Published in final edited form as:

Nature. 2010 December 16; 468(7326): 988–991. doi:10.1038/nature09580.

The mechanism of sodium and substrate release from the binding pocket of vSGLT

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Abstract

Membrane co-transport proteins that utilize a 5-helix inverted repeat motif have recently emerged as one of the largest structural class of secondary active transporters^{1,2}. However, despite many structural advances there is no clear evidence as to how ion and substrate transport are coupled. Here, we report a comprehensive study of the Sodium-Galactose Transporter from *Vibrio parahaemolyticus* (vSGLT) consisting of molecular dynamics simulations, biochemical characterization, and a new crystal structure of the inward-open conformation at 2.7 Å resolution. Our data show that sodium exit causes a reorientation of transmembrane helix 1 (TM1) opening an inner gate required for substrate exit, while also triggering minor rigid body movements in two sets of transmembrane helical bundles. This cascade of events, initiated by sodium release, ensures proper timing of ion and substrate release. Once set in motion, these molecular changes weaken substrate binding to the transporter and allow galactose to readily enter the intracellular space. Additionally, we identify an allosteric pathway between the sodium binding sites, the unwound portion of TM1, and the substrate binding site that is essential in the coupling of co-transport.

Secondary active transporters harness the energy stored in electrochemical gradients to drive the accumulation of specific solutes across cell membranes. This task is accomplished by the alternating access mechanism in which the substrate binding site is first exposed to one side of the membrane and, upon ion and substrate binding, a conformational change exposes the transported solute to the opposite face where it is released³. Sodium-glucose co-transporters are prototypes of secondary active transporters that drive the accumulation of sugars, amino acids, and other molecules into cells. In humans, these transporters play critical roles in the physiology of the brain, intestine, kidneys, and thyroid, where mutations in their genes are responsible for severe congenital diseases⁴. Furthermore, sodium-glucose co-transporters are

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Author Contributions Experiments were carried out and diffraction data collected by A.W., V.C. and J.A. Simulations were carried out by S.C. Data were analysed by A.W., S.C., V.C., J.M.R., E.M.W., M.G. and J.A. The manuscript was prepared by A.W., S.C., V.C., J.M.R., E.M.W., M.G. and J.A.

Author Information Coordinates and structure factors of the inward-open vSGLT structure have been deposited in the Protein Data Bank under accession number XXXX.

Supplementary Information accompanies the paper on www.nature.com/nature.

molecular targets for drugs to treat diabetes and obesity⁵. Elucidating the structural basis for co-transport will greatly facilitate our understanding of these diseases.

Over the last two years, there has been a surge of crystal structures^{6,7,8,9,10,11} displaying the 5-helix inverted repeat motif as originally reported for LeuT¹². Although the total number of transmembrane helices may differ, these structures share a common assembly of 10 TMs. This core domain is the structural hallmark of this family of transporters, referred to as the LeuT-superfamily, which include genetically diverse proteins that transport a wide range of substrates and differ in the number and type of driving ligand^{1,2}.

A general model for alternating access is being pieced together through comparisons of diverse structures from this family^{1,2,13}. However, the absence of sequence similarity between members and the chemical diversity of the transported substrates have prevented elucidation of the mechanistic basis for this process. This hurdle is being surmounted as *multiple structures of the same protein*--residing in different conformations throughout the transport cycle--are being solved, thus providing a comprehensive understanding of substrate binding^{7,14,15,16} and the transition from outward- to inward-facing conformations¹⁷. However, an atomic level understanding of sodium-coupled substrate co-transport is still absent but is required to explain the dynamics of alternating access utilized by this entire family of proteins.

To investigate the mechanism of sodium sugar coupling, we carried out molecular dynamics (MD) simulations on the galactose bound inward-occluded conformation of vSGLT (PDB ID: 3DH4)⁶ embedded in a 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) membrane¹⁸. All sodium co-transporters of the LeuT-superfamily share a common sodium binding site, termed the Na2-site, that adopts a different arrangement in the outward- as compared to the inward-facing conformation^{1,17}. In the inward-facing conformations of vSGLT and the hydantoin transporter Mhp1, TM8, which forms part of the sodium binding site, is displaced ~4 Å (relative to the outward conformation), generating a less favourable Na2-site that facilitates Na⁺ release (Fig. 1b). Na⁺ modelled at this site is loosely coordinated by the carbonyl oxygens of I65 (3.3 Å), A361 (3.2 Å) and the side-chain hydroxyl of S365 (3.1 Å) (vSGLT numbering). The carbonyl oxygen of A62 (3.6 Å) and the side-chain hydroxyl of S364 (3.6 Å) are also in close proximity (Fig. 1b). Previous MD simulations performed on vSGLT¹⁹ and Mhp1¹⁷ indicated that Na⁺ quickly leaves the Na2-site. Our results are in good agreement with these simulations, as we observe that the Na⁺ exits the Na2-site within 9 ns (Fig. 2a) and interacts with the hydrophilic pore-lining residue D189 on TM5 during exit. The importance of D189 was highlighted in the simulations of Li and Tajkhorshid¹⁹ and biochemical studies on human SGLT1 showed that mutating the D189 homolog (D204 in hSGLT1) reduces sodium affinity²⁰. All three MD simulations indicate that sodium exits the transporter to the intracellular space prior to galactose; however, additional conformational changes are still required to release the occluded galactose.

In the inward-occluded structure, galactose is located approximately halfway across the membrane bilayer in the centre of the core domain (Figs. 1a and 2b) where it is coordinated by extensive side-chain interactions from TMs1, 2, 6, 7, and 10. Subsets of these residues form two hydrophobic gates blocking galactose exit to the intracellular and extracellular spaces. The intracellular gate is primarily composed of Y263 from TM6, which stacks with the pyranose ring of galactose. Our MD simulations show that as Na⁺ exits the Na2-site, galactose undergoes significant fluctuations within the binding pocket, resulting in a root mean square displacement (RMSD) of ~2.8 Å with respect to the original position. At 52 ns, Y263 adopts a new and stable rotamer conformation that expands the exit pathway (from 52 to 110 ns) permitting the sugar to leave the binding site and enter the intracellular space

(Figs. 2a,b and 3a). After sugar release (at ~110 ns), Y263 returns to the original conformation.

To test the hypothesis that Na⁺ release stimulates an alternate conformation of Y263, we conducted a MD simulation where the sodium was lightly restrained in the Na2-site (see Supplementary Methods). Under these conditions, Y263 does not adopt the alternate rotamer conformation over the entire 200 ns of the simulation, thereby preventing galactose from exiting through the inner gate (Supplementary Fig. 1). This observation supports a model in which sodium release is required to drive conformational changes that disrupt the galactose-binding site and further suggests that interaction(s) between the Na2-site and Y263 are central to the transport mechanism.

The spontaneous release of galactose in the absence of applied forces makes possible the accurate determination of the binding free energy profile along the exit pathway (Fig. 3b). After Na⁺ release, galactose becomes weakly bound with a minimal energy barrier of ~2 kcal/mol, resulting from the interaction of the sugar with residues N64, S66, E68, and Q69 on TM1. N64 is of particular interest as it is located in the unwound segment of TM1 and has hydrogen bonds with the inner gate residue Y263 and the O2 hydroxyl of galactose linking the Na2-site with the galactose site. Thus, the interactions of N64 with Y263 and galactose may be critical to the transport mechanism.

To test the importance of these interactions, MD simulations and sodium dependent transport assays were performed on transporters with mutations at positions 64 and 263. Simulations of the N64A mutant show a momentary sodium departure from the Na2-site at 5 ns, but the ion rapidly returns and remains for the remainder of the simulation (100 ns). The failure of Na⁺ to leave the Na2-site prevents the unwound segment of TM1 from undergoing conformational changes, and Y263 remains in the orientation that blocks galactose exit (Supplementary Fig. 2a). In agreement with the simulation, sodium-dependent transport assays on the N64A mutant show no activity (Fig. 2c).

To further explore the role of N64, we introduced conservative mutations, N64Q and N64S, which, in principal, are both capable of maintaining the native hydrogen bonds to Y263 and galactose. All attempts to model the initial configuration of the N64Q mutant revealed substantial steric clashes that prevented further simulation, and this correlated well with the inability of N64Q transporters to carry out sodiumdependent transport (Fig. 2c). Modelling of the N64S mutation showed the protein was capable of forming a hydrogen bond with Y263 (3.3 Å) but not the O2 hydroxyl of galactose (4.3 Å). In the simulations, N64S releases the sodium from the Na2-site at 25 ns, and Y263 transiently adopts the alternate rotamer conformation before returning to its original position, preventing galactose exit through the inner gate (Supplementary Fig. 2c). Similarly, sodium leaves the Na2-site at 10 ns during the simulation of the Y263F mutant, but unlike tyrosine, the phenylalanine side chain does not progress to the alternate conformation and galactose remains in the binding site (Supplementary Fig. 2b). It is possible that longer simulation could reveal galactose release in these mutants since they both exhibit modest transport activity (~10% of wild type). Taken together, the sodium dependent transport assays and simulation data demonstrate that robust transport requires the precise orientation of N64 to stabilize galactose and the gating residue Y263.

Although the MD simulations and biochemical studies demonstrate a physical link between the Na2-site and the substrate, global details regarding the inward-open conformation (devoid of both ligands) remain elusive. To address this question we sought to determine the structure of vSGLT in the inward-open conformation. Two approaches were employed: the first was to screen for crystals of the wild-type protein in the absence of ligands, and the

second was to screen for crystals of the inactive K294A mutant⁶. Diffracting crystals were obtained with both approaches and exhibited the same overall configuration; the mutant crystals, however, diffracted to a higher resolution (2.7 Å) (see Supplementary Methods).

As in the original structure⁶, the inward-open conformation of vSGLT is composed of 14 TMs with a core domain of 10 TMs assembled through the association of an internal structural repeat of two 5 TM helical bundles. TMs 1–5 and TMs 6–10 are related by an approximate two-fold symmetry axis through the centre of the membrane plane. For vSGLT, there is an additional N-terminal TM (TM -1) and three additional C-terminal TMs (TM11–13). The inward-occluded and the inward-open structures exhibit a similar overall fold with an RMSD of 1.2 Å for all C α atoms. However, there are distinct structural differences between the two conformations, presumably due to changes resulting from the release of ligands (Fig. 4). With the exception of TM1, superimpositions of individual helices show that the transition between conformations occurs by rigid body movements of sub-domains (Supplementary Figs. 3 and 4). Consistent with the recent assignment for Mhp1, the ‘hash motif’ formed from TM3 and 4, and their inverted repeat equivalents TM8 and 9, align with a RMSD of 0.9 Å. TM2, 6, and 7 form another group of helices, termed the ‘sugar bundle’ for the extensive side-chain interactions with galactose (in the inward-occluded structure), and superimpose with an RMSD of 0.5 Å. TM5 and its inverted partner TM10 connect the hash motif with the sugar bundle. It appears that this new inward open structure of vSGLT is more similar to the recent inward facing conformation structure of Mhp1 (PDB ID: 2X79). Details regarding this analysis can be found in the Supplementary Methods.

The transition from the inward-occluded to the inward-open structure is presumably triggered by sodium release from the Na2-site and the alteration in the hydrogen-bonding network surrounding the unwound segment of TM1. In particular, the intracellular half of TM1 flexes $\sim 13^\circ$, modifying the coordination of N64 (Fig. 1b, c and 4a). In the absence of both ligands, N64 is coordinated to E88, previously hydrogen bonded to the hydroxyls (O2 and O3) of galactose, and Y263. This new conformation of TM1 is further stabilized by hydrogen bonds between S365 (a sodium-coordinating residue) and E68 on the unwound segment of TM1 (Supplementary Fig. 5). There are additional small rigid body rotations in the sugar bundle and the hash motif. When viewed from the intracellular side, each domain moves $\sim 3^\circ$ in opposite directions, thereby increasing the volume of the substrate-binding site by $\sim 1400 \text{ Å}^3$ (Fig. 4d compared to Fig. 4c). This 6° relative rotation likely disrupts protein-substrate coordination, and the increased volume permits water to enter the site. This interpretation is supported by our simulations in which we observe an increase in the number of water molecules in the substrate-binding site after sodium release (Supplementary Fig. 6). These waters effectively compete with the protein for hydrogen bonds, loosening galactose in the pocket and ultimately assisting its release to the intracellular space (Supplementary Movie and Supplementary Fig. 6).

How do sodium and galactose exit vSGLT? We propose the transition from the outward- to the inward-occluded conformation weakens the Na2-sodium binding site causing it to become metastable and release the ion on a short timescale ($\sim 9 \text{ ns}$ in our simulation). Upon exit, the carbonyl oxygens of the ion coordinating residues I65 and A62 undergo a conformational change in the unwound segment of TM1 producing a kink of $\sim 13^\circ$ (Figs. 1 and 4a). The simulation shows that movement of TM1 disrupts the hydrogen bond between N64 and Y263, allowing the side chain of Y263 to adopt a new conformation that opens a pathway to the intracellular space (Fig. 2b). Additional rigid body movements of the sugar bundle and hash motif widen the intracellular cavity allowing water penetration and further disrupting the substrate-binding site to enhance exit and prevent rebinding (Fig. 4b-d).

It is likely that the reaction scheme described here for vSGLT is broadly utilized by all sodium-dependent members of the LeuT-superfamily, since the Na2-site, the hydrophobic gates, and the unwound segments on TM1 and TM6 are all conserved^{11,21,22}. Furthermore, the physical architecture of the internal cavity of the protein and the means of substrate stabilization differ only slightly between the members of the superfamily. For proteins with a single sodium site (the Na2-site), vSGLT and Mhp1, the Na2-site is physically connected to the substrate site by interactions with a polar residue (N64 in vSGLT and Q42 in Mhp1), on the unwound segment of TM1. For proteins that harbour two sodium-binding sites (Na1- and Na2-sites) such as LeuT and putatively BetP, based on functional studies and structural homology to LeuT, the additional Na1-site is positioned on the opposite side of the unwound helix from the Na2-site. As seen for LeuT and BetP, interaction between the Na1- and Na2-sites are mediated by the unwound segment of TM1, and in both cases the sodium at the Na1-site is directly coordinated to the substrate. Regardless of whether the protein has one or two Na-sites, it is the conserved Na2-site, positioned most distal from the core of the protein, that regulates sodium and substrate release, and whose ordered reorientation (described herein) initiates said release, completing the reaction cycle. In summary, we have uncovered a primary structural feature coupling sodium and substrate co-transport in this important class of membrane proteins. These findings have fundamental implications for our understanding of membrane protein biology and for developing strategies to manipulate the alternating access mechanism therapeutically.

Methods

Molecular Dynamics Simulations

The vSGLT monomer (PDB ID: 3DH4) was embedded into a pre-equilibrated and solvated POPC bilayer using the OPM²³ and CHARMM-GUI¹⁸ software packages. Simulations were carried out using NAMD²⁴ with the CHARMM27 parameter set in a 150 mM sodium chloride bath. See Supplementary Methods.

Protein Expression and Purification

p3C423 plasmids carrying wild-type or mutant transporters were transformed and overexpressed in the TOP10 *Escherichia coli* cell line. Cell membranes were isolated, solubilized (2% w/v decyl- β -D-maltopyranoside) and tandemly purified using a Ni-NTA Superflow (affinity chromatography) and Superdex 200 (size exclusion chromatography) columns. See Supplementary Methods.

Transport Assays

Proteoliposomes were generated by reconstituting purified vSGLT protein with sonicated lipid at a protein:lipid ratio of 1:200. Transport activity was measured by monitoring the uptake of D-galactose, with ¹⁴C-D-galactose tracer, into proteoliposomes in the presence or absence of a 100 mM Na⁺ gradient (K⁺ replacing Na⁺). See Supplementary Methods.

Crystallization and Data Collection

Purified WT and K294A protein were concentrated to ~13 mg/ml and crystals were grown by the hanging-drop vapor diffusion method using the Mosquito nanoliter-dispensing robot. Data collected at the Advanced Light Source (beamline 5.0.2) were integrated and scaled and phases were calculated by molecular replacement. The model was built and refined to R_{work}/R_{free} of 25.1/27.4. See Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Thomas Vondriska and Dr. Kenneth Philipson as well as members of the Abramson, Wright, and Grabe labs for useful discussions and critically reading the manuscript. We would also like to thank Dr. So Iwata for advance release of the Mhp1 coordinates (PDB ID: 2X79), and Dr. Ralph Roskies for assistance with the computations. Simulations were carried out through a TeraGrid grant at the Pittsburgh Supercomputing Center and Texas Advanced Computing Center. This work was supported by NIH grants GM078844 (JA), RGY0069 (JA) and DK19567 (EMW) grant from the Human Frontier Science Program (JA). M.G. is an Alfred P. Sloan Research Fellow.

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Figure 1. Structure of the inward-open and an overlay with the inward-occluded structure
 a, A cartoon representation of vSGLT. The core domain of the inward-open conformation (TMs 1-10) is coloured by specific helix bundles involved in the transition from inward-occluded to inward-open conformation. The ‘hash motif’ formed from TMs3, 4, 8, and 9 is blue; the ‘sugar bundle’ formed from TMs2, 6, and 7 is green; TM1 is red; and TMs5 and 10 are magenta. The periphery TMs (-1,11,12,13) are yellow. The inset shows an overlay between the inward-open (coloured as above) and inward-occluded (grey) conformations illustrating the coordination at the Na²- and galactose-binding sites. b, c, Overlay of the inward-open and inward-occluded conformations with the same colouring as (a). Conformational changes in the inward-open structure reveals a ~13° kink in the unwound segment of TM1 preventing sodium coordination at the Na²-site (b). In the absence of galactose, the galactose-binding residue N64 hydrogen bonds to E88 and Y263 maintaining an open pathway from the intracellular space to the substrate-binding site (c).

Figure 2. Mechanism of galactose release

a, Sodium and galactose exit vSGLT. The RMSD of Na⁺ (green) rapidly increases at 9 ns indicating exit from the Na2-site followed by the release of galactose (red) at 110 ns. **b**, Y263 adopts two rotamers. (Upper panel) Y263 is shown in the conformation observed in the inward-occluded structure⁶ in which it blocks substrate exit through a hydrogen bond with N64 on TM1. (Lower panel) At 52 ns, Y263 adopts a rotamer conformation that expands the exit pathway. **c**, D-galactose uptake by WT and vSGLT mutants in proteoliposomes. Results are expressed as % uptake of WT protein in either 100mM NaCl or KCl showing the mutants N64A, N64S, N64Q and Y263F severely impair sodium-dependent transport.

Figure 3. The potential of mean force for galactose unbinding

a, After Na^+ exit from vSGLT, we observe galactose exit at 110 ns. Snapshots along this natural pathway are shown in different colours. Red corresponds to galactose in the binding site, and purple corresponds to a snapshot near 110 ns just as galactose is exiting the hydrophilic cavity to the bulk water. b, Galactose binding energy to vSGLT in the absence of Na^+ . Umbrella sampling along the natural, equilibrium pathway shown in (a) was used to determine the binding free energy. The distance from the binding site in the x-ray structure, along the pathway is shown along the x-axis. The coloured arrows correspond to the galactose snapshots shown in (a). Distance values greater than 15 Å correspond to galactose in the inner hydrophilic cavity. The largest barrier is ~ 2 kcal/mol at 5 Å, which corresponds to galactose interaction with residues in the kink region of TM1.

Figure 4. Conformational changes in the transition from inward-occluded to inward-open structure

a, TM1 superimposed between the inward-open (red) and inward-occluded (grey) structures showing a $\sim 13^\circ$ kink in TM1. b, Overlay of the inward-open (coloured as in Fig.1) and inward-occluded (grey) conformations. 3° rigid body rotations of the 'hash motif' and 'sugar bundle' in opposite directions expose the substrate-binding site to the intracellular environment. c, Accessibility cavity of the inward-occluded conformation is coloured blue. d, Accessibility cavity of the inward-open conformation is coloured gold. The conformational changes from TM1, hash motif, and sugar bundle cause an increase of $\sim 1400 \text{ \AA}^3$ in the accessible volume of the inward-open conformation aiding galactose release.