Ion-Releasing State of a Secondary Membrane Transporter

Jing Li and Emad Tajkhorshid*
Department of Biochemistry, Beckman Institute, and Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois

ABSTRACT The crystal structure of Na⁺-coupled galactose symporter (vSGLT) reports the transporter in its substrate-bound state, with a Na⁺ ion modeled in a binding site corresponding to that of a homologous protein, leucine transporter (LeuT). In repeated molecular dynamics simulations, however, we find the Na⁺ ion instable, invariably and spontaneously diffusing out of the transporter through a pathway lined by D189, which appears to facilitate the diffusion of the ion toward the cytoplasm. Further analysis of the trajectories and close structural examination, in particular, comparison of the Na⁺-binding sites of vSGLT and LeuT, strongly indicates that the crystal structure of vSGLT actually represents an ion-releasing state of the transporter. The observed dynamics of the Na⁺ ion, in contrast to the substrate, also suggests that the cytoplasmic release of the Na⁺ ion precedes that of the substrate, thus shedding light on a key step in the transport cycle of this secondary transporter.

Received for publication 14 June 2009 and in final form 3 September 2009.

*Correspondence: emad@life.uiuc.edu

Secondary membrane transporters couple electrochemical ionic gradients across the membrane to uphill translocation of their substrates. Despite diversity in sequence and substrate, accumulating evidence from biochemical, kinetic, and structural studies suggests that all ion-coupled transporters operate via the so-called alternating-access mechanism, in which the transporter alternates between two major conformational states, an inward-facing and an outward-facing one—thereby switching the substrate access between the two sides of the membrane (1,2).

Due to the lack of high-resolution structures of the same transporter in both states, however, the nature of the structural transitions involved in the process, and how they underlie the transport mechanism, are largely unknown. Furthermore, only limited information is currently available on the sequence of binding and unbinding events during the transport cycle for most transporters. In recent years, the crystal structures of several secondary membrane transporters have been resolved. Most of the structures, namely, leucine transporter (LeuT) (3–5), glucose transporter (GluT) (6), the benzyl-hydantoin transporter from Microbacterium liquefaciens (Mhp1) (7), and the latest, betaine transporter (BetP) (8), are either in the outward-facing or in an intermediate, occluded state. Capturing the first inward-facing conformation, the structure of a bacterial Na⁺-coupled galactose transporter (vSGLT) takes a critical step toward a better characterization of the alternating-access mechanism in secondary transporters (9).

Na⁺/substrate stoichiometry in vSGLT has been identified as 1:1 (10). However, in the reported crystal structure, no Na⁺ binding site could be verified by cocry stallization of the protein with electron-dense ions (9). Based on the structure of LeuT (3), another Na⁺-coupled secondary transporters with significant architectural similarity to vSGLT, a Na⁺ ion was modeled in a plausible Na⁺ binding site at the intersection of TM1 and TM8 (9). The observation that the S365A mutation completely abrogates Na⁺-dependent transport in vSGLT strongly supports the notion that the proposed binding site is indeed a Na⁺ binding site (9). Moreover, several residues in this binding site, i.e., A62, I65, A361, S364, and S365, correspond to conserved residues in the Na⁺-binding site of LeuT. However, whether this site is occupied by a Na⁺ ion in the inward-facing conformation of vSGLT reported in the crystal structure remains an open question. The occupancy of this site is of high relevance to the sequence of events in the transport cycle of vSGLT. Whereas the binding sequence of the substrate and ion(s) is characterized for vSGLT and hSGLT (human Na⁺/glucose transporter) (11–13), the unbinding sequence of these species into the cytoplasm continues to be unknown.

To address this question, we performed several equilibrium simulations of a membrane-embedded model of vSGLT (see the Supporting Material for details). Invariably, in all of the simulations, the Na⁺ ion unbinds spontaneously from the binding site within a few nanoseconds and diffuses into the cytoplasmic solution (Fig. 1). The reproducible, rapid unbinding of Na⁺ strongly suggests that the binding site does not provide an optimal configuration to retain the ion. In other words, the crystal structure appears to have captured an open Na⁺ binding site.

Interestingly, in all the simulations, after detaching from the binding site, the Na⁺ ion moves first to the region around D189, where it spends some time before completely leaving the protein (Fig. 1). It appears that the Na⁺ ion is somehow attracted by this residue, which is ~5 Å away from the Na⁺ ion in the crystal structure, and represents a highly conserved residue within the Na⁺/solute cotransporter family. The observed dynamics of the Na⁺ ion suggests that this residue...
might act as a fishhook above the Na\(^{+}\) binding site, facilitating the diffusion of the ion toward the cytoplasm in vSGLT. This residue, however, does not seem to constitute a binding site for the ion, as the ion only transiently interacts with it along its unbinding pathway.

The importance of D189 is supported by studies of homologous proteins, Na\(^{+}\)/proline transporter PutP and hSGLT1, where mutation of this residue results in complex effects ranging from significant decrease of the Na\(^{+}\)-coupled transport (14), or even altered Na\(^{+}\)/H\(^{+}\) selectivity (15).

To examine other possible Na\(^{+}\) binding sites that might be occupied in the crystal structure of vSGLT, a 10-ns simulation was done on a Na\(^{+}\)-free protein. Any site occupied with a Na\(^{+}\) ion under crystallization conditions would be expected to exhibit detectable, rapid local conformational changes in response to the absence of the ion in the simulation. Therefore, we should be able to identify potential Na\(^{+}\) binding sites through examination of fluctuations and structural deviation of individual side chains. The root mean-square deviation (RMSD) of the whole protein remains consistently <2.5 Å in this simulation, indicating a stable conformation in the absence of Na\(^{+}\). The individual RMSD of the majority of residues in vSGLT is <2.5 Å (Fig. S1). The residues with higher RMSD values are mostly facing outwards, e.g., facing the membrane (thus, irrelevant to specific Na\(^{+}\) binding). Furthermore, they are spatially too scattered to be able to cluster and form a Na\(^{+}\) binding site. Note that coordination of a Na\(^{+}\) ion requires multiple ligands, usually provided by oxygen atoms from two or more residues in proteins. The substrate and Na\(^{+}\) have been suggested to be transported with 1:1 stoichiometry (10) in vSGLT. Faham et al. report that no second Na\(^{+}\) binding site could be identified (9), i.e., the site constituted by A62, I65, A361, S364, and S365 is probably the only ion binding site. In the Na\(^{+}\)-free simulation, these residues except I65 demonstrate RMSD values <1.5 Å, without any apparent major conformational changes, consistent with the notion that the site was likely not occupied by a Na\(^{+}\) ion in the crystal.

Our proposal that the Na\(^{+}\) binding site in vSGLT cannot stably bind Na\(^{+}\) in the inward-open conformation captured in the crystal structure is strongly supported by the comparison of the Na\(^{+}\) binding sites of vSGLT and LeuT. At the sequence level, the residues in the Na\(^{+}\) binding sites of vSGLT and LeuT are very similar. In LeuT (3), residues coordinating the ion, i.e., G20, V23, A351, T354, and S355, form a tight, square pyramidal arrangement around the Na\(^{+}\) ion, with Na\(^{+}\)–O distances ranging between 2.1 and 2.4 Å (Fig. 2). All of the corresponding distances in the binding site of vSGLT, however, are above 3.1 Å. In particular the distances between the Na\(^{+}\) ion and A62:O or S365:OG are >3.6 Å, with angles that are too irregular for an optimal Na\(^{+}\) coordination (Table S1).

In such a configuration, stable ion binding is very difficult for the site in vSGLT to achieve. We note that for the Na\(^{+}\)-binding site of LeuT, Na\(^{+}\) was found to be completely stable in MD simulations of over tens of nanoseconds (16). Based on these results, we suggest that the Na\(^{+}\) binding sites of LeuT and vSGLT represent two distinct states, a closed and an open binding site, respectively. The configurational differences between these two binding sites seem to have originated from a shift in the interhelical angle of TM1 and TM8 in vSGLT (corresponding to TM1 and TM8 in LeuT), which becomes apparent by overlaying the structures of the two proteins (Fig. 2). The smaller interhelical angle of LeuT furnishes

**FIGURE 1** Spontaneous Na\(^{+}\) unbinding from its binding site in vSGLT. (A and B) Positions of the Na\(^{+}\) ion at t = 4 ns of simulations (Na1–Na6), with Na0 indicating the position of the ion in the crystal structure. TM1 (pink) and TM8 (orange) are the helices directly participating in the Na\(^{+}\)-binding site. D189 is close to the N-terminus of TM5 (blue). (C and D) Na\(^{+}\) trajectories in the first 4 ns of simulations Na2 (C) and Na4 (D) colored from red to blue according to the time. (E) Displacement of the Na\(^{+}\) ion from its position in the crystal structure. The gray bar highlights the region in the vicinity of D189.
a closed binding site, which binds Na\(^+\) tightly, whereas the larger angle in vSGLT results in the opening of the Na\(^+\) binding site. These conclusions are all reinforced by similar comparisons made with another transporter, Mhp1—which, similar to LeuT, exhibits a closed Na\(^+\)-binding site (Fig. S4).

Sequence comparison of LeuT, Mhp1, and vSGLT suggests that the Na\(^+\)-binding site proposed in the crystal structure of vSGLT is indeed a conserved ion binding site for divergent transporters and is critical for coupled substrate binding and symport (17). Our study, however, strongly suggests that this site is in an ion-releasing state in the crystal structure. This finding has important ramifications in our structural view of the alternating access mechanism. Viewing the Na\(^+\) binding site of vSGLT as an open site, and comparing it with the corresponding closed sites in LeuT and Mhp1, we can deduce an important functional feature from the crystal structures of these proteins. Structural comparison of these transporters provides the first example in which we clearly see how global structural changes (tilting and shift of the helices) that take place during the transition between the inward-facing and outward-facing states, propagate into specific binding site of the ion (expansion of the site), thus, allowing the protein to release the ion into the solution. Furthermore, the rapid release of the Na\(^+\) ion within a few nanoseconds observed in all simulations, in sharp contrast to the substrate which did not exhibit any appreciable displacement from its binding pocket even after extension of one of the simulations to 200 ns (Fig. S2), suggests that the cytoplasmic release of the Na\(^+\) ion precedes that of the substrate, thus, shedding light on a key step in the transport cycle of this secondary transporter.

**SUPPORTING MATERIAL**

A detailed methodology and additional results, including one table and four figures, are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01449-0.

**REFERENCES and FOOTNOTES**


