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The mechanism of substrate release by the aspartate transporter Glt_{Ph}: insights from simulations†

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Abstract

Glutamate transporters regulate excitatory amino acid neurotransmission across neuronal and glial cell membranes by coupling the translocation of their substrate (aspartate or glutamate) into the intracellular (IC) medium to the energetically favorable transport of sodium ions or other cations. The first crystallographically resolved structure of this family, the archaeal aspartate transporter, Glt_{Ph}, has served as a structural paradigm for elucidating the mechanism of substrate translocation by these transporters. Two helical hairpins, HP2 and HP1, at the core domains of the three subunits that form this membrane protein have been proposed to act as the respective extracellular and IC gates for substrate intake and release. Molecular dynamics simulations using the outward-facing structure have confirmed that the HP2 loop acts as an EC gate. The mechanism of substrate release at atomic scale, however, remained unknown due to the lack of structural data until the recent determination of the inward-facing structure of Glt_{Ph}. In the present study, we use this recently resolved structure to simulate the release of substrate to the cytoplasm and the roles of HP1 and HP2 in this process. The highly flexible HP2 loop is observed to serve as an activator (or initiator) prompting the release of a gatekeeper Na⁺ to the cytoplasm and promoting the influx of water molecules from the cytoplasm, which effectively disrupt substrate–protein interactions and drive the dislodging of the substrate from its binding site. The completion of substrate release and exit, however, entails the opening of the highly stable HP1 loop as well. Overall, the unique conformational flexibility of the HP2 loop, the dissociation of a Na⁺, the hydration of binding pocket, and final yielding of the HP1 loop 3-Ser motif emerge as the successive events controlling the release of the bound substrate to the cell interior by glutamate transporters.

Introduction

Nerve cell communication involves the stimulated release of a neurotransmitter by one cell following an action potential and its detection by another at junctions called synapses.¹ Neurotransmitter translocation through the host cell membrane is enabled by integral membrane proteins that usually couple the uphill translocation of their substrate (neurotransmitter) to the energetically favorable co-transport of sodium ions or other cations into the intracellular (IC) region.^{2–4}

Glutamate transporters (GluTs) belong to such Na⁺-coupled secondary transporters: they regulate the translocation of their substrate (glutamate or aspartate) into glial cells, along with some transport into pre- and post-synaptic neurons. GluTs have a major impact on developmental plasticity and long-term potentiation; and they have been implicated in many pathological conditions including stroke, epilepsy, cerebral ischemia, amyotrophic lateral

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sclerosis, Alzheimer's disease, and schizophrenia.⁵ The molecular mechanism of Na⁺-coupled transport by the family of GluTs has been a subject of investigation for the past fifty years.⁶

With the increase in the number of crystallographically resolved structures for Na⁺-coupled secondary transporters in recent years^{7–13} and the complementary biophysical and computational studies, we are now in a position to unravel the molecular mechanisms that control the various phases of the substrate transport cycle. In particular the availability of inward-facing and outward-facing conformers for a number of transporters now permits us to examine thoroughly the mechanisms of substrate/ion binding and release in different states. A recent such study performed on sodium-benzyl-hydantoin transporter Mhp1¹⁴ elucidated the molecular motions that enable the alternating access mechanism¹⁴ which is considered to be applicable to many Na⁺-coupled secondary transporters.⁶ Furthermore, two representative members of the respective families of GluTs and neurotransmitter sodium symporters (NSS), aspartate transporter from *Pyrococcus horikoshii*, Glt_{Ph},⁹ and leucine transporter from *Aquifex aeolicus*, LeuT,¹⁵ have served as structural paradigms for exploring the mechanism of Na⁺-coupled substrate transport.

The structures resolved to date are in support of the classical alternating access mechanism.^{16–20} Accordingly, the transporter periodically undergoes *global* transitions between an outward-facing (*viz.* open-to-the-extracellular (EC) region) and inward-facing (*viz.* open-to-the-IC region) structures, which we will shortly designate here as the respective macrostates E and I (Fig. 1). The macrostate E binds the substrate and ions from the EC region; it then isomerizes into to macrostate I to release the substrate and ions into the cytoplasm.^{6,21,22} In addition to this global change in structure, each macrostate undergoes conformational changes on a *local* scale between open (O) and closed (C) microstates, *i.e.*, the E macrostate needs to open up before substrate binding and then close down to ensure substrate recognition and its sequestration from the EC environment after binding. Likewise, local fluctuations in the macrostate I would enable the release of the substrate from the otherwise inward-facing but closed conformer. The fluctuations between open and closed conformers in the respective E and I macrostates are accepted to modulate the EC and IC gates of the transporter, as schematically illustrated in Fig. 1.

Glt_{Ph} is a homotrimer, each subunit being comprised of two domains, N-terminal and C-terminal. The two structures available for this protein represent the E (Fig. 2A, left panel) and I (Fig. 2A, right panel) states. The N-terminal cores of these two structures are very similar and are superimposable with a root-mean-square deviation of 0.6 Å.¹³ The C-terminal domain, also called core, on the other hand, does not align and shows a substantial movement (Fig. 2B, left panel). The core consists of the transmembrane (TM) helices TM7 and TM8, and two helical hairpins HP1 and HP2, which are involved in binding the substrate (aspartate, Asp[−]) and ions. The crystal structures each have two bound sodium ions, designated as Na⁺₍₁₎ and Na⁺₍₂₎,^{9,12} and Asp[−] uptake is coupled to these two ions.²³ The eukaryotic transporter, on the other hand, co-transportes three sodium ions and a proton, and the cycle is completed by the counter-transport of a potassium ion.²⁴ In Glt_{Ph} Na⁺₍₂₎ is located near the HP2 loop and TM7 residues A307 and N310 (Fig. 2B, right panel); it is referred to as a gatekeeper because of its propensity to stabilize the closed form of the HP2 loop that acts as an EC gate.^{25,26} Na⁺₍₁₎ is buried deeper in the binding cavity, coordinated by the carbonyl oxygens of L303 and G306 on TM7 and D405 on TM8.

The transition between the E and I states involves a 16–17 Å vertical (elevator-like) translation (Fig. 2B, left panel) of the core domain, which rigidly carries the bound substrate and ions from the EC side to the IC side. Although the structure is at modest resolution (3.5–3.8 Å) and obtained through the introduction of judicious mutant cross-links, the features of

the structure can be recapitulated by swapping conformations of inverted repeats, as shown by Forrest and co-workers.²⁷

The mechanism of Asp^- uptake by Glt_{ph} in macrostate E is now clearly established. Experimental¹² and computational^{28,29} studies have confirmed that the HP2 loop acts as an EC gate: it fluctuates between open and closed forms irrespective of substrate binding (local changes in the cycle) and its open form exposes polar and charged residues to the EC environment, which triggers the diffusion and binding of Asp^- , water molecules, and sodium ions. In particular, binding of $\text{Na}^+_{(1)}$ primes the substrate binding site to house the Asp^- by inducing a rotameric flip at the N401 side-chain.^{28,29} $\text{Na}^+_{(2)}$, on the other hand, stabilizes the substrate-bound closed (or occluded) microstate of the HP2 loop, thereby sequestering the substrate from the EC environment.^{12,28–31}

The mechanism of Asp^- release by Glt_{ph} , however, could not be established to date, due to the absence of structural data at atomic resolution for the IC-facing structure (macrostate I) of Glt_{ph} . While simulations for galactose transporter (resolved in macrostate I),^{32,33} a transporter structurally homologous to LeuT, provided insights into possible mechanisms of substrate release by LeuT structural homologs, the mechanism of substrate release by GluTs remained unknown. The recent determination of Glt_{ph} in the inward-facing macrostate¹³ permits us to explore, for the first time, the conformational dynamics that mediate the Na^+ -coupled release of substrate by a GluT, as well as to assess the role of particular structural elements in IC gating, as presented below.

Three possible local events could be envisioned during IC gate opening by Glt_{ph} in macrostate I: opening of HP2; opening of HP1; or opening of both HP1 and HP2. The event in each scenario would facilitate substrate/ion release to the cytoplasm since HP1 and HP2 are exposed to the IC medium in the macrostate I. The precise mechanism of sodium-coupled release, *e.g.*, the key interactions (hydrogen bonds, salt bridges) that are made or broken during this process or the role of sodium ions and solvation, is yet to be established. Given the size and complexity of this system, we performed extensive molecular dynamics (MD) simulations in the presence of explicit solvent, lipid molecules, substrate, and ions for a total run time of 0.16 μs in order to elucidate these time-resolved events at atomic resolution. Our simulations point to an almost spontaneous dissociation of $\text{Na}^+_{(2)}$ and opening of the HP2 loop at the early stages of the process, which prompt a flux of water molecules into the substrate-binding pocket within tens of nanoseconds. The influx of water molecules disrupts protein–substrate interactions and triggers the dissociation of the substrate from the ligating amino acids on HP2 and TM8. Despite the dislodging of substrate enabled by these events, its hydrogen bonds with the 3-Ser motif on the HP1 loop carry on as the most persistent interactions that prevent the complete release of the substrate. Only at the final stage of one of the runs (at ~ 50 ns) does the HP1 loop ‘open’ in one of three subunits to allow for the ‘exit’ of the substrate. Thus, substrate release requires the initial opening of the HP2 loop and $\text{Na}^+_{(2)}$ dissociation, the ensuing flood of water molecules to destabilize protein–substrate attractive interactions, and the final opening up of the HP1, which acts as an IC gate. Notably, the HP2 loop acts as an activator of the IC gate. This role of HP2 in macrostate I, along with its function as EC gate in the macrostate E shown in previous studies,^{12,28,29} highlights the versatility of this particular hairpin for enabling both substrate binding and release functions during synaptic transmission.

Materials and methods

System and MD simulations

The starting structure for MD simulations was the inward-facing crystal structure (PDB: 3KBC) of Glt_{ph} .¹³ The entire homotrimer transporter was embedded in a pre-equilibrated

lipid bilayer of 321 (palmitoylphosphatidylcholine) POPC molecules upon overlaying its N-terminal domain (also called scaffold) residues 141–148 in each subunit with those of the transporter in the E state, with a 3-fold z-axis symmetry perpendicular to the membrane; this ensured an optimal, compatible geometrical insertion in the lipid bilayer accurately capturing the global motion between the I and E states. The system was solvated with 27 459 water molecules, which spanned a width of 10 Å, on both the IC and EC sides of the membrane, sufficient to account for periodic boundary effects during simulations. The resulting simulation box of $11.8 \times 11.8 \times 10.5$ (nm)³ contained more than 10^5 atoms. Counter ions were added into the solvent to ensure electro-neutrality of the system. Periodic boundary conditions were applied. All simulations were carried out using the GROMACS 3.3.1 suite of programs³⁴ with the GROMOS 43a1 force field.³⁵ PRODRG2 server³⁶ was used to obtain the force field parameters for Asp[−]. Electrostatic interactions were treated using the particle mesh Ewald method,³⁷ and the LINCS algorithm³⁸ was used to constrain bond lengths enabling a 2 fs time step to be adopted. Isothermal conditions at 310 K were implemented by Berendsen temperature coupling to the substrate, ions, lipid and water molecules. The single point charge (SPC) model³⁹ was used for water molecules. The lipid parameters were based on previous MD studies^{40,41} with addition of GROMOS parameters for double-bonds in the acyl tail. Semi-isotropic pressure coupling was used to maintain a pressure of 1 bar. Each system was energy-minimized with the steepest descent algorithm, followed by an equilibration simulation of 3 ns, in which the backbone atoms were restrained by harmonic potentials, while the side-chain atoms, the water molecules and lipids were allowed to relax. This allowed the lipid molecules to optimally pack around the protein. This was followed by the production runs, where all the restraints were removed.

Three production runs were carried out in the presence of substrate and sodium ions at their respective binding sites in the core: two (MD1 and MD3) of 40 ns each and one (MD2) of 50 ns. We adopt below the nomenclature used in the PDB structure for the subunits, *i.e.*, designate them as subunits A, B and C. The three simulations each had the same starting configuration, but different starting velocities. In MD3, a constant electric field of −70 mV was applied across the membrane so as to mimic the resting potential of a living cell,⁴² in order to examine its effect on the observed transport mechanism. Finally, an additional run, MD4 was carried out for 33 ns, using as initial state the 16 ns snapshot from MD3 and removing the substrate, but including the two bound Na⁺ ions, to examine the dependence of the influx of water molecules (into the binding site) in the presence of substrate.

Results and discussion

Opening of HP2 loop, dissociation of Na⁺₍₂₎, and solvation of Asp[−] binding site

Fig. 3 illustrates the succession of events observed in MD1 for subunit B (subunit names are based on the PDB structure nomenclature). Originally, the substrate is tightly bound in the core domain, occluded from the IC solvent by the closed conformer of both the HP1 (yellow) and HP2 (pink) loops. We note in particular its close interaction with the conserved 3-Ser motif S277 at the HP1 loop, with G354 and G357 on HP2 loop, and with Na⁺₍₂₎ near G354. D394 on TM8 also contributes to the stabilization of the bound form by forming a hydrogen bond (2.3 Å) with the backbone amine group of G357. Likewise, a hydrogen bond between the S277 hydroxyl group and G354 carbonyl group (1.8 Å) and a close interaction between S279 and G354 (4 Å) assist in maintaining the sequestering of the substrate from the IC solvent. From *ca.* 1 to 15 ns the substrate and its close environment undergo minimal structural changes, as illustrated in the snapshot at 11 ns, except for a weakening in the interaction between S277 and G354, due to the fluctuations of the HP2 loop.

At *ca.* 16 ns, Na⁺₍₂₎ begins to dissociate out of its binding site. The separation between the tips of the HP1 and HP2 loops is momentarily increased at this point. The time dependence

of this separation is detected by monitoring the distance between (i) the hydroxyl oxygen of S277 on HP1 and the backbone carbonyl of G354 on HP2, and (ii) the hydroxyl oxygen of the S279 and the backbone N–H group of G354 as presented in the respective panels A and B of Fig. 4. However, these probed distances are not consistently elongated until *ca.* 24–26 ns. At *ca.* 20 ns, several water molecules have entered the binding site (see the snapshots taken at 21, 29, and 34 ns in Fig. 3). Water is concentrated at two areas of the binding site: below the substrate, near the vacated Na⁺₍₂₎ binding site; and above the substrate, deeper in the binding pocket, near the side-chains of D394 and R397 (of TM8). The hydrogen bond between D394 and G357 (of the HP2 loop) is disrupted by the water molecules, evidenced by the increase in their separation from 2.3 Å to *ca.* 6.5 Å, shown in Fig. 3 (at 29 and 34 ns) and Fig. 4C.

The opening of the HP2 loop and the solvation of the binding site are coupled phenomena. The opening of the HP2 loop is an intrinsic motion of the protein; however, its magnitude, as detailed later, is dependent on the extensiveness of binding site solvation, though the opening of the HP2 activates or promotes solvation initially. The coupling of amplified HP2 loop reconfigurations and nascent binding site solvation facilitate the weakening of Na⁺₍₂₎–protein interactions (detailed later), which often leads to a total, spontaneous dissociation of the ion from its protein binding-site.

Role of HP2 as an activator of substrate release

The increase in distance between HP1 and HP2, and between HP2 and TM8, is driven by the inherent flexibility of the HP2 loop. HP1 and TM8 remain stable, as demonstrated in Fig. 4D by the almost constant distance (7–8 Å) between S278 (HP1) and D394 (TM8). HP2 dynamics is also manifest in the trimodal distributions observed in Fig. 4, panels A–C. Notably, similar histograms have been obtained for the outward-facing state E as well,²⁶ supporting the view that the HP2 loop undergoes the same type of movements irrespective of its exposure to the EC or IC region. The opening of the HP2 loop exposes the bound substrate as well as other polar and charged groups, which attracts water molecules to the binding site. The exposure of substrate upon opening of the HP2 loop may be clearly seen in Fig. 5. Notably, the HP2 tip undergoes a displacement of 7.1 Å while HP1 remains almost unchanged (panel A), and HP2 opening alone is sufficient to expose the substrate (panel B) and attract water molecules to the substrate-binding pocket. Notably, the salt-bridges formed between Asp- and D349- and R397 would present formidable obstacles toward releasing the substrate into the cytoplasm, if it were not for the destabilizing effect of water molecules that effectively disrupt these interactions.

The high mobility of HP2 loop, the increase in the HP2-HP1 and HP2-TM8 distances and the destabilization of protein–substrate interactions by the influx of water molecules were also observed in MD3 where an electric field of –70 mV representative of the membrane potential was applied, as well as MD2. Fig. S1 and S2 in the ESI† display the counterparts of the respective Fig. 3 and 4A–C, in MD3. Observations made in MD2, which was the only run where the substrate was observed to exit the binding site, are presented in detail below.

Substrate release and involvement of HP1 3-Ser motif at the final step

At the early stages of the MD2 simulations, events similar to those observed in MD1 and MD3 were observed (not shown). More interesting were the later stages, which allowed for visualizing for the first time the exit of the substrate by a GluT family member. A substantial redistribution of substrate–protein interactions occurred in subunits A and B at *ca.* 35 ns in this run.

We illustrate in Fig. 6 the time evolution of atomic interaction in subunit B. We begin with a snapshot taken at 15 ns shown in Fig. 6A. At this time, the substrate (bright yellow) is still tightly coordinated by amino acids (labeled) on the HP1 loop (light yellow), HP2 loop (pink) and TM8 (green, on the *left*), while $\text{Na}^+_{(2)}$ is on the verge of leaving its binding site near HP2 tip.

A major change in structure occurs however between $t = 34.5$ ns and 35 ns, as may be seen from the comparison of the panels B and C in Fig. 6. While in MD1, HP2 alone was observed to move away from the substrate and from the remainder of the core domain, in MD2, the motion of HP2 appears to be accompanied by that of the substrate, which moves away from TM8 and HP1 residues. The dislocation of Asp^- was assisted by water molecules that wedged between the substrate and the side-chains of D394 and R397 on TM8, which effectively disrupted protein–substrate hydrogen bonds and electrostatic interactions, as compiled in Table 1 for all three subunits (see Fig. 6A for the spatial positions of the listed amino acids and their functional groups at the substrate-binding site). Both subunits A and B exhibited significant disruptions in their substrate–protein interactions (highlighted in bold). Whereas the substrate in subunit C remained highly stable. The entries displayed in pink refer to interactions that were either fully maintained, or occasionally disrupted but restored, within the course of simulations.

The significant changes in substrate–protein interatomic distances and substrate orientation observed within a relatively short time span (500 ps) in Fig. 6B and C are suggestive of the crossing of an energy barrier (transition state) between these two snapshots. At $t = 34.5$ ns, the ammonium group of the substrate flips nearly 180° around an axis normal to the membrane, and its interactions with D394 and T398 on TM8, and R276 on HP1 are irreversibly broken. The α -carboxylate group is observed at this time to interact even closer with N401 (TM8) and S277 (HP1) sidechain N–H and O–H groups, respectively (subunit B in Table 1). Within the succeeding short time-frame, the interactions of the substrate β -carboxylate oxygens with R397 NH_3^+ group (on TM8), and G359 and A358 N–H groups (on HP2) are also weakened, while a hydrogen bond between the substrate NH_3 group and V355 carbonyl group forms (see the corresponding distance of 1.8 Å in panel C) and the interaction with the HP1 3-Ser motif S277 hydroxyl group becomes even stronger (1.6 Å).

In view of the extensive rearrangements of Asp^- within the binding cavity, we extended this run beyond 40 ns. Although longer simulations are, in principle, desirable to observe multiple events and/or verify the observations as shown elegantly in a recent study,¹⁴ the extension of the simulation duration to 50 ns in the present system allowed us to visualize substrate unbinding and release. The tight interactions of the substrate with HP2 loop residues in subunit A (Table 1) prevented its translocation, and subunit C continued to maintain its original occluded form. Subunit B core domain, on the other hand, exhibited remarkable events. Fig. 6D illustrates its conformation at $t = 44$ ns. Notably, the substrate has diffused out of the binding site at this time; but a strong association with the 3-Ser motif S278 persists, even as Asp^- has left the binding pocket! Surprisingly, further extension of this run showed that this interaction drags the substrate back toward the binding pocket at $t = 45$ ns (Fig. 7), but the entropic drive for the dissociation of the substrate eventually predominates, as can be seen from the snapshot at $t = 49$ ns, where the substrate moves further away from the protein towards the cytoplasm. Notably, during this last time frame (between $t = 44$ and 49 ns) HP1 loop is seen to undergo an opening towards the IC solvent (Fig. 7), which may indeed be viewed as the final step in the gating motion of the transporter in the macrostate I.

Spontaneous dissociation of Na⁺₍₂₎ vs. stability of Na⁺₍₁₎

Na⁺₍₂₎ ion spontaneously dissociates from its binding site to the bulk IC solvent in two of the three subunits (A and B) in both MD1 and MD2 (Fig. 3 and 6); and in one subunit (B) in MD3. In sharp contrast to Na⁺₍₂₎, Na⁺₍₁₎ remains tightly bound to its binding site on the transporter in all runs. Fig. 8A shows how the positions of the three Na⁺₍₁₎ ions (in all three subunits/protomers) remain unchanged near 6 nm along the *z*-axis (perpendicular to the membrane; shown in Fig. 8B) in MD1 and MD2, while Na⁺₍₂₎ shows substantial displacements toward the cytoplasm (higher *z*-values) in subunits A and B in both runs MD1 and MD2 (while the Na⁺₍₂₎ in subunit C remains practically fixed in both runs).

The origin of this difference is manifest in the characteristics of the respective binding sites. The binding site for Na⁺₍₂₎ is composed of the backbone carbonyl groups of T308 on TM7, and S349 and T352 near the HP2 tip, whereas the site for Na⁺₍₁₎ contains the negatively charged side-chain of D405 and the backbone of G306 and N310 (Fig. 8B). The paired, ionic interaction, unique to the Na⁺₍₁₎ binding site, holds the ion to the transporter. The relative stability of Na⁺₍₁₎ in its binding site compared to that of Na⁺₍₂₎ has also been observed in the outward-facing conformation.²⁶

Smaller HP2 fluctuations and reduced water influx in the absence of substrate

As an additional test, the MD3 snapshot at *t* = 16 ns was adopted as starting point for a new run, MD4. In this simulation, the substrate was removed from each subunit, to determine if/how the presence or absence of substrate would affect HP2 loop dynamics. In accord with previous runs, Na⁺₍₂₎ rapidly dissociated out of its binding site (this time in subunits B and C). Fig. S3 (ESI[†]) shows the time evolution of two metrics that gauge the opening of the HP2 loop: the distance between S277 of HP1 and G354 of HP2 (panel A), and that between D394 of TM8 and G357 of HP2 (panel B). Comparisons of these two panels with their counterparts derived from the runs conducted in the presence of substrate (Fig. 4A and C for MD1, and Fig. S2B and A, ESI[†], for MD3) reveals two interesting features: (i) the HP2 loop fluctuations in MD4 obey a unimodal distribution for each subunit rather than bimodal or trimodal, (ii) in particular the 'long-distance' peak around 8 Å (Fig. 4 and Fig. S2, ESI[†]) is absent in the absence of substrate (Fig. S3, ESI[†]). This is despite the fact that the HP2 loop is in an open conformation in subunits B and C at the beginning of MD4.

The large HP1–HP2 and HP2–TM7 distances refer to the wide opening of the HP2 loop. Such extensive rearrangements are indeed observed only when there is an influx of water molecules into the binding cavity, and the influx itself is strongly enhanced in the presence of a bound substrate, the charged/polar groups of which are partially exposed to the IC medium upon occasional opening of the HP2 loop. Therefore, in the absence of substrate, which acts as an attractor for water molecules, no extensive reconfiguration of the HP2 (otherwise necessary for accommodating the water molecules) is observed.

Motions accessible to HP2 in the outward-facing and inward-facing states

Fig. 9A shows conformations of the HP2 loop observed in MD simulations of the macrostates E and I resolved for Gltp_h. The structure of the HP2 loop as an EC gate is similar to that of the putative IC gate. Also of interest is the distinct rigidity of the HP1 loop compared to HP2 during the early stages of the simulations. The dynamic character of the IC gate (HP2) is also captured by averaged structures of various time segments from the MD simulation (Fig. 8B). The high flexibility of HP2 loop compared to that of HP1 may be easily explained by the lengths and amino acid compositions of the two loops. HP1 loop is composed of four residues, including the conserved 3-Ser motif: R276, S277, S278, and S279, all of which are polar or charged and closely interact with the substrate. HP2, however, is much longer (nine residues) and composed of hydrophobic side-chains of

(A353, A358, V355), four glycines (G351, G354, G357, G359) and a proline (P356).^{9,13} In the macrostate I, the HP2 loop is packed against TM8, but this does not impede the ability of the HP2 loop to open; indeed, neighboring TM5 residues are observed to adjust as the conformation of the HP2 loop opens as the simulation progresses, as shown in Fig. 9B. It is important to note that the inward-facing crystal structure was forced into the inward conformation by the introduction of mutant cysteine cross-links. It remains unclear whether HP2 is as tightly packed against TM5 as predicted by the biased crystal structure; nevertheless, the HP2 loop of the inward-facing X-ray structure is predicted in present simulations to open up, and its outward motion is accompanied by an accommodating shift in TM5 position. The high mobility of this loop, shown above to be an intrinsic property imported by its overall topological and chemical characteristics, is instrumental in activating the IC gating process. This observation is in accordance with the paradigm wherein protein function is governed by the intrinsic dynamics favored by its architecture.^{43,44}

Conclusion

Structural analysis of Glt_{ph} in multiple states is key to the construction of a reaction scheme for ion-driven substrate transport. Much has been learned by experimental^{9,12,13} and theoretical efforts²⁶ on the interactions/events controlling the substrate uptake portion of the transport cycle, *e.g.*, the identification of the EC gate (HP2 loop) and key residues enabling the stabilization of substrate and Na⁺ ions. It has been hypothesized that the structurally similar HP1 loop acts as the IC gate.^{6,9,12,13,21} Our results suggest that substrate release proceeds *via* a more elaborate mechanism, initiated by the fluctuations of the intrinsically flexible HP2 loop and the dissociation of the gatekeeper Na⁺₍₂₎ near HP2 tip, which trigger an influx of water molecules, which in turn weaken substrate–protein interactions and promote the dislocation of the substrate. The last barrier to the release of the substrate is the strong interaction with the 3-Ser motif on HP1. In this respect, HP1 is the IC-gate controlling element, although access of the substrate to the cytoplasm would not occur if it were not for the activation of this sequence of events by the HP2 loop. These new findings will need to be further confirmed by more extensive simulations and experiments.

The examined process of substrate release represents only one portion of the complete transport cycle presented in Fig. 1, that along the lower horizontal portion. The upper horizontal portion of the cycle, *i.e.*, events leading to substrate and sodium uptake in the outward-facing state (macrostate E), has also been thoroughly examined in previous simulations. The two remaining portions are the horizontal changes along the vertical axes, the time scale of which would be expected to be several orders of magnitude slower, describe the collectivity of the involved global motion. Computational examination of these changes is possible by adopting coarse-grained models and analytical tools, such as elastic network models and normal mode analyses.^{45,46} The time scale of the present simulations is not long enough to provide statistically reliable information on the collective motions of the three subunits.⁴⁶ Instead, it permits us to investigate local events that take place near the substrate and ion binding sites of each subunit, as discussed in our earlier MD study²⁸ of the outward-facing Glt_{ph}. With existing structural data, advances in spectroscopic and biochemical measurements and computational models and methods, we expect to gain a full understanding of the various stages of the transport cycle in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic representation of the substrate transport cycle by Na⁺-coupled secondary transporters. The transport cycle involves two half-cycles distinguished by different conformations (macrostates) of the transporter: outward-facing (E) and inward-facing (I), each also being subject to local conformational changes (or microstates) between the *open* and *closed* conformers of the respective EC and IC gates. The cycle begins from the E state on *top, left* which is subjected to local fluctuations (between open and closed conformers of the EC gate while the IC gate is presumably closed), and to possible binding/unbinding of a sodium ion, Na⁺₍₁₎. Stabilization of a Na⁺₍₁₎-bound open conformer binding primes the subunit for substrate binding, succeeded/accompanied by the binding of a second sodium ion, Na⁺₍₂₎. The presence of bound S and Na⁺₍₂₎ induces a preference for the closed state of the EC gate. Upon enclosure of S and ‘sealing’ of the EC gate by Na⁺₍₂₎, a global change in structure (vertical arrow) from macrostate E to I takes place, while the EC and IC gates probably maintain their closed forms. This transporter in the I macrostate is also subject to local conformational fluctuations that enable the opening of the IC gate and release of the gatekeeper Na⁺₍₂₎ and substrate, succeeded by the possible release of additional cations. Finally, another global change in structure restores the transporter back to its E macrostate and the cycle is poised to repeat.

Fig. 2.

Structures of the homotrimeric aspartate transporter, Glt_{Ph} in the outward-facing (E) and inward-facing (I) macrostates. (A) Comparison of the outward-facing (*left*; PDB: 2XFH) and inward-facing (*right*; PDB: 3KBC) structures resolved by X-ray crystallography. Each subunit consists of two domains: the core domain (TM8, TM7, HP1, HP2) colored blue in the EC-facing (*left*) and magenta in the IC-facing (*right*) structures and the N-terminal domain, colored cream and light teal in the respective EC-facing and IC-facing conformations. The bound substrates are shown in space-filling representation. In the E state, the subunits form a bowl-like structure open to the EC region. The bowl-like structure is diminished in I state. (B) Overlay of the core-domain of a given subunit in the E and inward I states, upon superimposition of their N-terminal domains, shows that the substrate translates by approximately 16–17 Å normal to the membrane upon transition of the transporter from E to I state. The substrate-binding site in the core domain is magnified on the *right*, to display the positions of the substrate and two sodium ions with respect to the helical hairpins HP2 (red; residues 338–373) and HP1 (yellow; 259–291), and the TM helices TM8 (purple; 377–416), and TM7 (orange; 297–329). Note that TM7 is broken near the substrate-binding site, hence the notation TM7a and TM7b for its C- and N-terminal portions. The helices that form the hairpin HP1 are designated as HP1a and HP1b, and a similar notation is adopted for the HP2 helices.

Fig. 3.

Succession of events leading to HP2 opening, solvation of the binding site and spontaneous release of a sodium ion in subunit B, MD1. Snapshots at different times illustrate the time-resolved events associated with the opening of the HP2 loop. Distances between the pairs S277-G354 and S279-G354 at the tips of the respective hairpins HP1 (yellow) and HP2 (red), and between D394 (on TM8) and G357 (on HP2) are reported in Ångströms in all panels. At $t = 0$, the bound substrate is occluded from the IC solvent by the closed loop conformations of HP2 and HP1. At $t = 11$ ns, the sodium ion $\text{Na}^+_{(2)}$ begins to dislodge from its initial binding site and the distance between S277 and G354 at the tips of the respective hairpins HP1 and HP2 increases from 1.8 (original) to 4.1 Å. At $t = 21$ ns, $\text{Na}^+_{(2)}$ is totally solvated and a number of water molecules have wedged between D394 and G357, thereby disrupting the hydrogen bond that initially existed. Snapshots 21 to 34 ns show that the HP2 loop undergoes a large opening, as demonstrated by the increase in the distance between

D394 and G357 to $>6 \text{ \AA}$ and the increased separation of the HP1 and HP2 loops. Overall, the binding site becomes increasingly solvated.

Fig. 4.

Time evolution of interactions associated with the IC gate opening, MD1. Results for subunits, A, B, C are colored red, green, and blue, respectively. Panels A–D (*left*) refer to the time evolution of distances between specific pairs of atoms, and (*right*) show the corresponding histograms. (A) D394 (side-chain carboxylate carbon) and G357 (amide hydrogen). (B) S277 (hydroxyl oxygen) and G354 (carbonyl oxygen). (C) S279 (hydroxyl oxygen) and G354 (amide hydrogen). (D) S278 (hydroxyl oxygen) and D394 (side-chain carboxylate carbon).

Fig. 5. Exposure of substrate upon opening of the HP2 loop in macrostate I. (A) Overlay of an open (29 ns) and closed (1 ns) states of the HP2 loop in MD1; TM7 and TM8 were used for superposition. HP1 exhibits minimal changes, if any, while HP2 undergoes a significant opening as indicated by the loop displacement of 7.1 Å. (B) Surface representation of these structures illustrating the exposure of the substrate to the IC region upon HP2 opening: closed state (top) and open state (bottom).

Fig. 6.

Succession of events leading to the dislocation of substrate in subunit B, MD2. Snapshots at different times illustrate the time-resolved events associated with the release of the substrate in subunit B. Distances between substrate atoms and the closest atoms on the labeled amino acids are reported in Ångstroms. At $t = 15$ ns, the bound substrate is sequestered from the IC solvent by the closed loop conformation of HP2 and HP1, and $\text{Na}^+_{(2)}$ begins to dislodge from its binding site. Snapshots at 34.5 and 35 ns demonstrate the rapid reorientation of the substrate, induced by the severed hydrogen bonds to D394 and R397, which arise from binding site solvation following the opening of the HP2 loop. At $t = 44$ ns, the substrate is dislodged from the binding cavity. Note that the only remaining substrate–protein hydrogen bond (1.8 Å) is that between the aspartate β -carboxylate oxygen and the hydroxyl group of S277 on the 3-Ser motif at the HP1 loop. The substrate is completely dissociated from HP2, as evidenced by the increase in the distance between the aspartate NH group and HP2 G354 backbone hydroxyl from 1.8 Å at $t = 35$ ns to 8.5 Å at $t = 44$ ns. See Table 1 for a detailed list of lower and upper bounds observed for particular interatomic distances in all three subunits.

Fig. 7. Release of Asp⁻ to the cytoplasm by Glt_{Ph} in the inward-facing state. Panel A displays the original structure of the core domain in one of the subunits, color coded by the secondary structural elements, including both the bound substrate and two sodium ions. Panel B displays three snapshots of the same subunit taken from MD2 at 44, 45 and 49 ns, succeeding the influx of water molecules and dislocation of substrate from two different perspectives. Panel B (where the substrate is deleted for clarity) has same perspective as panel A, and permits us to view how HP2 loop is 'open' or binding pocket is exposed, compared to the initial snapshot in panel A. Panel C, on the other hand, is identical to panel B, but rotated around the vertical (z-) axis; TM8 is removed, and the neighborhood of the substrate is enlarged to display the opening of the HP1 loop. Notably, the substrate is partially released at *t* = 44 ns (while the HP1 loop was closed), attracted back toward the binding pocket at 45 ns, but moves back towards to the cytoplasm this time enabled by the substantial 'opening' of the HP1 loop while the hydrogen bond between Asp-β-carboxylate oxygen and S277 hydroxyl group is maintained. S277 and S278 are displayed in green stick representation.

Fig. 8.

Comparison of the stabilities of bound $\text{Na}^+_{(2)}$ and $\text{Na}^+_{(1)}$. (A) The ordinate displays the position of the sodium ions along the z -direction (normal to the membrane plane) as a function of time, observed in MD1 and MD2. $\text{Na}^+_{(2)}$ and $\text{Na}^+_{(1)}$ are initially located at $z \approx 5.8$ nm and $z \approx 6.0$ nm. Results for subunits A, B, and C are colored red, green, and blue, respectively. $\text{Na}^+_{(2)}$ dislodges out of the transporter into solution, whereas $\text{Na}^+_{(1)}$ remains bound (flat curves at $z \approx 6.0$ nm). (B) The positions and the interactions the ions make with the protein are illustrated, as defined by the crystal structure (PDB: 3KBC).

Fig. 9. Intrinsic flexibility of the HP2 loop. (A) Shown are snapshots from MD1 at 1, 16 and 29 ns; and for comparison, a snapshot at 20 ns from a simulation of the substrate bound to the EC-facing conformation of Glt_{Ph} (from Shrivastava *et al.*, 2008); TM7 and TM8 were used for the superposition in both panels. (B) Shown are average structures from 9–15, 20–30, and 31–40 ns overlaid to the average structure from 0–6 ns of MD1. Distances are reported in Ångstroms. Note that the opening of HP2 loop is accommodated by a rigid-body translation of about 2.2 Å in TM5.

Table 1

Changes in key interactions at the substrate binding site

Interacting atoms ^a	Subunit A			Subunit B			Subunit C		
	Average	Min	Max	Average	Min	Max	Average	Min	Max
(TM8) D394 _{COO-} —AspNH3+	5.6 ± 2.1	3.1	10.1	4.5 ± 1.7	3.2	13.6	<i>4.2 ± 0.5</i>	<i>3.2</i>	<i>6.5</i>
(TM8) T398 _{OH} —AspNH3+	5.1 ± 2.3	2.6	11.6	4.1 ± 2.2	2.6	11.4	<i>3.0 ± 0.4</i>	<i>2.6</i>	<i>6.2</i>
(TM8) R397 _{CN3+} Asp _β COO-	6.3 ± 2.1	3.8	11.4	5.0 ± 1.6	3.6	16.3	<i>4.3 ± 0.2</i>	<i>3.8</i>	<i>5.0</i>
(TM8) N401 _{(ss)CONH-} Asp _α COO-	5.8 ± 2.1	3.3	13.0	4.5 ± 1.7	3.1	12.9	<i>3.7 ± 0.2</i>	<i>2.8</i>	<i>5.0</i>
(TM7) T314 _{OH} —Asp _β COO-	6.0 ± 2.4	2.8	11.8	4.4 ± 2.1	2.8	16.4	<i>3.5 ± 0.2</i>	<i>2.9</i>	<i>4.2</i>
(HP1) R276 _{(bb)CONH-} Asp _α NH3+	4.4 ± 1.5	2.6	9.3	3.6 ± 1.4	2.6	9.3	<i>3.7 ± 0.4</i>	<i>2.7</i>	<i>5.6</i>
(HP1) S277 _{OH} —Asp _α COO-	4.3 ± 1.0	3.0	8.6	<i>4.4 ± 0.9</i>	<i>2.9</i>	<i>7.1</i>	5.8 ± 0.5	3.5	7.2
(HP1) S278 _{OH} —Asp _α COO-	<i>4.0 ± 0.9</i>	<i>3.0</i>	<i>9.3</i>	5.0 ± 1.3	3.0	9.6	<i>3.5 ± 0.2</i>	<i>3.0</i>	<i>4.2</i>
(HP1) S278 _{CONH-} Asp _α COO-	<i>2.8 ± 0.5</i>	<i>2.1</i>	<i>7.8</i>	<i>2.7 ± 0.4</i>	<i>2.0</i>	<i>7.0</i>	<i>2.7 ± 0.2</i>	<i>2.1</i>	<i>3.5</i>
(HP2) G359 _{CONH-} Asp _β COO-	4.2 ± 1.4	2.0	10.4	4.0 ± 2.3	2.0	16.2	<i>2.7 ± 0.3</i>	<i>2.0</i>	<i>3.8</i>
(HP2) A358 _{CONH-} Asp _β COO-	<i>3.4 ± 0.6</i>	<i>2.1</i>	<i>7.4</i>	3.2 ± 0.7	2.1	11.1	<i>3.6 ± 0.4</i>	<i>2.4</i>	<i>5.4</i>
(HP2) G357 _{CONH-} Asp _β COO-	<i>3.5 ± 1.1</i>	<i>1.9</i>	<i>8.8</i>	3.6 ± 0.9	2.1	7.9	<i>4.5 ± 0.4</i>	<i>3.0</i>	<i>7.3</i>
(HP2) V355 _{CONH-} AspNH3+	<i>3.8 ± 1.0</i>	<i>2.6</i>	<i>8.1</i>	4.1 ± 1.3	2.7	13.8	<i>3.8 ± 0.4</i>	<i>2.7</i>	<i>5.6</i>
(HP1) R276 _{CN3+} —D394 _{COO-}	4.2 ± 0.6	3.1	7.3	4.0 ± 0.4	3.1	6.4	3.8 ± 0.3	3.1	4.9

^aResults are taken from MD2 (snapshots are taken every 10 ps). Average distances are reported in Ångstroms. The atoms shown in bold italic denote the monitored distance (column 1). Bold entries represent the interactions that are broken over the course of the simulation; italic entries represent those that remain—although some of them are occasionally disrupted and restored. A broken hydrogen bond, however, is ultimately determined by an analysis of distance and geometry. Thus, some bold italic entries shown above can contain distances conducive to hydrogen-bonding. All interactions refer to the coordination of the substrate, except for the last row added to illustrate the stability of HP1.