Point of VIEW

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Gate Movements in Glutamate Transporters

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ABSTRACT Sodium-coupled glutamate transporters are essential for efficient excitatory transmission in the brain and function by exposing their binding sites alternately to either the synapse or the interior of the cell. After the recent determination of the crystal structure of an archaeal homologue of the eukaryotic glutamate transporters, corresponding to the substrate occluded form, now the same has been achieved for the outward-facing conformation. These structures provide important insights into the molecular mechanism of ion-coupled transporters.

Communication between nerve cells in the brain is achieved predominantly by neurotransmitters. When an electrical impulse (action potential) spreads along the axon of a nerve cell and reaches the nerve terminal, neurotransmitter molecules are released into the synapse. After diffusion across the synapse, the neurotransmitter activates receptors on the postsynaptic nerve cell, and as a result the chemical signal is converted back into an electric signal. For this process to be efficient, the neurotransmitter must be removed from the synapse soon after release, usually by transporters located in the plasma membranes of neurons and/or glial cells surrounding the synapse. Glutamate is the predominant excitatory neurotransmitter in the mammalian brain. A little over two years ago, Gouaux and coworkers (*1*) described a first crystal structure of an archaeal homologue of the eukaryotic glutamate transporters. A very recent paper by Boudker *et al.* (*2*) reports the crystal structure of another conformation of the same transporter, providing novel clues on the mechanism of this important class of transporters.

The synaptic glutamate levels must be reduced to concentrations much below the $K_{0.5}$ of the glutamate receptors. On the other hand, inside the cell the glutamate concentration is typically 10 mM or more, and therefore the transmitter must be transported against a concentration gradient of at least 4 orders of magnitude. The energy for this is provided mainly by the electrochemical gradient for sodium ions, which is in turn generated by the Na^+,K^+-ATPase. Given the magnitude of the electrochemical sodium gradient, thermodynamics dictates that such a huge glutamate gradient can be sustained only by coupling the transport of glutamate with multiple sodium ions. In fact, studies have shown that in eukaryotic glutamate transporters three sodium ions and a proton are co-transported per glutamate anion and the transport cycle is completed by transporting a potassium ion in the opposite direction (*3*–*6*). Transporters generally function by exposing their binding sites alternately to either side of the membrane. This enables them to catch their cargo, such as glutamate and co-transported ions, on one side and then release it on the other. A widely accepted theory proposes that two gates can be used to accomplish this, with only one open at a time, just like locks in a waterway (*7*). Support for this idea comes from crystal structures from several transporters, which invariably show a cavity closed off from the aqueous space on either one side or both sides of the membrane.

No functional information on the archaeal homologue, named Glt_{Ph}, was available when its first structure was reported (*1*). However, it was clear that Glt_{Ph} belonged to the solute carrier 1 (SLC1) family of glutamate and other transporters and not just because of sequence conservation. The features revealed in the structure were in excellent agreement with functional studies on eukaryotic glutamate transporter mutants and with the experimentally determined membrane topology of the glutamate transporter GLT-1 from brain and its counterpart GltT from *Bacillus stearothermophilus* (reviewed in ref 1). This first Glt_{Ph} structure

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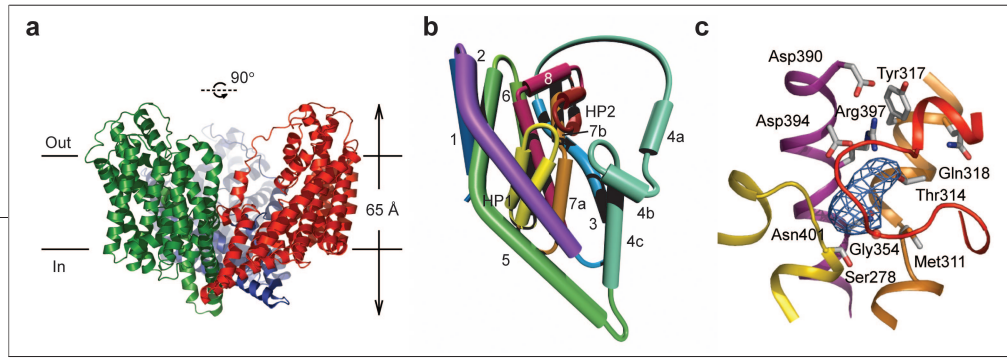

Figure 1. Structure of Glt_{Ph}. a) View of the trimer parallel to the membrane. b) Schematic representation of the protomer fold. c) A close-up view of the substrate-binding site, with some of the residues implicated in glutamate and ion binding shown in stick representation, together with the non-protein density. Adapted with permission from Macmillan, copyright 2004 (*1*).

revealed a bowl-shaped structure, formed by a trimer of the transporter, with a solvent-filled extracellular basin extending halfway across the membrane bilayer (Figure 1, panel a). At the bottom of the basin are three independent binding sites, one in each transporter monomer. Each of these binding sites is cradled by two helical hairpins, HP1 and HP2, reaching from opposite sides of the membrane (Figure 1, panels b and c). The non-protein electron density, apparently corresponding to bound substrate, was found in proximity with conserved amino acid residues critical for function in the corresponding transporters from brain, including an arginine residue implied in the binding of one of the two carboxyl groups of the substrate (*1, 8*). The location of this buried substrate site is reminiscent of the occluded leucine site in LeuT (*9*). LeuT belongs to a different transporter family, SLC6, and is a bacterial homologue of the transporters for many other neurotransmitters and other solutes. On the basis of the initial Glt_{Ph} structure, the authors (*1*) proposed that glutamate transport is achieved by movements of the hairpins that allow alternating access to either side of the membrane.

The new paper by the Gouaux group (*2*) represents an important mechanistic and structural follow-up of the paper describing the initial structure of the glutamate transporter homologue (*1*). Glt_{Ph} was reconstituted into liposomes and shown to be a sodium-coupled aspartate transporter. In contrast with its eukaryotic counterparts, which transport L-glutamate and L- and D-aspartate with similar apparent affinities, Glt_{Ph} transported aspartate with an apparent affinity that was several orders of magnitude higher than that of glutamate. Despite the relatively low resolution of the Glt_{Ph} structure, the authors were able to get more detailed information on how the substrate is liganded by the binding pocket of the transporter by using anomalous diffraction with sulfur- or bromide-containing analogues of substrate and non-transportable substrate analogues. This revealed additional amino acid residues interacting with the transporter, in addition to the above-mentioned arginine residue. Important follow-up challenges will be to understand the structural basis for the discrimination between glutamate and aspartate, as well as the stereospecificity for glutamate in the eukaryotic transporters, which prefer the L- over the D-isomer.

Particularly interesting results were obtained when Gouaux and colleagues (*2*) solved the crystal structure of Glt_{Ph} in complex with D,L-*threo*-β-benzyloxyaspartate (TBOA), a non-transportable substrate analogue in the eukaryotic transporters (*10*), as well as in Glt_{Ph} (*2*). TBOA basically locks the transporter in an outward-facing conformation (Figure 2, panels a and b). The overall structure of the transporter was found to be similar in the aspartate-bound and TBOA-bound complexes, except that in the TBOA-bound structure HP2 adopts an "open" conformation. HP2 moves ~10 Å from its position in the aspartate-bound complex toward the extracellular loop connecting transmembrane (TM) helices 3 and 4. This loop itself also moves closer to HP2, enabling direct contacts. These movements expose the substrate binding site to the extracellular solution (Figure 2, panel a) and support the hypothesis that HP2 represents the external gate of the transporter (*1*). Similar proof for a role of HP1 as the internal gate (or part of it) will have to await the crystallization and structure determination of Glt_{Ph} or another family member in the inward-facing conformation. Nevertheless, it is of interest to note that a very recent cysteine-scanning study of the glutamate transporter GLT-1 from glial cells supports the idea that the inward movement of HP1 results in the opening of a pathway between the binding pocket and the cytoplasm, which is lined by parts of TM domains 7 and 8 (*11*).

In addition to transport by Glt_{Ph}, monitored upon its reconstitution into proteoliposomes, the authors used two types of binding assays to monitor the interaction of substrate and ions with the transporter. One assay was isothermal titration calorimetry, and the other was tryptophan fluorescence. Fluorescence measurements were done on transporter constructs with a single tryptophan, introduced at the top of TM helix 4 (L130W). These types of studies showed that aspartate binding is coupled to the binding of at least two sodium ions, whereas the binding of TBOA is coupled to the binding of one sodium ion. The latter is consistent with the observation that binding of a radiolabeled TBOA analogue to eukaryotic glutamate transporters requires only one sodium ion (*12*). Moreover, the authors observed that lithium and thallium (Tl^+), but not potassium, can substitute for sodium in the binding assay, albeit with a very low apparent affinity. Also, thallium, but not potassium, could diminish sodium-dependent aspartate transport.

Because Tl^+ has a robust anomalous scattering signal, this cation was used as a potential sodium analogue to define the binding sites of the latter cation. Despite the above-described data indicating that Tl^+ can interact functionally with the transporter, this cation could not support aspartate transport (*2*). Therefore, the assumption that Tl^+ faithfully mimics the action of sodium (*2*) must be treated with caution. Examination of anomalous difference Fourier maps of crystals soaked in thallous nitrate

revealed two strong peaks per subunit (*2*). Only the presence of sodium during the soaking of the Glt_{Ph} crystals, but not that of lithium or potassium, prevented the appearance of the two peaks, an observation consistent with the above assumption. The two putative sodium sites are near the bound aspartate, but they are not in direct contact with the substrate. Site 1 is buried deeply within the protein, "below" the aspartate (more toward the intracellular side). This site is formed by carbonyl oxygens from TM helices 7 and 8 and apparently also by three side-chain oxygens, two of which are provided by Asp405 from TM 8. Site 2 is close to HP2 and seems to have coordinating main-chain carbonyl oxygens located in TM 7 and HP2. In the presence of TBOA, only the Tl^+ peak corresponding to site 1 is observed. This result is in nice agreement with the fact that TBOA binding was found to be coupled to that of one sodium ion, because the movement of HP2 induced by TBOA would disrupt site 2. Conversely, crystals of the D405N mutant soaked in Tl^+ exhibited only the strong peak corresponding to site 2. The functional data on Glt_{Ph} indicate that two sodium ions are transported per aspartate but do not exclude a stoichiometry of >2 (*2*). In fact, the brain glutamate transporters use three sodium ions per glutamate (*3, 4*) (see Figure 2, panel b). One possibility is that the third sodium site of Glt_{Ph} has a higher specificity and therefore cannot be labeled by Tl^+. Functional studies indicate that two conserved residues, an aspartate and an asparagine in the unwound part of TM domain 7, may interact, directly or indirectly, with the third sodium ion (*13, 14*). Intriguingly, in the substrate-bound form of Glt_{Ph}, the side chains of these residues face away from the binding pocket (*1*). However, the binding of three sodium ions, a proton, and a glutamate to the eukaryotic transporters, depicted as a single step in Figure 2, panel b, obviously is a multistep process, and the side chains of the two residues may be accessible to the external aqueous phase in one or more of the intermediate conformations. These two residues have also been implicated in the reorientation of the binding sites of the empty eukaryotic transporters by potassium (*14*). This step is probably potassium-independent in the case of the bacterial glutamate transporters, because they lack the glutamate residue shown to be critical for the interaction with potassium in the eukaryotic transporters (*15*).

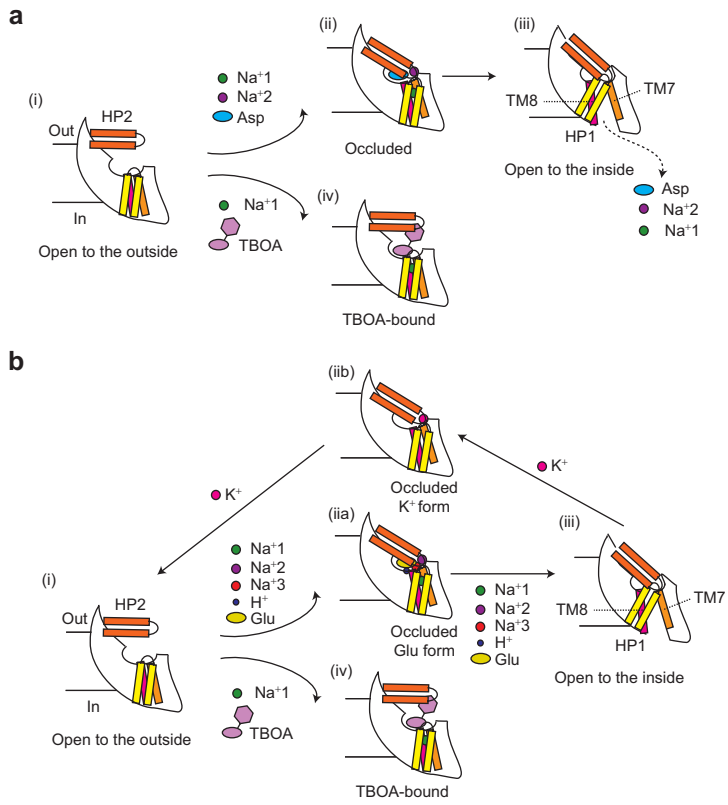

Figure 2. Schematic mechanism of glutamate transport. a) Glt_{Ph}: (i) Apo state with HP2 in an open conformation. (ii) Sodium ions 1 and 2 and aspartate bind and induce closure of HP2, yielding the occluded state. (iii) Opening of the internal gate allows the release of sodium and aspartate to the cytoplasm, probably involving the movement of HP1 and perhaps also TMs 7 and 8. HP2 remains closed. The transport cycle is completed by the closure of HP1 to yield the empty occluded state, followed by the opening of HP2. (iv) TBOA binding blocks transport by stabilizing HP2 in an open conformation, in which the sodium 2 site is disrupted. b) Eukaryotic glutamate transporters: (i), (iia), and (iii) are equivalent to (i), (ii), and (iii) of panel a, except that glutamate (or aspartate) is transported with three sodium ions and a proton. (iib) The completion of the transport cycle requires the binding and translocation of potassium *via* the occluded potassium-bound state. The TBOA-bound state (iv) is similar to that depicted in panel a. Adapted with permission from Macmillan, copyright 2007 (*2*).

The elucidation of two Glt_{Ph} structures, representing the TBOA-locked outward-facing conformation and the substrate-occluded conformation (Figure 2), is a major step toward the unraveling of the molecular mechanism of this important class of transporters. Important future steps

will be the determination of the structures of the other conformations, as well as improvement of the resolution of the present structures. This could determine unambiguously if the phenomenon that one or more of the co-transported sodium ions are in physical contact with the transported amino acid, as observed in the LeuT transporter (*9*), is a widely used mechanism in ion-coupled transport. In addition to studies at the structural level, in-depth mechanistic studies, employing biochemical and electrophysiological approaches to wild-type and mutant transporters, will be required to address the significant question of how the closing of one gate is tightly coupled to the opening of the other.

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