Transport mechanism of a glutamate transporter homologue GltPh

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Abstract
Glutamate transporters are responsible for uptake of the neurotransmitter glutamate in mammalian central nervous systems. Their archaeal homologue GltPh, an aspartate transporter isolated from Pyrococcus horikoshii, has been the focus of extensive studies through crystallography, MD simulations and single-molecule FRET (smFRET). Here, we summarize the recent research progress on GltPh, in the hope of gaining some insights into the transport mechanism of this aspartate transporter.

Introduction
Glutamate transporters, also known as excitatory amino acid transporters (EAATs), belong to the dicarboxylate/amino acid:ammonium (Na+ or H+) symporter (DAACS) family [1]. In the mammalian central nervous system, neuronal and glial EAATs transport glutamate, the main neurotransmitter, from the outside to the inside of the nerve cells, removing excessive excitotoxic glutamate, which may cause neurotoxicity [2,3]. Various human diseases, such as Alzheimer’s disease, epilepsy and strokes, have been linked to dysfunction of EAATs [4,5].

In humans, there are five subtypes of glutamate transporters (EAAT1–5) [6]. The transport of glutamate is driven by energy derived from ion gradients, mostly Na+ [2,3,6]. In EAATs, three Na+ ions and one proton are co-transported with glutamate and the transport cycle is completed by the counter-transport of one K+ ion [7]. In addition to the ion-coupled transport, EAATs also display uncoupled chloride conductance [8–11] and have different preferences towards ions [10]. Therefore, glutamate transporters function both as secondary active transporters and anion-selective ion channels [8,10,12].

Despite the importance of glutamate transporters in mammalian systems, there are currently no crystal structures of a mammalian EAAT. One archaeal homologue of the glutamate transporter, GltPh, isolated from Pyrococcus horikoshii glutamate transporter, has however been extensively studied over the past ten years. It shares 37% sequence identity with human EAAT2 [13,14] and many functionally important amino acid residues are highly conserved between GltPh and its human homologues [13], making it an excellent model system for researchers to use. GltPh transports aspartate together with three Na+ ions into the cytoplasm [15], accompanied by a stoichiometrically uncoupled Cl− conductance as well [16]. There are thus three major differences between it and the human EAATs: first that no proton is symported with aspartate [17], second that K+ ion counter-transport is not required to complete the transport cycle [17] and third, a strong preference for aspartate over glutamate [18]. In contrast, EAATs require one proton for co-transport [7], one K+ ion counter-transport to complete the transport cycle [7] and transport glutamate and aspartate with similar affinity [8,11,19]. In this review, we summarize the current state of structural studies, MD simulations and single-molecule FRET (smFRET) studies of GltPh that have provided insights into its transport mechanism – and by extension, the mechanism of the EAATs as well.

Overall structure and domain motions of GltPh

The outward-facing state, captured in the first crystal structure of GltPh [13], revealed a homotrimer (Figure 1a) with a bowl-shaped extracellular-facing basin whose surface is hydrophilic and as deep as half of the trimer’s height. Each wedge-shaped protomer (Figure 1b) consists of two domains: a trimerization domain formed by four transmembrane (TM) helices (TM1, TM2, TM4 and TM5) providing interactions between subunits in the trimer; and a transport domain formed by four TM helices (TM3, TM6, TM7 and TM8) and two re-entrant loops [helical hairpin (HP) structures, HP1–2] [13,20].

Comparison of the aspartate-bound structure and the structure with the competitive inhibitor DL-threo-β-benzylxoxaspionate (TBOA)-bound shows that HP2 serves...
Figure 1 | Structures of GltPh

(a) Trimer of GltPh viewed from the extracellular side in the outward-facing state. Each monomer, in cartoon, is coloured differently. (b) Cartoon representation of a monomer of GltPh, shown parallel to the membrane in the outward-facing state with aspartate and two Na\(^+\) ions bound. The TM helices and re-entrant loops are labelled. The substrate is shown as stick and the two Na\(^+\) ions are shown as purple spheres. The black dashed lines between TM3 and TM4a represent the loop connecting the helices. (c) View of the Na\(^+\)-binding sites. (d) View of the aspartate-binding site. Dashed lines show the hydrogen bonds between amino acid residues and Na\(^+\) or aspartate.

as the extracellular gate [18]. GltPh can adopt an ‘open’ conformation (solved with TBOA bound), which allows substrate access from the outside to its binding site, at which point it switches to the ‘closed’ conformation (solve with aspartate bound). This role of HP2 has also been verified by MD studies [21,22]. HP1 was therefore proposed to function as the intracellular gate as its movement is involved in the dissociation and release into the cytoplasm of the substrate and ions [20]. However, this remains the subject of some controversy in recent MD studies, as will be discussed below (Transport Mechanism).

As the substrate-binding site in both the aspartate- and TBOA-bound structures is approximately 5 Å (1 Å = 0.1 nm) beneath the extracellular surface, these two structures are called [20] the outward-facing closed (or occluded) state and outward-facing open state respectively. The inward-facing state is obtained by cross-linking of a double-cysteine mutant introduced into GltPh [20] (Table 1). For example in the structure of GltPh–K55C–A364CHg, aspartate is bound approximately 5 Å beneath the intracellular surface [20].

Biochemical, crystallographic and double electron–electron spin resonance [DEER (also called PELDOR)]
spectroscopy data all demonstrate that the trimerization domain serves as a scaffold and stays in almost the same conformation during ligand binding and transport [20,23,24], whereas the transport domain, stabilized by the scaffold, undergoes large conformational changes involving a TM translation and rotation [20]. Various studies with different techniques performed on EAATs show that individual subunits in the homotrimer function independently [25–28]. Although there is no direct evidence about how the subunits in GltPh function, it should be similar to the EEATs, given the high level of similarity between GltPh and the EAATs.

Rigid body movement (called ‘elevator-like’ motions [29]) of the transport domain can be observed when comparing the structures of apo or holo outward-facing and inward-facing GltPh, respectively [20,30]. The elevator-like motions of the transport domain can be observed when comparing the uptake and are pivotal steps of the transport cycle [31,32]. When compared to the outward-facing holo crystal structure, the HP2 loop is collapsed into the aspartate binding and Na2 sites [18]. In the outward-facing apo crystal structure, the HP2 conformation resembles the inward-facing conformation [30] and the mutant bound aspartate more weakly [18]. In the outward-facing apo crystal structure, Na2 is below the re-entrant helical HP2, coordinated by the carbonyl groups of Gly306 and Asn310 (TM7), of Asn401 (TM8) and the Asp405 γ-carboxylate group, and analysis of data from the GltPh D405N crystals soaked in Tl+ solution (an Na+ mimic) found a strong peak only at the Na2 position where it is in the outward-facing holo structure [18].

Steered molecular dynamics (SMD) simulations suggested that the breaking of the hydrogen bond between Pro 304 and Thr308 destabilizes the last turn of the TM7a helix and therefore cannot coordinate an Na+ ion at Na2 [18]. In the outward-facing holo crystal structure, Na1 is located below the aspartate, coordinated by the main chain carbonyls of Gly306 and Asn351 (TM7), of Asp405 (TM8) and the Asp405 side chain (TM8) (Figure 1c). Of these residues, Asp405 is the most important: it coordinates Na1 bidentately via the γ-carboxylate group, and analysis of data from the GltPh D405N crystals soaked in Tl+ solution (an Na+ mimic) found a strong peak only at the Na2, not the Na1, position and the mutant bound aspartate more weakly [18]. In the outward-facing holo crystal structure, Na2 is below the re-entrant helical HP2, coordinated by the carbonyl groups of Thr308 and Met311 (TM7) and of Ser349 and Thr352 (HP2) [18] (Figure 1c).

In both the outward-facing and inward-facing holo crystal structures, the distance between the hydroxy group of Thr308 side chain and the backbone carbonyl of Pro304 is approximately 4.8 Å, which is too far to form a hydrogen bond. This allows Thr308 to coordinate the Na+ ion at Na2. However, the Pro304-Thr308 hydrogen bond exists in the outward-facing apo crystal structure [30] and the outward-facing crystal structure of GltPh with TBOA bound [18]. In the outward-facing apo crystal structure, the HP2 loop is collapsed into the aspartate binding and Na2 sites as well [30]. In the structure of GltPh in complex with TBOA, HP2 moves approximately 10 Å away from the position where it is in the outward-facing holo structure and therefore cannot coordinate an Na+ ion at Na2 [18].

Steered molecular dynamics (SMD) simulations suggested that the breaking of the hydrogen bond between Pro304 and Thr308 destabilizes the last turn of the TM7a helix and allows readjustment of the backbone carbonyl oxygen atoms, between these two Na+ ions and the bound aspartate [18].

The positions of two Na+ ions (Na1 and Na2) have been experimentally identified: there is no direct interaction

<table>
<thead>
<tr>
<th>PDB ID code</th>
<th>Inward- or Outward-facing Description</th>
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<tbody>
<tr>
<td>1XFH</td>
<td>Outward-facing 7 histidine introduced and GltPh7H used for crystallization</td>
</tr>
<tr>
<td>2NWL</td>
<td>Outward-facing GltPh7H with aspartate</td>
</tr>
<tr>
<td>2NNW</td>
<td>Outward-facing GltPh7H with TBOA</td>
</tr>
<tr>
<td>2NXW</td>
<td>Outward-facing GltPh7H with aspartate and Na+</td>
</tr>
<tr>
<td>3V8G</td>
<td>Intermediate outward-facing Cross-linked GltPh7H V198C-A380CHg with aspartate and Na+. One protomer in intermediate outward-facing state</td>
</tr>
<tr>
<td>3V8F</td>
<td>Inward-facing Cross-linked GltPh7H V216C-M385CHg with aspartate and Na+</td>
</tr>
<tr>
<td>3KBC</td>
<td>Inward-facing Cross-linked GltPh7H K55C-A364CHg with aspartate and Na+</td>
</tr>
<tr>
<td>4IZM</td>
<td>Outward-facing Cross-linked GltPh7H L66C-S300CHg with aspartate and Na+</td>
</tr>
<tr>
<td>4P1A</td>
<td>Inward-facing Cross-linked GltPh7H K55C-A364CHg with thallium bound (apo conformation)</td>
</tr>
<tr>
<td>4P19</td>
<td>Inward-facing Apo cross-linked GltPh7H K55C-A364CHg</td>
</tr>
<tr>
<td>4P3J</td>
<td>Inward-facing Apo cross-linked GltPh7H K55C-A364CHg in alkali-free conditions</td>
</tr>
<tr>
<td>4P6H</td>
<td>Inward-facing Cross-linked GltPh7H K55C-A364CHg with thallium bound (bound conformation)</td>
</tr>
<tr>
<td>40YE</td>
<td>Outward-facing GltPh6H R397A with no ligands bound</td>
</tr>
<tr>
<td>40YF</td>
<td>Outward-facing GltPh R397A with Na+ bound</td>
</tr>
<tr>
<td>40YG</td>
<td>Outward-facing GltPh7H R397A with aspartate and Na+</td>
</tr>
<tr>
<td>4X2S</td>
<td>Inward-facing GltPh7H R276S-M395R with aspartate and Na+</td>
</tr>
</tbody>
</table>
Asn 310 and Asp 312 are both part of the highly conserved NMDGT motif [18]; Thr 314 in the motif is involved in aspartate binding [18] and mutations of the equivalent residue (Thr 400) in EAAT2 abolish its function [39].

Substrate affinity and binding
Although GltPh is a glutamate transporter homologue, it exhibits a strong preference for aspartate as a substrate in the presence of an Na⁺ gradient. It shows 60000-fold higher affinity for aspartate (with Kₐ values for aspartate and glutamate of approximately 2 nM and 122 μM respectively) than GltPh. The aspartate-binding site consists of the tips of HP1 and HP2, the conserved NMDGT motif of TM7 (see above) and hydrophilic residues on TM8 [18] (Figure 1d). The α-carboxyl group of the substrate interacts with the side chain of Asn 65 (TM8) and the main chain amide nitrogen of Ser 278 (TM1). The γ-carboxyl group interacts with the side chains of Thr 314 (TM7) and Arg 397 (TM8). The substrate amino group interacts with the side chain of Asp 594 (TM8) and the backbone carbonyl groups of Arg 276 (HP1) and Val 355 (HP2) (Figure 1d).

Transport mechanism of the aspartate transporter GltPh
Binding thermodynamics studies show that aspartate binding and release, rather than TM movements of the transport domains, is coupled to the chemical potential of sodium ions in solution [42].

Uncoupled chloride ion conductance
A stoichiometrically uncoupled Cl⁻ conductance is observed along with aspartate transport in GltPh [16]. This Cl⁻ conductance can partially neutralize the membrane potential caused by the electrogenic substrate transport. The anion selectivity of GltPh is almost the same as that of EAATs. Mutation of a conserved amino acid (S65V in GltPh, located in TM2) strongly affects the chloride conductance with almost no effect on the Na⁺: aspartate symporter [16], similar to results observed in EAAT1 (S103V) [40]. Clearly, Cl⁻ permeates through a specific pathway [16] and Ser 65 is somehow involved in the process. In a recent MD simulation [41], however, researchers were unable to find any evidence showing that Ser 65 interacts directly with Cl⁻. Combined with experimental evidence obtained from both GltPh and EAAT4, they proposed that Ser 65 exerts its effect on anion permeation by altering the rates of conformational changes leading to the open anion channel.

A recent study combined MD simulations with fluorescence spectroscopy of GltPh and patch-clamp recordings of mammalian EAATs [41]. The authors suggested that lateral movement of the transport domain triggers formation of the anion-selective permeation pathway only if the domain sampled intermediate transporter conformations, rather than outward- or inward-facing states. They predicted residues that line the ion permeation pathway by simulation and verified these predictions through fluorescence spectroscopy and functional studies on mutant transporters. Of the residues lining the pathway, the side chain of Arg 276 protrudes from the tip of HP1 into the Cl⁻ permeation pathway and this resulting positive charge contributes to the anion selectivity for both GltPh and the EAATs [41]. This residue is also involved in the binding of substrates [18,30]. Interaction with the substrate does not compromise its role in anion permeation and selectivity [41].
The GltPh transport cycle

Model of the GltPh transport cycle for a monomer based on available crystal structures and MD simulations on the binding and release order of the ligands. Protein data bank (PDB) codes are in parentheses. The helical HP structure in red is HP2 and the blue one is HP1. The purple circles represent Na⁺ ions binding at Na1, Na2 and Na3. The grey ellipse represents aspartate. Starting from the upper left corner, in the outward-facing apo structure, Na⁺ ion binding at Na1 triggers structural changes in the transport domain and HP2, which opens the aspartate and Na2 sites to conformations similar to that in the holo transporter [30]. After aspartate and Na⁺ ion bind to their corresponding binding sites, there is a further, unknown conformational change linked to the binding of Na3 before movement across the membrane. Once the transport domain reaches the intracellular side, through opening of the intercellular gate, the substrates release into cytoplasm. The transport domain stays compacted with collapsed ligand-binding sites, which make it suitable for TM movement, thus completing the transport cycle [30]. There are as yet no experimental data on the position of the third Na⁺ ion-binding site or the binding order of the ligands.

Conformational changes dominate the process and remain to be elucidated by further research [42]. After the ligands are fully bound to the transport domain and occluded from the solvent by the closure of both HP1 and HP2, the transport domain moves across the membrane as a rigid body [20] (Figure 2).

Simulations based on the inward-facing crystal structure of GltPh have provided preliminary insights into the process of substrate release into the cytoplasm. DeChancie et al. [45] suggested that release is initiated by dissociation of Na⁺ from the Na2 site and, almost simultaneously, opening of the HP2 loop exposes the substrate and other polar and charged groups. This attracts water molecules to the substrate-binding site, which further destabilizes interactions between substrate and protein residues on HP2 and TM8. The HP1 loop then opens, disrupting the strong hydrogen bonds between the SSS motif (Ser277–Ser279) on the HP1 loop and the substrate, allowing the aspartate to dissociate. In this model, HP2 serves as an activator of the intracellular HP1 gate [45]. However, a previous simulation suggested that HP2 is in fact the intracellular gate in the inward-facing state [46]. In this model, HP2 opening is a prerequisite for substrate release into the cytoplasm. Understanding the mechanism of substrate release requires further research.

Following substrate release, the transport domain undergoes a series of conformational changes to prepare itself...
for the TM movement. The conformational changes in the inward-facing apo structure are that though all of the ligand-binding sites are distorted, the apo transport domain is as closed and compact as in the fully bound structure [30] (Figure 2). This may be critical for the transport domain to transit to the outward-facing state.

**Outlook**

Although crystallographic, MD simulations and smFRET studies have greatly increased our understanding of the GltPh transport mechanism, there are still many questions yet to be answered, including a definitive answer to the position of the third Na⁺ ion, the mechanism of substrate binding and release, and how the transport cycle is completed. Single-molecule and structural studies should yield definitive insights into the mechanism of substrate release and the transition to the outward-facing state in GltPh. However, to understand the differences between it and the EEATs, for instance the differing substrate and ion transport specificity, will require high-resolution structures of the EEATs, either by X-ray crystallography or – possibly – by EM using the new generation of microscopes.

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**References**

25. Grewer, C., Balani, P., Weidenfeller, C., Bartusel, T., Tao, Z. and Rauen, T. (2009) Functional analysis of the third Na⁺ ion, the mechanism of substrate binding and release, and how the transport cycle is completed. Single-molecule and structural studies should yield definitive insights into the mechanism of substrate release and the transition to the outward-facing state in GltPh. However, to understand the differences between it and the EEATs, for instance the differing substrate and ion transport specificity, will require high-resolution structures of the EEATs, either by X-ray crystallography or – possibly – by EM using the new generation of microscopes.

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