for the serotonin (5-HT) substrate [22], as well as a variety of antidepressants, including TCAs [24] and the SSRI citalopram [23,24]. hSERT is of particular clinical significance as it is both a common target of psychostimulants, such as cocaine and MDMA (ecstasy), as well as a target for SSRIs used in the treatment of mood disorders (Prozac, Zoloft, Celexa, etc.) [17]. Notably, unlike the LeuT-TCA [19,20] and LeuT-SSRI [21] crystal structures, in which the drugs bind in the extracellular vestibule (S2) above the central substrate-binding site (S1), all the proposed drug binding sites in the hSERT model overlap with the proposed 5-HT substrate-binding site, suggesting a competitive mechanism for inhibition, which is supported by experimental evidence [24,33,34]. Beuming et al. [35] built a homology model for the dopamine transporter DAT from LeuT. In their model, the binding sites of cocaine and substrates dopamine and amphetamine also overlap.

2.2. Coupling between the substrate and sodium ions in Na^+-coupled secondary transporters

Na^+-coupled secondary transporters require Na^+ for the transport of their main substrates [3,4,14]. The facilitating roles of Na^+ in these transporters may be divided to two main categories: to facilitate the binding of the substrate, and to facilitate the conformational dynamics necessary for the substrate transport cycle. While we

Table 1
Affinities for three TCAs (Clomipramine, Imipramine, and Desipramine) binding to LeuT computed with FEP/MD with restraining potential [25].

Drug	G_{rep}	G_{disp}	G_{elec}	G_{pos}	G_{conf}	G_{tot}(bind)	Exp. relative affinity
C	−9.4 ± 0.9	−19.4 ± 0.6	2.0 ± 0.6	8.8 ± 0.1	4.4 ± 0.5	**−13.6 ± 1.4**	**0**
I	−7.8 ± 1.4	−15.3 ± 0.6	−3.9 ± 0.9	9.3 ± 0.1	4.3 ± 0.8	**−13.4 ± 1.0**	**+1.5**
D	−9.3 ± 0.4	−18.3 ± 0.1	2.6 ± 0.1	9.9 ± 0.1	3.0 ± 1.2	**−12.1 ± 1.4**	**> +1.5**

The values are reported in kcal/mol. G_{rep}, G_{disp}, G_{elec}, G_{pos}, G_{conf} are the binding free-energy contributions from repulsive VDW, attractive VDW, electrostatic, translational and rotational restraints (positional restraint), and conformational restraint respectively. G_{tot}(bind) (highlighted) is the total binding free energy. The experimental relative binding affinities (highlighted) are deducted from the half-maximal inhibitory concentration (IC50) of these TCAs [20] which should strongly correlate to their binding affinities for non-competitive inhibitors. Clomipramine has an IC50 of inhibition of about eight fold lower than Imipramine. Thermodynamically, this corresponds to a free-energy decrease of about 2 kT (~1.5 kcal/mol at a temperature of 315 K). It was also shown that desipramine is a less potent inhibitor compared to Imipramine (personal communication from S. Singh).

discuss these roles separately, we note that a Na^+ ion most probably have both effects at the same time. The structures of bacterial homologue of NSS family transporters LeuT reveal two Na^+ binding sites: site Na1 and site Na2 [5,19,20]. The Na2 ion is ~8 Å away from the bound substrate and does not directly bind to the substrate. By contrast, the Na1 ion directly binds to the carboxyl group of the bound leucine substrate [5]. This Na1 site is assumed to be conserved in mammalian NSS transporters including hSERT, and the GABA transporter GAT1 from brain [16,36,37]. To account for this probable direct coupling between Na^+ and substrate in the EAATs, a 3-ion model of the glutamate transporter Glt has been developed in a recent experimental and computational study [36] (more details in Section 3.2 of this review). In this model, a third Na^+ binding site, named Na3, is identified for the bacterial aspartate transporter Glt in addition to the two Na^+ binding sites revealed by the crystal structure [38]. This putative Na3 site, similar to the site Na1 of LeuT, directly binds to the substrate [36].

The direct structural coupling between Na^+ and the co-transported substrate in the crystal structure of LeuT [5,20] and the developed 3-ion model of Glt [36] makes it tempting to hypothesize that Na^+ binding may be required prior to the loading of the binding pocket. To investigate the role of Na^+ in substrate recognition, we computed [36,39] the free energy of binding as a function of the ion occupancy. The relative binding free energies for LEU and aspartate (ASP) to the corresponding transporters are reported in Table 2 as a function of ion occupancy [36,39]. It should be stated that the overall convergence in binding free-energy simulations is very slow and binding affinities obtained for a single conformational state are hard to compare with experimental measurements reporting affinities usually averaged over the whole conformational cycle. Thus, we focused our computations on the relative binding free energies, e.g., those relative to the LeuT-LEU and Glt-ASP complexes with and without Na^+ ions in the binding pockets. In both cases, the relative free energies of the substrate binding to the transporter suggest that removal of the ion directly coupled to the co-transported substrate will have an inhibitory effect on the binding affinity [26]. The proposed direct coupling of the cation at site Na3 of Glt to the transported amino acid could explain the reported experimental Na^+/Li^+ selectivity of the apparent affinity for the transported amino acid in homologous transporter EAAT3 [36]. However, it is important to note that this direct coupling is not uniquely conserved among different transporters [3,4,14].

2.3. Chloride ions provide an additional level of support for substrate binding

The human serotonin transporter hSERT is responsible for recycling 5-HT (a.k.a. serotonin) molecule from the synaptic cleft. hSERT belongs to the SLC6 family and is a eukaryotic homologue of the prokaryotic LeuT. Although LeuT and hSERT have a low overall sequence similarity, the similarity approaches 50% at the substrate and ion-binding site. Like LeuT, hSERT consists of 12 trans-membrane (TM) helices, with an inverted symmetry. Helices 1, 3, 6 and 8 form the substrate-binding cavity, with TM1 and 6 unwound in the middle to accommodate the 5-HT molecule and expose the backbone carbonyl oxygen and amide nitrogen for ion/substrate coordination. In close proximity to this site are other residues responsible for coordinating ions required for transport. Several homology models for hSERT, derived from LeuT, have been reported [17,22,46]. These homology models have helped to identify residues forming the Na^+, Cl^-, substrate, or drug binding sites [17,22,46]. The proposed transport stoichiometry is 1-5-HT in: 1-Na^+ in: 1-Cl^- in: 1-K^+out [40], which yields a transporting cycle that is electroneutral. However, this transport stoichiometry has not yet been conclusively confirmed. To complicate things even more, at times there is a non-stoichiometric flux where additional 5-HT-induced charge movement occurs, which is referred to as an uncoupled current [41]. Uncoupled currents do occur under physiological conditions in wild-type proteins. However, the frequency and magnitude of the uncoupled current are increased in certain mutants.

The intriguing phenomenon of the coupling between the transport of chloride and the transporter's function has been recorded for a number of secondary transporters including hSERT and hDAT [16,37]. In hSERT, for example, the involvement of a chloride ion is important for the 5-HT transport cycle [42-44]. The chloride ion has also been shown to enhance the binding affinity of 5-HT and several antidepressants [34,45]. The mechanisms for the correlation between Cl^- and substrate transport and substrate/drug binding remains somewhat controversial. Forrest et al.[16] and Zomot et al.[37] showed that the Cl^- binding sites in SERT and GABA (gamma-aminobutyric acid) transporter GAT-1 from rat brain share some residues with the Na1 site and provides direct coupling to 5-HT binding/transport. In a recent work by Tavoulari et al., it was shown that Cl^- binding enhances the binding affinity of fluoxetine (Prozac) by providing direct favourable physical interaction to antidepressants as well as affecting the conformational equilibrium of SERT [45].

In summary, Cl^- provides an additional level of control and its presence is absolutely crucial to proper function. How this coupling could possibly be achieved? In a recent study [47], the experimental data along with our simulation data suggest that an asparagine residue (N101) in TM1 is important for the coupling of Cl^- to 5-HT transport [47]. This residue is directly responsible for coordinating Na^+, equivalent to residue (N27) in LeuT. However, rather than directly coordinating Cl^- binding, we found that N101 translates Cl^- binding into the preparation of the active site for 5-HT and Na^+. We also showed that the S336 of TM6 is important for efficient coupling between 5-HT and Na^+. Although LeuT transport is Cl^- independent, evolutionarily there is still evidence of negative charge coordination [16]. LeuT has an E290 residue [16] at the position corresponding to the proposed Cl^- binding site, which provides a negative charge that can substitute for the Cl^- (see Fig. 2A/B). In the eukaryotic hSERT, this residue is replaced by a neutral residue S372, making the presence of Cl^- a necessity [16]. A combined MD simulation and electrophysiology approach was used to assess the effects of the mutation N101A on the substrate/ion coupling. The mutant did not show the Cl^- dependence of the wild-type system, suggesting that Cl^- was not required for 5-HT transport by the mutant. As previously mentioned, “leak current” does occur, but in mutants, these currents have a greater magnitude [47]. This indicates that N101 is also important for maintaining proper stoichiometry.

It is logical to assume direct coordination of Cl^- by N101. However, Cl^- does not shield inactivation of N101C mutants [48], indicating a lack of close proximity. Furthermore, two recent studies that identified the Cl^- binding site in SERT and GAT1 do not implicate N101 as one of the coordinating residue [16,37]. Our MD simulations [47] further support that N101, like N27 in LeuT, exclusively coordinates Na^+, not Cl^-. The models for both wild-type hSERT and the N101A mutant were built [46] with combination of homology and de-novo (ROSETTA) modeling. The binding sites for the substrate (5-HT) [49,50] and the Na1 and Na2 ions [5] were determined based on data available in literature. To assess stability of bound substrate and co-transported ions we used all-atom MD simulations for these proteins embedded into lipid bilayer both in the absence and presence of Cl^-. Results from these analyses for Cl^- are displayed in Fig. 2B/C. In hSERT, Na^+, Cl^- and 5-HT are co-localized around the Na1 binding pocket through their coordination by residues in TMs 1, 2, 6 and 7. These four helices form a bundle whose relative movement within the protein allows for opening and closing of the permeation pathway [17]. Our structures predict that Cl^- and Na^+ coordination is linked via residues S336 (TM6) and N368 (TM7). 5-HT interacts with the active site residues via a salt bridge provided by its ethylamine nitrogen. Our MD simulation results [47] indicate that a backbone shift at residue S336 in response to Cl^- removal is responsible for the loss of hydrogen-bonding interactions between the OH of the S336 side chain and the NH of the N368 side chain, uncoupling the

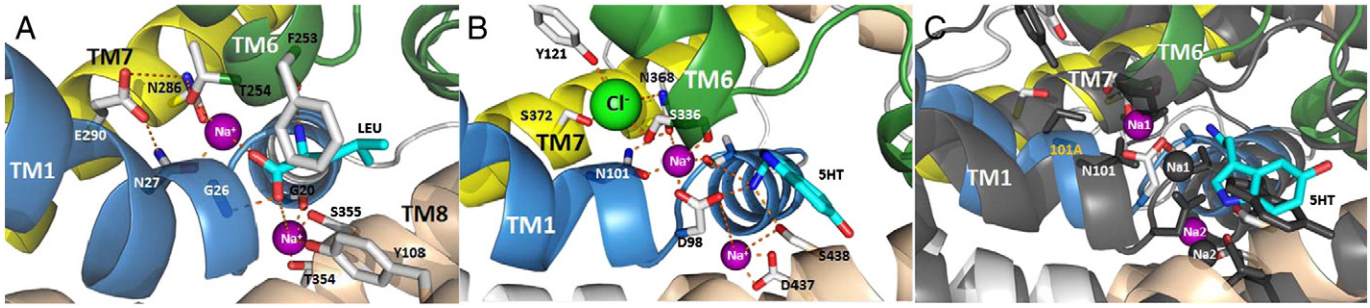

Fig. 2. Comparative look at the similarities and differences of the LeuT (A) and hSERT (B, C) binding sites. Both sodium ions (magenta) are shown in their respective Na1 and Na2 binding sites with interacting residues. Chloride ion (green) is shown in the hSERT NaCl system (B) only, as LeuT does not have a coordination network to accommodate the anion. However a negative charge is provided at this site by the Glu290 residue of LeuT (A). Panel (C) shows the hSERT WT (gray) and N101A mutants superimposed onto one another. Both systems are Cl− free and the creation of the new Na1 coordination network for the mutant system is achieved by the Na+ (purple sphere) shift [47].

interactions of TMs 1 and 6 with TMs 2 and 7 (Fig. 2C). Though indirect, the interactions of N101 observed in hSERT are likely critical to coupling: They lead to an extensive hydrogen bond and coordination network around the bound ions/substrate. The N101A mutation considerably disrupts the H-bond network found in the hSERT substrate-Na1 ion-binding pocket. Coordination of Na^+ by the N101 side chain amide oxygen is lost, as is the H-bond between 5-HT and S336. However, the N101A side chain, being considerably smaller than that of N101, permits a local repacking of the Na1 binding site in the absence of Cl^- that displaces the Na^+ ion by ~2.0 Å. This allows for the formation of a novel Na^+ coordination site (see Fig. 2C) in which the S336 side chain coordination is reinstated. These interactions in the mutant effectively mimic the relative positioning and interactions of the same residues found in the WT hSERT Cl^- ion coordination models, therefore allowing for 5-HT transport in the absence of Cl^-.

To further our understanding of the complex role of Cl^- in the binding of Na^+ and 5-HT, we computed [47] binding enthalpies using an MM/PBSA approximation [51]. The evaluation of binding enthalpies for N101A helps to elucidate the exact role of the anion in the transport cycle (Table 3). The ΔΔH for the Cl^--free WT protein shows significant inhibition in both Na^+ and 5-HT binding to the transporter, which indicates an important role of Cl^- in the stabilization of the entire binding pocket. The analyses of the ion coordination within the Na1 site showed that the Cl^--free transporter displayed different ion coordination for Na1 than the anion bound complex. The coordination number of Na1 is reduced from 6 to 5 in this case. Removal of Cl^- from the N101A mutant led to the re-establishment of Na1/Na2 sites (see Fig. 2C) with the energetics comparable to that of the WT system. On the other hand, binding of Cl^- to the N101A mutant destabilizes ion coordination at the Na1 site such that the affinity of Cl^- for the transporter is reduced relative to that of a wild-type transporter. In summary, our analysis [47] suggest that N101 may play an important role in modulation of binding affinity to sites Na1 and Na2, as well as participate in the regulation of Cl^- binding. It is clear that Cl^- is essential to transport by properly prepping the active site for subsequent ion and substrate binding. We have also demonstrated that Cl^- binding translates to proper N101 positioning, allowing for the most energetically favorable ion/substrate binding.

Table 3
Enthalpies of binding for Na^+/Cl^-/5HT in the WT and N101 mutant of the hSERT system [47].

Site	WT (NaCl) ΔH	WT (Cl^- free) ΔΔH	N101A (NaCl) ΔΔH	N101A (Cl^- free) ΔΔH
Na1	−17.0	+6.0	0	+0.9
Na2	−16.0	+5.0	+5.5	+1.7
Cl^-	−7.4	–	+2.0	–
5HT	−37.0	+15.0	−1.8	+1.0

Enthalpies of binding (kcal/mol) for the substrate 5HT and co-transported ions Na^+/Cl^- relative to the wild-type system.

3. Cation selectivity in secondary transporters

3.1. One molecule—two mechanisms: The case of LeuT and Glt transporters

The selective binding of Na^+ ions is a requirement for the robust directional transport that fuels substrate uptake. The mechanism of selective ion binding to membrane proteins and subsequent ion regulation of protein function is a subject that has been hotly debated over the last fifty years [52–54]. Recent progress in the structural studies of secondary amino acid transporters provides us with a unique opportunity to address the molecular mechanism of cation selectivity [3]. In the case of a transporter, it is reasonable to expect that relative ion selectivity between ions *i* and *j* ($\Delta\Delta G_{i/j}$) will be thermodynamically governed by the differences in equilibrium binding to the site and the hydration free energies, because the lifetimes of the various conformational states of the protein are extremely long compared to the relevant dynamics of ion-binding sites. Free-energy simulations (FEP) allow for the direct computation of the property of interest using the following equation [55]:

$$\Delta\Delta G_{i,j} = \left[\Delta G^{i}_{site} - \Delta G^{j}_{site}\right] - \left[\Delta G^{i}_{bulk} - \Delta G^{j}_{bulk}\right]$$

where the first term is evaluated by a free-energy perturbation between ions *i* and *j* in the protein site and the second term is a similar perturbation in the bulk (i.e., water). The details of the simulations can be found in previously published studies [36,56].

To study the preference of two binding sites found in the structural studies of two different transporters, LeuT and Glt, we performed FEP computations for Na^+/K^+ and Na^+/Li^+ pairs [36,56]. The results for monovalent cation selectivity are listed in Table 4. Interestingly, the selectivity of the pairs of ion-binding sites from the two unrelated

Table 4
ΔΔG for Na^+/K^+ and Na^+/Li^+ selectivity in the crystallographic sites Na1, Na2 for two different transporters Glt and LeuT [36,39].

	$\Delta\Delta G_{Na/K}$	$\Delta\Delta G_{Na/Li}$
Glt		
Na1	1.9	−0.6
Na2	−0.5	2.1
LeuT		
Na1	6.1	−0.8
Na2	3.2	1.5

Reference bulk water values for this set of potential parameters for ΔG_{bulk}(K^+ to Na^+) and ΔG_{bulk}(Li^+ to Na^+) is -18.56 kcal/mol (CHARMM-27 nbfix) and 22.90 kcal/mol [39], respectively. FEP simulations were run for all-atom systems containing lipids, water, counterions and a protein. By convention, a positive sign in the table reflects a binding site preference for Na^+.

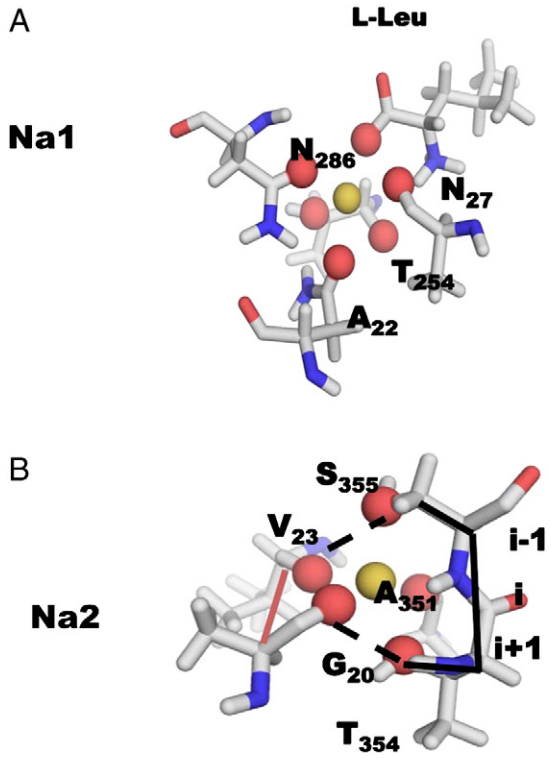

Fig. 3. A) and B) Ball-and-stick representation of the two binding sites (Na1 and Na2). The covalent and hydrogen bonds in the site Na2 are shown as solid and dashed lines, respectively.

transporters differs substantially. For instance, neutral site Na2 is unable to select for Na^+ over K^+ in Glt, but does provide high selectivity against Li^+. This is in good accord with experimental data [36]. At the same time, site Na1, which contains a charged substrate (carboxylate), has the opposite specificity sequence. This site displays substantial preference for Na^+ over K^+, yet completely lacks the ability to discriminate Li^+. It should be addressed that our studies also indicate that this site (Na2) is likely to be ambivalent in Glt, and the Tl^+ labeling (a common marker for K^+) of this site was consistent with these simulations [57].

What is the reason for these apparent differences in the sites' preference for a given cation? Results of atomistic simulations may provide some grounds for discussion. In one case (site labeled as Na1 in Glt and LeuT transporters), the presence of a charged ligand enables preference to be given to a smaller cation, such as Na^+, while the site is still intrinsically flexible. The selectivity of this site is an emergent property that depends on the balance of interactions between the ion and coordinating ligands. The presence of a charged moiety helps to overcome the penalty for ion dehydration [39]. At the same time, it is unlikely that this binding site provides substantial selectivity against competing Li^+. This seems to be a generic trend for many Na^+-selective sites. A PDB survey [39] suggests that Na^+-binding sites contain 1.1 charged ligand on average, as compared to 0.7 for K^+. Therefore, Na1 in both transporters can serve as a prototypical Na^+ site for membrane proteins.

In the Na2 site from LeuT, a local connectivity exploring *i* and *i + 1* covalent binding (where i stands for residue number), may play an important role in the exclusion of smaller cations [39]. This connectivity pattern (illustrated in Fig. 3A/B) has been found to be a unique feature of many Na^+ sites in proteins, including binding sites in two unrelated transporters, LeuT and vSGLT. Hydrogen bonding also contributes to the build-up of the local molecular stiffness. To explore their importance, we performed a statistical analysis of the hydrogen bonding of the residues forming the Na1 and Na2 binding sites [39]. The difference in hydrogen bonding and covalent connectivity between the two binding sites in LeuT is striking. The Na1 binding site contains only a single long-lived hydrogen bond between the non-coordinating backbone nitrogen group of the T254 and the carbonyl oxygen of G250. Site Na2 displays two long-living H-bonds between the G20–A19–V23 motif and the S355–G352–A351 motif. This hydrogen bonding, supplemented by the covalent bonds between residues, forms a ring-like structure that coordinates the ion. In addition, there are at least three bifurcated bonds. The backbone atoms of S355 and T354 residues coordinating the ion in the Na2 binding site are covalently bound. With its high connectivity, the Na2 binding site is reminiscent of the organization of cyclic ionophores, such as valinomycin (K-selective) or monensin (Na-selective).

3.2. Location of a third ion-binding site in the aspartate transporter Glt

What if the crystal structure is lacking the resolution of all functionally important ion-binding sites for Na^+? A particular example of this challenge can be found in the Glt transporter. Experimentally, the stoichiometry of this transport is 3:1 [63,64]. Published structures identified two binding sites and put forward a tentative mechanism of transport [57]. To circumvent this problem, we developed a blinded search algorithm enabling the identification of putative ion-binding sites [36]. Briefly, the probe particle insertion method, based on the Grand Canonical Monte Carlo method, was used to identify the location of a third sodium-binding site in Glt and EAAT3-Glt chimera proteins. Fig. 4A shows the region with highest insertion probability of probes. A number of sites identified by these methods are shown in Fig. 4B/C. To validate the location of the proposed sites, we ran free-energy simulations combined with MD simulations to assess the stability and selectivity of the identified sites. Two of the

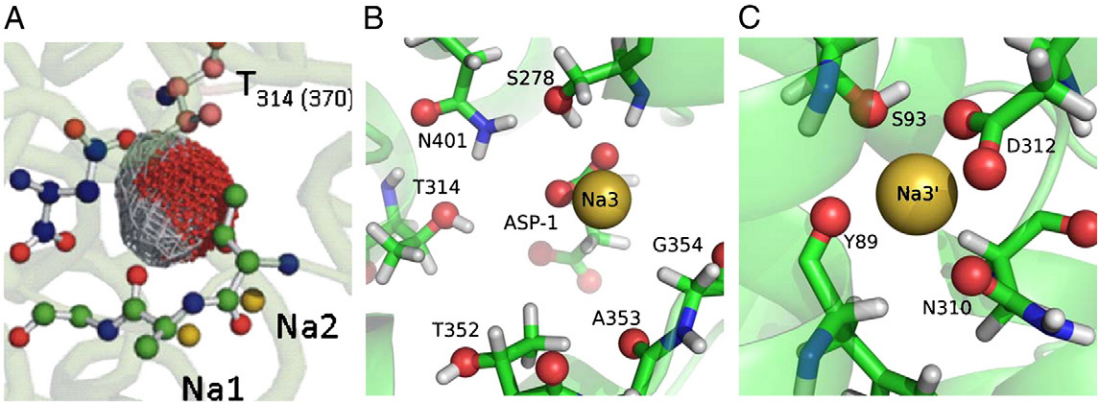

Fig. 4. Model with 3 Na^+ sites (Na1 -Na2 -Na3) suggested by the grand canonical Monte Carlo simulations. A) Region with highest insertion density of probe particles (water). Na1 and N2 are shown as yellow balls. B) and C) The proposed Na3 and Na3′ sites respectively.

sites found satisfied the requirements for selective Na^+ binding from combination of MD and FEP computations. First of the proposed binding site labeled Na3 [36] is formed by a substrate bound, the side chains of residues T314 and N401 along with A353 and G354 (Glt) (Fig. 4B). The combination of biophysical and electrophysiological studies showed that both of the identified residues (T314 and N401) are involved in the modulation of ion-dependent transport and ion preference of the transporter [36]. The second (Fig. 4C) tentative sodium-binding site refined by MD simulation involves ion coordination by the Y89, S93, N310 and D312 (Glt) side chains [36]. All of the residues forming these two sites were experimentally confirmed to be involved in sodium-binding/or transport cycle modulation. The ion bound to Na3′, however, was found to be unstable in MD simulations. Therefore, it was proposed that Na3′ serves an important functional role as a transitional binding site providing energetically favorable access for an ion to the binding pocket. Simulations suggest that the simultaneous occupation of this binding site and crystallographic site Na1 is impossible due to strong electrostatic repulsion.

Separate studies regarding the location of the third binding site based on water accessibility to the Glt interior have been performed by the Tajkhorshid group [65]. The results of the accessibility studies and extensive MD simulations placed Na^+ next to the D312 side chain. Furthermore, it also suggested a "knock-on" mechanism of the subsequent binding site occupation, where the ion occupying Na1 is proposed to jump to the Na3 binding site. The proposed Na3 site is formed by residues T92, D312 and N310 [65]. Despite apparent differences, independent studies [36,65] mapped D312, T92 and N310 as important residues for ion binding and conductance.

In review, there probably is a unique Na3, but experimentally and computationally, it is a low-affinity site that binds Na^+ with mM affinity. This renders unambiguous mapping of the site very difficult. Therefore, the ion-binding/transport picture emerging from these theoretical studies suggests the presence of several low-affinity sites separated by low barriers between different conformational sub-states of the system in which ions can slide from one site to another, shifting the stability of the gates and, perhaps, affecting substrate-binding affinity.

3.3. Studies of ion selectivity in secondary transporters based on reduced models

While computations including all-atom systems are required to establish a direct link to experimental data, reduced models [53,58,59] comprised of only the ion and the coordinating ligands may still offer insights when selectivity is related to the thermodynamic binding equilibrium in a well-defined site, like in secondary transporters. Several important assumptions must be made before invoking reduced models. First, it is assumed that long-range interactions are not expected to directly affect the relative free-energy differences between two monovalent cations. Second, the charge of the ion remains unchanged, as is the case with Na^+ and K^+. Finally, the difference in the non-electrostatic interactions between an ion and the environment is expected to decay rapidly. The problem, however, is not necessarily reducible to the toy model treatment. The rigidity of the protein matrix surrounding the binding pocket, which controls the binding site confinement by indirectly bounding its dynamics, may still be important.

To address this problem, we developed a theoretical approach [60,61] that allows us to include the local structural determinant of ion selectivity in protein binding sites into a reduced description of the toy model. First, a local subsystem with the bound ion and the N coordinating ligands is defined. Next, the influence of the rest of the system (ΔW_{site}) onto the subsystem is rigorously expressed as the sum of two separate contributions:

$$\Delta W^{site} = \Delta W_c^{site} + \Delta W_g^{site}$$

The first term in the equation above tracks all of the molecular forces confining the ion and the ligands within a microscopic subvolume. It is important to emphasize that this term does not prevent the system from adapting to an ion of a different size. The second term accounts for the actual rigidity of a site's scaffold by controlling the precise geometry of the coordinating ligands best adapted to the bound ion with the parameter λ, which has the dimensions of the force constant. This decomposition allows us to examine the role of local interactions and structure in the control of ion selectivity of the binding site. By virtue of construction, one can identify two distinct selectivity regimes. In the first regime, the geometric forces or matrix rigidity are the dominant factors behind the site's selectivity. For the second regime, selectivity emerges despite the positional fluctuations of the coordinating ligands. In the first limit, the geometric forces imposed by a protein matrix are negligible ($\lambda_g \to 0$) and the ion and ligands are free to fluctuate within the volume of the binding site. That is, the environment acts as a confinement, and the complete disintegration of the site or conformational changes greater than that of standard thermal fluctuation would destroy the site's selectivity. In this case, the selectivity of this site is expressed as:

$$\lim_{\lambda_g \to 0} \Delta G_{ij}^{site} \approx \left\langle U_i^{il} + U_i^{ll} \right\rangle_i - \left\langle U_j^{il} + U_j^{ll} \right\rangle_j$$

The free-energy difference ($\Delta\Delta G_{Na,K}$) is set by the interplay between the mean ion-ligand (U^{il}) and the ligand–ligand interactions (U^{ll}).

In the other limit

$$\lim_{\lambda_g \to \infty} \Delta G_{ij}^{site} \approx \left\langle U_i^{il} \right\rangle_i - \left\langle U_j^{il} \right\rangle_j$$

The difference in the ligand–ligand interactions as a response to an ion bound to the site is approaching zero, and the selectivity depends on the difference in ion–protein interactions. While both limits treat selectivity in an idealized manner, they seem to provide a simple illustration of the factors governing the selectivity of sites Na1 and Na2. The results of the selectivity analysis of these sites in LeuT are shown in Fig. 5. Robust selectivity for Na^+ could potentially be achieved without additional architectural forces stabilizing the site's geometry (site Na1). This relies on a balance of interactions between the protein and ions, as well as modifications of the ligand–ligand interactions in the site itself. On the other hand, site Na2 can only be selective in the presence of architectural forces created by covalent

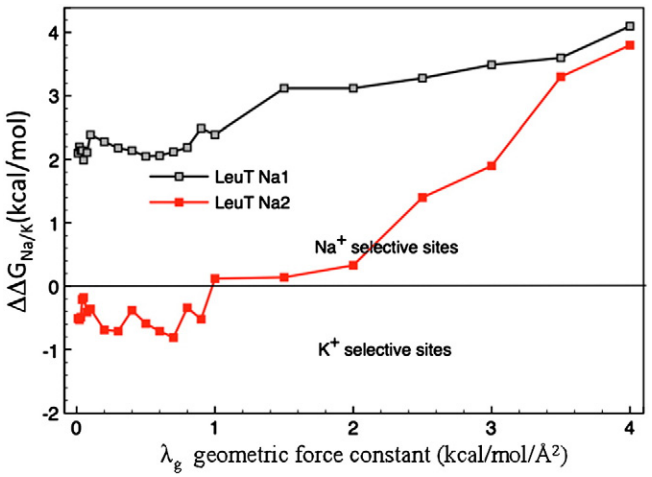

Fig. 5. Relative free energy of selectivity for two sites in LeuT as function of the site's geometrical rigidity (λg) from the reduced-model analysis [60].

bonding between coordinating ligands and a supplementary network of hydrogen bonds, consistent with the results of simulations using the entire protein. Interestingly, it is site Na2 that is conserved among different transporter families. This may suggest local structure-dependent selectivity, indicating a "trigger-like" role for this site in the transporting cycle [39,62]. Of course, this analysis of molecular mechanisms for ion selectivity in transporter sites based on toy models is a simplification of a single binding site. Other factors, such as coupling between two adjacent binding sites in LeuT, may play a significant role in the determination of the binding/selectivity properties of this two ion-binding pocket. This is a topic for future investigations.

4. Molecular insights into gating dynamics: A big role for small molecules

4.1. Conservation of the LeuT Na2 ion in secondary transporter of the LeuT fold

The recent discovery of the LeuT (structural) super family of transporters reveals striking similarities in the topological organization of these proteins, despite the fact that these transporters belong to different families and their sequence similarities are low. Although the sodium-binding stoichiometry for these transporters varies, one sodium-binding site, namely the Na2 sodium-binding site in LeuT, is conserved across LeuT (Na2), vSGLT, and Mhp1. In BetP, the Na2 site has been assigned to a different site [9], but there are speculations that the site might be at the same position as LeuT Na2 [4,66]. This conserved Na^+ site is composed of residues from two TM helices that belong to the "bundle" and the "scaffold" [17], respectively. For example, in LeuT, the Na2 site (Fig. 1) [5] is formed by the two backbone carbonyl oxygen atoms from G20, V23, and A361 and two side chain hydroxyl oxygen atoms from T354 and S355. These residues belong to either TM1, part of the "bundle", or TM8, part of the "scaffold". It should be noted that this Na^+ does not directly coordinate to the main substrate as the Na1 Na^+ (in LeuT or BetP) does. Despite the differences among the main substrates of these transporters, the conservation of this sodium-binding site (Na2) indicates that there are some general mechanisms that govern the coupling between this Na^+ and the main substrate.

4.2. Role of the sodium ions in modulating the gating dynamics of LeuT-fold Na^+-coupled transporters

The general mechanism for ion-coupled substrate transport is the alternating access mechanism [3,4,15]. Briefly, the ion(s) and substrate bind to one face (extracellular) of the transporter, which then induces conformational changes, followed by translocation of the ion(s) and substrate to the opposite face (intracellular) of the membrane. The transporter then changes its conformation from inward-facing to outward-facing, thereby resetting the cycle. Ion/substrate binding and dissociation change the free-energy landscape of the ion/substrate/protein complex that drives this translocation cycle over and over. One version of the alternating access for the LeuT-fold Na^+-coupled secondary transporters is the "rocking bundle" model proposed by Forrest et al. [17] The essence of this model, in the instance of LeuT, is that the rigid body movement of the four-helices "bundle", relative to the "scaffold", captures the major conformational changes needed for the protein to change its conformation between open-out and open-in states. Na2, located in the interface of the "bundle" and the "scaffold", may play an important role in modulation the transport cycle. Although modulation of the transporter's conformational dynamics via the binding and unbinding of Na^+ to Na2 is conceptually simple, the exact role of the ion and the mechanisms that lead to the destabilization of the thermodynamically stable occluded state are still poorly understood [3]. Many studies have devoted efforts to understanding the coupling mechanism between the main substrate and the co-transported sodium ion(s).

Celik et al. [67] studied the binding of the leucine substrate to LeuT and its coupling to the two sodium ions. Using steered molecular dynamics implemented in NAMD, they elucidated the unbinding path of the leucine substrate from the occluded LeuT. By analyzing the pulling forces, they concluded that the Na1 ion may bind earlier than the leucine substrate. The presence of a Na^+ in Na1 contributes to the electrostatic attraction of the leucine substrate toward the binding pocket during its translocation from the extracellular vestibule into the central binding pocket. As for the Na2 ion, they proposed that it may not be coupled to the substrate transport and may only play a structural role, at least for this stage of transport (binding of the substrate and Na1 ion) [67]. In a study carried out by Caplan et al. [39], it was shown that the binding of the Na^+ ion to LeuT Na2 is required to enhance the ion selectivity of the two ion-binding motif in LeuT and prevent the binding of both K^+ and Li^+ [39]. Shi et al.[27] found that the presence of Na^+ in the Na1 site of LeuT reorganizes TM3 and TM8 and facilitates the leucine binding site's opening to the extracellular milieu. However, this result is inferred from simulations of LeuT in the *0:0:0* state (with the Na1 and Na2 ions and substrate removed) and the *Na1:Na2:0* state (with the substrate removed). Therefore, the presence of the Na2 sodium might also contribute to this effect.

In order to focus on the role of the conserved Na2 site, we recently conducted a detailed analysis of the *Na1:0:Leu* (with Na2 ion removed) and *Na1:Na2:Leu* (fully loaded occluded) states of LeuT using a combination of molecular dynamics, free-energy computation and quasi-harmonic analysis [62]. These studies revealed that the removal of Na^+ from Na2 encourages rapid local hydration of the protein core between the "bundle" and "scaffold" sub-domains and modulates the gating dynamics. The removal of the Na2 ion reinforces the D404-R30 extracellular thin gates but promotes an opening of the R5-D369 intracellular thin gate. The tightening of the extracellular thin gate is achieved primarily by the ion-induced movement of D404, which belongs to TM10. Interestingly, TM10 forms the proposed extracellular thin gate of Mhp1 [8]. MD simulations combined with principal component analysis suggest that the ion-coupled opening of the R5-D369 thin gate is achieved by the movement of TM1I. This is in accord with the vSGLT transporter [68], where TM1I flexes by approximately 13° at the kink in the helix–break–helix region to release the substrate to the intracellular side. Interestingly, our simulation results are consistent with the single molecular FRET experiments carried out by Zhao et al. [69] and EPR experiments carried out by Claxton et al. [70]. Zhao et al. [69] showed that with the nominal absence of Na^+, the probability of intracellular gate opening is promoted through a reciprocal motion of TM1, while Claxton et al. [70] showed that the binding of Na^+ induces an outward-facing conformation. Shi et al.[71] further proposed that the Na2 sodium of LeuT and the corresponding charged side chain of K158 in ApcT modulates the intracellular gating dynamics through the initial modulation of a backbone dihedral in TM1, namely V23 in LeuT and I22 in ApcT. It is clear that ion selectivity and binding is important, as this leads to substrate binding, but how is this all coupled together to induce substrate translocation? The current understanding is that all ions and substrate bind to the open-out conformation, followed by a conformational change, the transporter into an open-in conformation. At this point, the affinity for the substrate and ions is lowered, releasing them to the cytoplasm. In order to reset the transport cycle, the conformation of the transporter needs to be reverted back to an open-out state. To achieve this, the binding of Na^+ may play a role in LeuT [62]. For Glt, this conformational change may be driven by stochastic dynamics of the transporter with relatively low barriers between conformational states [72]. For SERT, a potassium ion may be counter transported. The mechanism of K^+ binding is hard to deduce, but hopefully our results and future work on selectivity at the atomic level can guide us in the future and challenge current theories.

4.3. Not only ions and a substrate: Water as an active player in the transporter cycle

Water transport across the membrane may occur through many proteins; however, the only permeation mechanism resolved with atomic resolution thus far is that for aquaporins. One of the most striking illustrations of the physiological importance of water transport across secondary transporters can be found in the sugar transporters family. Sugar transporters couple the uptake of one sugar molecule to the co-transport of a sodium ion, also translocating approximately two hundred water molecules playing an important role in the regulation of the cell osmolarity. It is well accepted that co-transporters facilitate water permeation by either or both of two independent mechanisms: osmotic flow through a water channel in the protein and flow driven by ion/substrate co-transport. However, the molecular mechanism of transport-linked water flow is controversial. The crystal structure of vSGLT [6] has served as a model for the exploration of water permeation across sugar transporters via computational studies. In an attempt to shed some light on the molecular basis for water transport across vSGLT, Choe et al. [76] performed a 200 ns MD simulation of the transporter. Their results showed that a significant number of water molecules cross the protein through the sugar-binding site in both the presence and absence of galactose. This result is in accordance with the hypothesis of a passive channel mechanism. On the other hand, the authors noted that approximately 75 water molecules follow the escape of galactose from its binding site into intracellular space. Based on this observation, Choe et al. argued that galactose also acts as a piston that rectifies the flow of water, thus supporting the idea of the co-transporter as an active pump. We have also investigated [73] putative water permeation pathways through vSGLT and through the homologous human Na-glucose co-transporter (hSGLT1) by means of Grand Canonical Monte-Carlo, Potential of Mean Force (PMF) and MD simulations. The vSGLT structure captured in the occluded-in [6] conformation and a homology model of hSGLT1 made from this structure have been used for our study [73]. For vSGLT, our study showed the presence of a water-filled pathway, which was in accordance with results from Choe et al. However, the path is interrupted in the middle of the protein by a hydrophobic constriction made of protein side chains instead of the sugar. In contrast, hSGLT1, which lacks one of the hydrophobic residues in the constriction zone, features a continuous water permeation pathway going through the sugar-binding site. Additionally, the hSGLT1 model displayed an alternative water path that runs parallel to the putative substrate pathway. We performed MD and PMF simulations on the WT form of the two transporters. Both display a well-defined constriction zone that controls/limits water transport through the transporter. The energetic cost of filling any of the observed water paths are in accordance with passive flow. Moreover, the energetic profile of a water molecule going through the proposed permeation routes is asymmetric, suggesting unidirectional flow [73]. The results on a water pathway in the hSGLT1 transporter, however, should be taken with a grain of salt. The exact barrier heights are sensitive to packing of residues forming the path. The use of homology model, even refined with all-atom simulations allowing for structural relaxation, may take its toll on a predictive power. Nevertheless, the combination of theoretical and experimental studies reported in Ref. [73] shows qualitatively consistent molecular mechanism of water transport. The water transport across secondary transporters may not be a unique feature of the sugar transporters family. Existence of the water pathways from cytoplasm to the substrate-binding site for sodium-coupled LeuT-fold transporters has been proposed for rSERT [17], PutP [74], vSGLT [68], Mhp1 [7], and LeuT [62,75]. Computational studies allow for the examination of the role that water may play in transporting cycle at an atomic resolution. In our MD simulation of LeuT [62], we showed that water molecules could access the Na2 ion from cytoplasm, even in the occluded state. The removal of Na^+ from Na2 encourages further hydration (Fig. 6) and may set up a series of events that lead to conformational transition with a rocking of the "bundle" relative to the "scaffold". We also proposed that the local hydration of the polar residues of the Na2 sites might compensate for the energy penalty due to the release of the Na^+. Therefore, slow but certain hydration of the binding pocket may provide a sufficient driving force to move the transporter from one state to another by binding/unbinding events.

5. Conclusions

The main purpose of this mini-review is to show that computational approaches, although limited in time-scale, can play a role of middleman between structural and functional studies of ion-coupled secondary transporters [16,17,27,36,47,62,68]. Free-energy simulations and theoretical developments have demonstrated that ion binding and selectivity in these proteins can rely on two distinct mechanisms: exploiting local stiffness (site Na2 of LeuT) or a complex network of interactions (sites Na1 in LeuT and Glt transporters) [56]. Molecular simulations also suggest that "structural" sites such as Na^+ binding sites in vSGLT [68], Mhp1, and LeuT (Na2) may be involved into modulation of the gating transitions in secondary transporters. The studies based on homology models for hSERT suggest that the actual mechanism of substrate stabilization may rely not only on the direct interactions between the co-transported substrate (5HT) and the binding pocket in the transporter, but also on indirect effects due to the presence of Cl^- ions in the adjacent binding site [45,47]. It was shown that Cl^- is required for the orientational stabilization of several residues involved in coordination of the bound substrate [47]. We argue that water may play an active role in transport being a substrate itself (vSGLT) [73,76] and a potential "catalyst" for the gating dynamics in LeuT [62]. The "wetting" of the binding pocket for the solute and unbinding of an ion coupled to the solute, are both

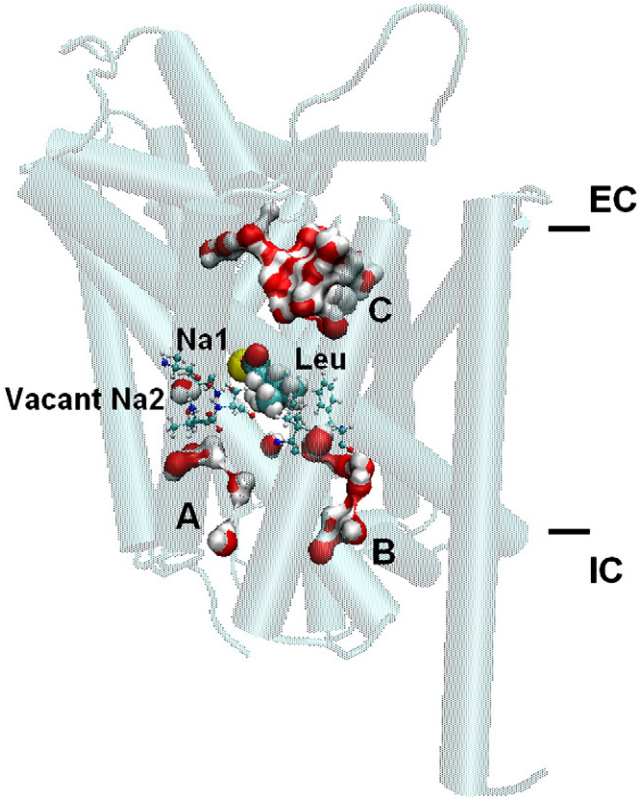

Fig. 6. Water pathways from intracellular (IC) and extracellular (EC) sides to the protein core of LeuT with Na^+ in the Na2 sodium-binding site removed. The pathways from IC lead to the Na2 site (pathway A) and the substrate-binding site (pathway B) respectively [62].

expected to have unnegligible effects on the stability of the occluded states [62,76].

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