Distinct roles of the Na⁺ binding sites in the allosteric coupling mechanism of the glutamate transporter homolog, Glt<sub>Ph</sub>

Erika A. Riederer, Pierre Moënne-Loccoz, and Francis I. Valiyaveetil

Edited by Ernest Wright, David Geffen School of Medicine at UCLA, Los Angeles, CA; received November 29, 2021; accepted March 18, 2022

Glutamate transporters carry out the concentrative uptake of glutamate by harnessing the ionic gradients present across cellular membranes. A central step in the transport mechanism is the coupled binding of Na⁺ and substrate. The sodium coupled Asp transporter, Glt<sub>Ph</sub>, is an archaeal homolog of glutamate transporters that has been extensively used to probe the transport mechanism. Previous studies have shown that hairpin-2 (HP2) functions as the extracellular gate for the aspartate binding site and plays a key role in the coupled binding of sodium and aspartate to Glt<sub>Ph</sub>. The binding sites for three Na⁺ ions (Na1-3) have been identified in Glt<sub>Ph</sub>, but the specific roles of the individual Na⁺ sites in the binding process have not been elucidated. In this study, we developed assays to probe Na⁺ binding to the Na1 and Na3 sites and to monitor the conformational switch in the NMDGT motif. We used these assays along with a fluorescence assay to monitor HP2 movement and EPR spectroscopy to show that Na⁺ binding to the Na3 site is required for the NMDGT conformational switch while Na⁺ binding to the Na1 site is responsible for the partial opening of HP2. Complete opening of HP2 requires the conformational switch of the NMDGT motif and therefore Na⁺ binding to both the Na1 and the Na3 sites. Based on our studies, we also propose an alternate pathway for the coupled binding of Na⁺ and Asp.

Significance

Glutamate transporters harness ionic gradients present across the membrane for the rapid removal of glutamate from the synaptic space. Normal function of glutamate transporters is required for efficient synaptic transmission and preventing excitotoxicity. Central to the transport mechanism is the coupled binding of Na⁺ and the substrate. While structural studies have identified the Na⁺ and the substrate binding sites, the mechanism by which Na⁺ and substrate binding is coupled is not known. In this study, we developed assays to monitor Na⁺ binding and to track key conformational changes in Glt<sub>Ph</sub>, an archaeal homolog of glutamate transporters. We use these assays along with previously developed assays to describe the specific roles of the Na⁺ sites in the coupling mechanism.

Transport of molecules across cellular membranes is essential for life. Na⁺ coupled transporters use the existing Na⁺ gradient to carry out transport across cell membranes of a variety of substrates such as amino acids, sugars, nucleosides, neurotransmitters, and vitamins (1, 2). High-resolution structural studies have revealed the molecular architecture of a number of these transporters. However, the mechanism by which the transport of the substrate is coupled to the Na⁺ gradient is not well understood. Here, we investigate this mechanism of Na⁺ substrate coupling in the context of glutamate transporters.

Glutamate transporters, also known as excitatory amino acid transporters or EAATs, are responsible for the uptake of glutamate following release into the synaptic space during synaptic transmission (3–5). Glutamate transporters harness energy from pre-existing gradients of Na⁺, K⁺, and H⁺ across the neuronal membranes to drive the rapid uptake of glutamate (6, 7). Rapid removal of glutamate from the synaptic space is required for efficient synaptic transmission and for preventing excitotoxicity (3).

Studies on the archaeal homologs Glt<sub>Ph</sub> and the closely related Glt<sub>TK</sub> have greatly contributed to our present knowledge of the structure, dynamics, and functional mechanisms of glutamate transporters (8, 9). Glt<sub>Ph</sub> and Glt<sub>TK</sub> are archaeal sodium coupled Asp symporters in which the transport of Asp is coupled to the movement of three Na⁺ ions (10). Glt<sub>Ph</sub> is a homotrimer in which each subunit consists of two domains: a trimerization domain and a transport domain (Fig. 1 A and B) (9, 11). The binding sites for Asp and the three Na⁺ ions (labeled Na1, 2 and 3) are contained within the transport domain (Fig. 1 C) (12, 13). Studies on Glt<sub>Ph</sub> have shown that the transport mechanism involves the elevator-like movement of the transport domain to ferry the bound Asp and Na⁺ ions across the membrane (11, 14–17).

An essential feature of the transport mechanism in Glt<sub>Ph</sub> is that the binding of Na⁺ and Asp are tightly coupled (18, 19). Structures of Glt<sub>Ph</sub> and Glt<sub>TK</sub> show no direct contact between the Na⁺ ions and the bound Asp, which indicates that the mechanism of coupling involves allosteric changes in the transporter (Fig. 1 C). The transport domain consists of two hairpin loop regions referred to as HP1 and HP2 (12). HP2 acts as a gate to control access to the substrate binding site when the transporter is in the outward-facing state. Structural studies show that HP2 can be closed or open. The structure of Glt<sub>TK</sub> in the Apo state shows that HP2 is closed, a structure of Glt<sub>Ph</sub> with only Na⁺ bound shows an open HP2 while the structures of Glt<sub>Ph</sub> and Glt<sub>TK</sub> with Asp and Na⁺ bound show a closed HP2 (Fig. 1 C) (12, 13, 20).
biophysical investigations suggest the following sequence of events in the coupled binding of Na\(^+\) and Asp (Fig. 1D) (13, 19, 21, 22). Central to the coupling mechanism is the movement of HP2. In the Apo state, HP2 is closed thereby preventing access of Asp to the binding site. The binding of Na\(^+\) ions to the Na1 and the Na3 sites results in opening HP2. The opening of HP2 is accompanied by a remodeling of the binding site for high affinity coordination of Asp. The binding of Asp to the transport domain is followed by the closure of HP2 and the binding of the third Na\(^+\) ion to the Na2 site. The transport domain with the bound Asp and three Na\(^+\) ions, undergoes an elevator movement to an inward-facing state from which Asp and Na\(^+\) ions are released into the cell. A variation of this scheme, based on computational studies, suggests that HP2 is dynamic in the Apo state and that the binding of the first two Na\(^+\) ions stabilizes the HP2 in the open state (20). There are important aspects of the binding mechanism of Na\(^+\) and Asp to GltPh that are not well understood. A key uncertainty is the mechanism by which Na\(^+\) binding to the Na1 and the Na3 sites is linked to HP2 movement.

Here, we assess the roles of the Na1 and the Na3 sites in GltPh. We develop fluorescence-based assays to track Na\(^+\) binding and to monitor a conformational change in the highly conserved NMDGT motif. We perturb the Na1 and the Na3 sites and use these assays along with a previously developed assay to track HP2 movement to define the specific functional roles of Na\(^+\) coordination at Na1 and the Na3 sites. We find that Na\(^+\) binding to the Na1 site results in a partial opening of HP2 while Na\(^+\) binding to the Na3 site results in a conformational switch in the NMDGT motif. Na\(^+\) binding to both the Na1 and the Na3 sites is required for a full opening of HP2 and for high affinity binding of Asp to GltPh.

**Results**

**Tyr Fluorescence of GltPh Reports on Na\(^+\) Binding to the Na1 and the Na3 Sites.** We initially identified an assay for monitoring Na\(^+\) binding to the Na1 and the Na3 sites in GltPh. It has been reported that Na\(^+\) binding to GltPh can be monitored by changes in protein fluorescence (23). GltPh does not contain any Trp residues but contains 18 Tyr residues that are distributed throughout the protein (Fig. 2A) (9). The Tyr fluorescence of GltPh shows a \(\sim 10\%\) increase in emission intensity with Na\(^+\) (Fig. 2B and C). This effect is selective for Na\(^+\) as no change in fluorescence is observed with K\(^+\) (Fig. 2C). The change in fluorescence is also specific for Na\(^+\) binding as addition of Asp following Na\(^+\) binding (which causes closure of HP2) does not result in a further change in fluorescence (Fig. 2C). Of the 18 Tyr residues in GltPh, three residues, Y88, Y89, and Y247 are in the vicinity (<7 Å) of the Na1 and the Na3 sites (Fig. 2A). To probe whether the fluorescence changes in GltPh reflect Na\(^+\) binding to the Na1 and the Na3 sites, we substituted these Tyr residues with Phe. As a control, we also generated a Phe substitution at Y317 that was distant from the Na\(^+\) binding sites. We observed that the Y88F and the Y89F substitutions reduced the fluorescence change with Na\(^+\) while no fluorescence changes with Na\(^+\) were observed for a Y88F/Y89F double mutant (Fig. 2D). We could not evaluate the effect of Y247 on the fluorescence change due to poor biochemistry of the Y247F mutant while the Y317F substitution, which is distant from the Na1 and the Na3 sites, did not affect the fluorescence changes observed with Na\(^+\) (Fig. 2D). These results indicate that the changes in Tyr fluorescence of GltPh reflect Na\(^+\) binding to the Na1 and the Na3 site and that changes in Tyr fluorescence provides a facile assay to monitor Na\(^+\) binding to these sites.

Using the Tyr fluorescence assay, we measured a \(K_{\text{D}}\) of 45 mM for Na\(^+\) binding to GltPh (Fig. 2B). A \(K_{\text{D}}\) of 90 mM has been reported using a dye-based assay (18). Na\(^+\) binding to GltPh has also been evaluated by the HP2 movement assay to give a value of 178 mM (19) and 120 mM by using a F273W substitution (24). The affinity of Na\(^+\) was increased to 1.2 mM in the presence of 100 μM Asp, as expected for a coupled transporter (Fig. 2E).

To perturb the Na1 and the Na3 sites, we substituted the amino acid side chains that coordinate Na\(^+\) (Fig. 2F and G). At the Na3 site, Na\(^+\) is coordinated by the side chains of T92, S93, N310 and D312. We introduced both conservative and nonconservative substitutions at these positions and evaluated the effect on Na\(^+\) binding. For the conservative substitutions T92S and S93T, we observed an increase in Na\(^+\) binding affinity (Fig. 2F and SI Appendix, Table S1). The nonconservative substitutions showed two distinct effects. Substitutions at T92, N310 and D312 showed a dramatic shift in \(K_{\text{D}}\) and the maximal fluorescence response observed was roughly half the value observed for the wild-type protein (Fig. 2F and SI Appendix, Table S1). For the S93A/C mutants, we also observed a shift in the Na\(^+\) binding affinity but the maximal fluorescence response was similar to the wild type. These data support the T92C,
D312N, and N310D/S substitutions abrogating Na\(^+\) binding to the Na3 site while the S93 substitutions decreases the Na\(^+\) binding affinity at the Na3 site.

At the Na1 site, D405 is the only sidechain involved in Na\(^+\) coordination (Fig. 2G). It has been previously demonstrated using structural approaches and Asp binding assays that the D405N substitution perturbs Na\(^+\) binding to the Na1 site (12). We tested Na\(^+\) binding to the D405N substitution using the Tyr assay. We observed a decrease in the maximal response and a shift in the K\(_{Na}\) to 214 mM confirming the perturbation of Na\(^+\) binding to the Na1 site in the D405N mutant (Fig. 2G and SI Appendix, Table S1). We also used the dye-based assay to monitor Na\(^+\) binding in these Na1 and Na3 site mutants (SI Appendix, Fig. S1). The dye-based assay also reported perturbed Na\(^+\) binding in the Na3 site mutants consistent with the data obtained by the Tyr fluorescence assay. These experiments provide us with GlpH mutants in which Na\(^+\) binding to the Na1 or the Na3 sites is perturbed.

Perturbation of the Na3 Site Affects HP2 Movement. Next, we investigated the effect of perturbing the Na3 site on HP2 movement. We have previously developed a fluorescence assay to monitor the movement of HP2 (Fig. 3A) (19). This assay, which we refer to as the HP2 assay, involves the incorporation of two probes into GlpH, a Trp in HP2 and a PheCN in HP1. We substituted V355 in HP2 with Trp and used nonsense

---

**Fig. 2.** Using Tyr fluorescence to monitor Na\(^+\) binding to GlpH. (A) A GlpH protomer with Tyr residues shown in stick representation. Inset shows a close-up of the Na1/3 sites and the Tyr residues in close proximity to these sites. (B) Fluorescence emission spectra of wild-type GlpH on excitation at 289 nm in the Apo state and after the addition of 300 mM NaCl. (C) Fluorescence emission at 308 nm (excitation = 289 nm) for wild-type GlpH with the addition of 300 mM NaCl (purple), 300 mM NaCl/100 \(\mu\)M Asp (green), 300 mM KCl (blue), and 100 \(\mu\)M Asp (teal). The fluorescence values are normalized to the Apo protein. (D) Change in fluorescence from the Apo state for the wild-type GlpH and Phe mutants after the addition of 300 mM NaCl/100 \(\mu\)M Asp. Error bars in C and D are SEM, \(n = 3\) to 5. For C and D, the significance of the differences was evaluated by using a Student t test and marked with a single (\(P < 0.05\)), double (\(P < 0.01\)), triple asterisk (\(P < 0.001\)), or as not significant (NS, \(P > 0.05\)). (E) Na\(^+\) titration of wild-type GlpH. The normalized fluorescence change at 308 nm on addition of Na\(^+\) (black) is plotted. Sample Na\(^+\) titrations in the presence of 10 \(\mu\)M TBOA (red) and 100 \(\mu\)M Asp (green) are also shown. The solid line represents a fit to the Hill equation used to determine the K\(_{Na}\) (Na\(^+\), 45 mM; Na\(^+\) with TBOA, 4 mM; Na\(^+\) with Asp, 1.2 mM). (F) Close up view of the Na3 site and sample Na\(^+\) titrations of the various Na3 site mutants. (G) Close up view of the Na1 site and Na\(^+\) titration of the D405N mutant is shown. In F and G, Na\(^+\) titration for the wild-type GlpH is indicated by the solid black line.
suppression approaches to substitute S279 in HP1 with cyanophenylalanine (PheCN). In the Apo state, HP2 is (predominantly) closed and the probes are close together. In this state PheCN quenches the Trp fluorescence due to the proximity of the two probes (Fig. 3B) (25). The binding of Na\(^+\) opens HP2, which increases the separation between the probes and increases Trp fluorescence due to reduced quenching of Trp by PheCN. Binding of Asp closes HP2, which decreases the separation between the probes and results in a decrease in fluorescence (Fig. 3B). Monitoring the changes in fluorescence therefore provides us with a means to evaluate the opening of HP2 with Na\(^+\) and the closure of HP2 with Na\(^+\)/Asp.

We tested the effect of the Na3 site mutants on HP2 movement. For the N310S and the D312N mutants, we observed very small (2 to 5% compared to 35% for the wild-type control) fluorescence changes with Na\(^+\) in the HP2 assay (Fig. 3C and G). The lack of a fluorescence change in the HP2 assay can be attributed to either HP2 not opening or to HP2 not being propped open on the binding of Na\(^+\). To distinguish between these possibilities, we tested the effect of TBOA on HP2 opening. TBOA binds to GltPh similarly to Asp but keeps HP2 propped open due to the presence of the benzyloxy group in the side chain (12, 26). Correspondingly, the addition of Na\(^+\)/TBOA to the wild-type GltPh gives an additional 9% increase over the fluorescence changes seen with Na\(^+\) alone (Fig. 3B and G). Addition of Na\(^+\)/TBOA to the N310S or the D312N mutants showed fluorescence changes that were only slightly higher (∼2%) than the changes observed with Na\(^+\) alone, which indicates that HP2 opening is severely perturbed in these mutants (Fig. 3E and G). For S93A and T92C, we observed fluorescence changes with Na\(^+\) and with Na\(^+\)/TBOA indicating HP2 movement (Fig. 3D and F). The changes observed for
these mutants (~10% with Na+ and 21 to 24% with Na+/TBOA) were, however, smaller than the changes observed in the wild-type control indicating impaired HP2 opening in these mutants (Fig. 3G). These results indicate that Na+ binding to the Na3 site is required for HP2 opening and that the magnitude of the effect depends on the specific side chain that is perturbed. We see a severe effect for substitutions in the N310 and D312 residues that are in the NMDGT motif compared to substitutions at T92 and S93 in the TM3 helix.

**A Conformational Switch in the NMDGT Motif Couples Na+ Binding to HP2 Movement.** A highly conserved sequence in EAATs is the NMDGT motif (4). This motif forms the unwound region present in the middle of TM7 and contributes to the Na1 and Na3 sites (Fig. 4A) (12). The NMDGT region shows a different conformation in Apo-GltPh compared to Na+ or Na+/Asp-GltPh and therefore we speculated that the NMDGT motif couples Na+ binding to the opening of HP2 (13, 20, 21, 27). To investigate, we developed an assay to...
monitor the conformational switch in the NMDGT motif. We identified that residue L99 in the vicinity of the NMDGT motif undergoes a change in environment with the conformational switch in this motif. We substituted L99 with Trp to serve as a fluorescence reporter for the NMDGT conformational switch. The L99W-GltPh showed similar biochemistry to the wild-type transporter and was functional in Asp uptake (SI Appendix, Fig. S2). We observed that the fluorescence emission of L99W-GltPh was sensitive to Na$^+$ and showed a 21% decrease in fluorescence intensity and a blue shift in the emission maximum on the addition of Na$^+$ (Fig. 4 B and C). The blue shift in the fluorescence intensity indicates a shift to an environment with a lower polarity. There was only a small (1.5%) increase in fluorescence on the addition of Asp, which is anticipated as the NMDGT region has a similar structure in the Na$^+$ bound and the Na$^+$/Asp bound states (Fig. 4 B and C). The decrease in fluorescence for L99W-GltPh on the addition of Na$^+$ was fit with a Hill equation to give a $K_{Na}^{opt}$ of 172 mM, which decreased to 2 mM in the presence of 100 μM Asp (Fig. 4C and SI Appendix, Table S2).

The structures of GltPh in the apo and Na$^+$ bound states indicate that the change in the environment around L99 with the NMDGT conformational switch is due to a change in the relative positioning of the L316 side chain (Fig. 4D). To test, we investigated the fluorescence changes in a L99W/L316A mutant. We observed that the fluorescence changes were greatly attenuated compared to the L99W mutant (Fig. 4E). In control experiments, we confirmed that the L316A substitution did not affect HP2 movement (SI Appendix, Fig. S3). These experiments confirm that the fluorescence changes of L99W are indeed reporting on the NMDGT conformational switch.

We introduced various Na3 site substitutions into the L99W-GltPh and determined the effect on the NMDGT conformational switch (Fig. 4F and SI Appendix, Fig. S4 and Table S2). For residues N310 and D312 in the NMDGT motif, we found that the fluorescence changes in the N310T mutant were greatly attenuated compared to the control, L99W-GltPh, while the D312N substitution eliminated any fluorescence changes with Na$^+$ or Na$^+$/Asp addition. For the substitutions in T92 in the TM3 helix, we observed substantially reduced fluorescence changes in the T92C mutant while a similar extent of change to the wild-type control was observed for the T92S substitution. Consistent with the improved Na$^+$ binding observed for the T92S mutant in the Tyr assay, the changes in the NMDGT motif also took place at a lower Na$^+$ concentration compared to the control. For the S93A mutant, we observed that the extent of change was similar to the wild-type control although the changes were observed at a much higher Na$^+$ concentration. We find that substitutions that perturb Na$^+$ binding to the Na3 site also affect the NMDGT conformational switch thereby indicating a requirement of Na$^+$ binding to the Na3 site for the conformational switch. Further, we find that the effects of the Na3 mutants on the NMDGT conformational switch mirror the effects on HP2 opening, which suggests that the NMDGT conformational switch couples Na$^+$ binding at the Na3 site and HP2 opening.

An S93-M311 Steric Clash Assists in Propping HP2 Open. M311 in the NMDGT motif is a key residue for coupling Na$^+$ and Asp binding in GltPh (13, 21). We have previously shown that on Na$^+$ binding, M311 acts as a wedge to keep HP2 propped open (19). In GltPh, structures, the M311 side chain has been visualized in two conformations (Fig. 5A) that we refer to as the extended and the flipped conformation. In the apo state, the M311 side chain is dynamic and samples both these conformations while in the Na$^+$ or the Na$^+$/Asp bound state, the M311 side chain is in the flipped conformation. A structural inspection suggests that Na$^+$ binding at the Na3 site can influence the conformation of the M311 side chain. This occurs because in the Na$^+$ bound conformation, the S93 side chain sterically clashes with the extended conformation of the M311 side chain. To confirm this steric clash mechanism, we probed the effect of substitutions at S93 and M311 that changed the steric properties of these side chains. We substituted S93 with T to increase the steric bulk of this side chain. For the S93T mutant, we observed that the opening of HP2 took place at a lower Na$^+$ concentrations and to a larger extent than the wild type (Fig. 5B). At M311, a Leu substitution, which has a similar side chain volume (28) to Met but with different steric, showed HP2 opening in a relatively WT-like manner (Fig. 5B). In the M311L/S93T double mutant, we observed that the opening of HP2 took place at a substantially lower Na$^+$ concentration and the extent of opening was greater than observed for the single mutants (Fig. 5B). These results are in accordance with a steric interaction between the S93 and the M311 side chains when Na$^+$ binds to the Na3 site. This steric interaction helps switch the M311 side chain into the flipped conformation, wherein it acts as a wedge to keep HP2 open on Na$^+$ binding.
The D405N Substitution at the Na1 Site Uncouples HP2 Opening from Na⁺ Binding. We used the D405N substitution to evaluate the effect of perturbing the Na1 site on HP2 movement (Fig. 6A). In the HP2 assay, we did not observe a fluorescence change upon the addition of Na⁺ for the D405N mutant although a decrease in fluorescence was observed with Na⁺/Asp (Fig. 6B). The decrease in fluorescence indicates Asp binding and the closure of HP2. The lack of a fluorescence signal corresponding to the opening of HP2 with Na⁺ could stem from HP2 not being stable in the open state (as previously described) or due to HP2 already being open. We used TBOA binding to distinguish between these possibilities (as previously described). We found that the addition of Na⁺/TBOA did not result in a significant fluorescence change from the Apo- or the Na⁺/D405N GltPh (Fig. 6B). For the wild-type control, we observed an additional 9% change in intensity when comparing the fluorescence response to Na⁺ versus Na⁺/TBOA. We confirmed that TBOA binds to the D405N-GltPh in the presence of Na⁺ by a shift in the Asp affinity in the presence of TBOA (SI Appendix, Fig. S5). The lack of an effect of Na⁺ or Na⁺/TBOA in the HP2 assay for the D405N-GltPh, suggests that HP2 is already open in the Apo state. We also investigated a D405A substitution and observed a similar result (SI Appendix, Fig. S6). We speculated that if HP2 is already open in D405N-GltPh, then the mutant should be able to bind Asp in the Apo state. We tested Asp binding by the Apo-D405N transporter and observed a decrease in fluorescence indicating that Asp binding and closure of HP2 took place in the absence of Na⁺ (Fig. 6B). Asp binding to the Apo D405N transporter took place with a K_{A,DP} of 124 μM compared to a value of 0.033 μM in the presence of 200 mM Na⁺ (Fig. 6C). As anticipated, we did not detect Asp binding to the wild-type control in the absence of Na⁺.

We also used isothermal titration calorimetry (ITC) to evaluate Asp binding to the D405N-GltPh. In the presence of 10 mM Na⁺, we observed strong Asp binding with a K_D of 0.19 μM to the wild-type transporter while the Asp binding to the D405N transporter took place with a K_D of 3.4 μM and a smaller heat change (Fig. 6D and E). We tested Asp binding in the absence of Na⁺ and observed no binding to the Apo wild-type control (Fig. 6F). In contrast, we observed Asp binding to the Apo D405N transporter with a K_D of 11 μM. The magnitude of the heat change on Asp binding to the Apo D405N transporter was smaller than the change observed for Asp binding in the presence of Na⁺ (Fig. 6G). The ITC studies confirm that D405N-GltPh can indeed bind Asp without Na⁺. However, the lower magnitudes of the heat changes and the shifted affinities indicate differences in the conformational change upon Asp binding in the WT- and the D405N-GltPh. The combined data from the HP2 assays and the ITC experiments suggest that D405N substitution perturbs the Na⁺ coupled opening of HP2 and allows the binding of Asp to the Apo transporter.

Continuous Wave-Electron Paramagnetic Resonance Experiments Confirm an Open HP2 in D405N-GltPh. We used continuous wave-electron paramagnetic resonance (CW-EPR) experiments to further probe the movements of HP2 when the Na1 site was perturbed. We introduced spin probes at V355 and S279 as previously described to monitor the distance...
of HP2 (red) movement during the coupled binding of Na\(^{+}\) in HP2. (Fig. 7A) Cartoon representation of HP2 (red) movement during the coupled binding of Na\(^{+}\) ions (yellow spheres) and Asp (green oval). Sites for introducing the MTSL spin probes (yellow stars) for monitoring HP2 movement are 279 in HP1 (orange) and 355 in HP2. (B) Continuous wave EPR spectra of wild-type and D405N-Glt\(_{\text{ph}}\) under the following conditions: Apo, 200 mM NaCl, 200 mM NaCl/500 μM Asp. (C) Relative amplitude for the central peak in the EPR spectra in 200 mM NaCl (n = 5), 200 mM NaCl/100 μM TBOA (3), 200 mM NaCl/500 μM Asp (3) normalized to the Apo value. Error bars are SEM. The significance of the difference was evaluated by a test and reported with the symbols as used in Fig. 2C.

The D405N Substitution Partially Mimics an Na1-Only Bound State. We have established that HP2 opening in the wild-type Glt\(_{\text{ph}}\) on Na\(^{+}\) binding involves a conformational switch of the NMDGT motif. Next, we probed whether changes in the NMDGT motif are involved in the HP2 opening seen in the Apo-D405N mutant transporter. We were unable to use the L99W substitution for this purpose as the L99W/D405N mutant showed poor biochemistry. A key aspect of the NMDGT switch is a flip of the M311 side chain and in this flipped conformation, the M311 side chain acts as a wedge to keep HP2 propped open on Na\(^{+}\) binding. To infer involvement of the NMDGT conformational switch in HP2 opening in the Apo-D405N transporter, we evaluated the role of the M311 residue in this process. For this purpose, we investigated the effect of the M311A substitution on HP2 opening in D405N Glt\(_{\text{ph}}\). In the M311A/D405N Glt\(_{\text{ph}}\)(Phe\(_{\text{CN+}}\),W), there is no observable signal for HP2 opening as in the D405N mutant. We inferred the state of HP2 by investigating Asp binding since Asp binding can only take place if HP2 is open. We observed Asp binding to M311A/D405N mutant in the Apo state and the binding took place with a higher affinity than observed for the D405N mutant (SI Appendix, Fig. S7A). Asp binding to the M311A mutant in the Apo state was not detected (19). Asp binding to the M311A/D405N mutant in the Apo state indicates that the M311 side chain is not necessary for HP2 opening in the Apo-D405N transporter. This result indicates that the changes in the NMDGT motif and therefore the conformational changes corresponding to Na\(^{+}\) binding to the Na3 site are not involved in the HP2 opening seen in the Apo-D405N transporter. In the presence of Na\(^{+}\), we observe that the D405N+M311A Glt\(_{\text{ph}}\) shows a ~100-fold lower Asp binding affinity compared to D405N Glt\(_{\text{ph}}\) (SI Appendix, Fig. S7B). The change in the affinity with the M311A substitution indicate the participation of the M311 side chain and thereby the NMDGT conformational switch in the Na\(^{+}\) coupled binding of Asp to the D405N-Glt\(_{\text{ph}}\).

The opening of HP2 in the wild-type Glt\(_{\text{ph}}\) requires Na\(^{+}\). HP2 opening in the D405N transporter in the absence of Na\(^{+}\) indicates that the D405N substitution partially mimics the effect of Na\(^{+}\) binding (SI Appendix, Fig. S7C). Our experiments with the M311A/D405N mutant have ruled out a participation of the conformational changes corresponding to Na\(^{+}\) binding to the Na3 site. As D405 contributes to the Na1 site, we expect that the Asn substitution partially mimics the effects of a Na\(^{+}\) ion bound at the Na1 site. Therefore, we conclude that the Apo-D405N Glt\(_{\text{ph}}\) resembles a transporter with Na\(^{+}\) binding to only the Na1 site. Binding of Asp by the Apo-D405N Glt\(_{\text{ph}}\) suggests that HP2 opening and Asp binding can take place in the absence of Na\(^{+}\) and confirm the findings from the HP2 assays.
of Asp however, takes place in a low affinity mode that is likely distinct from the Asp binding mode seen in the structures of GltP\textsubscript{h} and GltP\textsubscript{vK} (SI Appendix, Fig. S7C). Further, the HP2 assays and the EPR studies indicate a limited HP2 opening when Na\textsuperscript{+} is bound to only the Na1 site in contrast to the full opening of HP2 that is observed when Na\textsuperscript{+} is bound to both the Na1 and the Na3 sites.

**Discussion**

The coupled binding of Na\textsuperscript{+} and Asp is central to the transport mechanism in GltP\textsubscript{h}. In this study, we developed assays to probe Na\textsuperscript{+} binding to the Na1 and Na3 sites in GltP\textsubscript{h}, and to monitor the conformational switch in the NMDGT motif. We used these assays along with the previously developed HP2 movement assay to show that Na\textsuperscript{+} binding to the Na3 site is required for the NMDGT conformational switch while Na\textsuperscript{+} binding to the Na1 site is responsible for the partial opening of HP2. Complete opening of HP2 requires the conformational switch of the NMDGT motif and therefore Na\textsuperscript{+} binding to both the Na1 and the Na3 sites.

Based on our results we propose the following mechanism for HP2 opening by Na\textsuperscript{+} (Fig. 8). In the Apo state HP2 is closed. The process of HP2 opening is initiated by Na\textsuperscript{+} binding to the Na1 site. It has been previously suggested that Na\textsuperscript{+} initially binds to the Na3 site as the Na3 site is predicted to have the highest Na\textsuperscript{+} binding affinity (13, 30, 31). However, the Na3 site is buried within the protein core and Na\textsuperscript{+} access to the Na3 site involves passage through the Na1 site. Computational studies have also previously suggested Na\textsuperscript{+} binding initially to the Na1 site (31). Occupation of the Na1 site by Na\textsuperscript{+} results in a partial opening of HP2. We anticipate that the partial open conformation of HP2 resembles the HP2 conformation seen in the structure of GltP\textsubscript{h} R397A with Na\textsuperscript{+} present (pdb: 4oyf) (21). The partial opening of HP2 now provides a pathway for Na\textsuperscript{+} ions to access the Na3 site as suggested (20). Binding of Na\textsuperscript{+} to the Na3 site can take place through this pathway or alternately, the Na\textsuperscript{+} ion at the Na1 site can transition to the Na3 site while the Na1 site is reoccupied by another Na\textsuperscript{+} ion (31). Occupation of the Na3 site results in a conformational switch of the NMDGT motif that is required for full opening of HP2.

Our data suggests that the binding of Asp to GltP\textsubscript{h} can take place through two different pathways. A recent study of a mutant D40N/S279E and the wild-type GltP\textsubscript{h} transporter has also reported heterogenous Asp binding consistent with two binding modes for Asp (32). The conventional pathway consists of Na\textsuperscript{+} binding to the Na1 and the Na3 sites prior to Asp binding. In the alternate pathway, the binding of Asp takes place after the initial binding of Na\textsuperscript{+} to the Na1 site. The binding of Asp is followed by Na\textsuperscript{+} binding to the Na3 site and then to the Na2 site. In the alternate pathway, we anticipate that initial binding mode of Asp with only Na1 bound is a low affinity binding mode that transitions to a high affinity binding mode when Na\textsuperscript{+} ions bind to the Na3 site. We expect that this alternate pathway (Na1→Asp→Na3→Na2) pathway dominates at low Na\textsuperscript{+} and high Asp concentrations, while the conventional pathway (Na3→Na1→Asp→Na2) dominates at high Na\textsuperscript{+} and low Asp concentrations, the physiological conditions under which GltP\textsubscript{h} operates. The presence of this alternate pathway explains how the transporter can bind Asp at low Na\textsuperscript{+} concentrations. A question to be addressed in future studies is visualizing the alternate mode of Asp binding to GltP\textsubscript{h} that dominates at low Na\textsuperscript{+} concentrations. We anticipate that structural studies of the Apo D40S/GltP\textsubscript{h} transporter with Asp bound should reveal this alternate binding mode. Further, we anticipate that the roles Na\textsuperscript{+} binding to the Na1 and the Na3 site on HP2 movement and the pathways for Asp binding elucidated for GltP\textsubscript{h} in this investigation are also operative in EAATs.
Experimental Procedures

Wild-type GltPh and the variants were expressed in *Escherichia coli* TOP10 cells and the unnatural amino acid PhF$_2$N$_2$ was incorporated using the in vivo nonsense suppression approach as previously described (9, 19, 33, 34). The tyrosine fluorescence based Na$^+$ binding assay, the NMDGT conformational switch assay and the hairpin 2 movement assay were carried out as described in the SI Appendix. EPR spectroscopy and ITC studies were carried out as previously described (18, 22, 35). Detailed descriptions of materials and methods are provided in the SI Appendix.

1. D. Drew, O. Boudker, Shared molecular mechanisms of membrane transporters. Annu. Rev. Biochem. 85, 543-572 (2016).
2. Y. Shi, Common folds and transport mechanisms of secondary active transporters. Annu. Rev. Biophys. 42, 51-72 (2013).
3. N. C. Dankoht, Glutamate uptake. Prog. Neurobiol. 65, 1-105 (2001).
4. R. J. Vandenberg, R. M. Ryan, Mechanisms of glutamate transport. Physiol. Rev. 93, 1621-1657 (2013).
5. J. Jiang, S. G. Amara, New views of glutamate transporter structure and function: Advances and challenges. Neuropharmacology 60, 172-181 (2011).
6. L. M. Levy, O. Warr, D. Attwell, Stoichiometry of the glial glutamate transporter GLT-1 expressed in neurons. Proc. Natl. Acad. Sci. U.S.A. 116, 15939-15946 (2019).
7. A. Alleaume et al., NAD$^+$-dependent gate dynamics and electrostatic attraction ensure substrate coupling in glutamate transporters. Proc. Natl. Acad. Sci. U.S.A. 110, 12486-12491 (2013).
8. N. Reyes et al., The tyrosine movement in single trimeric sodium-coupled aspartate transporters. J. Biol. Chem. 289, 15962-15972 (2014).
9. A. J. Scopelliti, J. Font, R. J. Vandenberg, O. Boudker, R. M. Ryan, Structural characterization reveals insights into substrate recognition by the glutamine transporter ASCT2/SLC1A5. Nat. Commun. 9, 38 (2018).
10. N. Reyes et al., Large domain movements through the lipid bilayer mediate substrate release and inhibition of glutamate transporters. eLife 9, e58417 (2020).
11. I. Hänel, S. Jensen, W. Grundke, Structural flexibility of the glutamate transporter GltPh. J. Biol. Chem. 289, 634-640 (2014).
12. P. I. Valiyaveetil, F. I. Vanlync, O. Boudker, E. Gouaux, Structural transitions along the transport cycle of glutamate transporters. eLife 3, e02283 (2014).
13. D. Ewers et al., Structural flexibility of the glutamate transporter homologue. Proc. Natl. Acad. Sci. U.S.A. 110, 12486-12491 (2013).
14. M. Groeneveld, D. J. Slotboom, Crystal structure of a substrate-free aspartate transporter. Nat. Struct. Mol. Biol. 20, 1224-1226 (2013).
15. G. Verdon et al., Coupled ion binding and structural transitions along the transport cycle of glutamate transporters. eLife 3, e02283 (2014).
16. A. Guskov et al., Aspartate coupling stoichiometry in the glutamate transporter homologue. Biochemistry 54, 6374-6387 (2015).
17. I. Weyand, C. Fahlke, Induced fit substrate binding to an archetypal glutamate transporter homologue. Proc. Natl. Acad. Sci. U.S.A. 110, 12486-12491 (2013).
18. D. Drew, O. Boudker, Shared molecular mechanisms of membrane transporters. Annu. Rev. Biochem. 85, 543-572 (2016).
19. E. A. Roderer, F. I. Valiyaveetil, Investigation of the allosteric coupling mechanism in a glutamate transporter homolog via unnatural amino acid mutagenesis. Proc. Natl. Acad. Sci. U.S.A. 116, 15939-15946 (2019).
20. P. J. Focke, P. Mornie-Loccoz, H. P. Larsson, Opposite movement of the external gate of a glutamate transporter homolog upon binding cotransported sodium compared with substrate. J. Neurosci. 31, 6255-6262 (2011).
21. G. Verdon, O. Boudker, Y. Jin, E. Gouaux, Structure of the glial glutamate transporter GLT-1 expressed in a Chinese hamster ovary cell line selected for low endogenous Na$^+$-dependent glutamate uptake. J. Neurosci. 18, 9626-9648 (1998).
22. G. B. Erkens, I. Hanelt, D. J. Slotboom, Crystal structure of a substrate-free aspartate transporter. Nat. Struct. Mol. Biol. 20, 1224-1226 (2013).
23. D. Yernool et al., Position of the third Na$^+$ site in the aspartate transporter GltPh and the human glutamate transporter, EAAT1. PLoS One 7, e33058 (2012).
24. M. Kawai, R. B. Altman, S. C. Blanchard; O. Boudker, Transport dynamics in a glutamate transporter homologue. Nature 502, 114-118 (2013).
25. G. B. Eken, I. Hanelt, J. M. Goudsmits, D. J. Slotboom, A. M. van Oijen, Unsynchonised subunit motion in single trimeric sodium-coupled aspartate transporters. Nature 502, 119-123 (2013).
26. E. R. Georgiev, P. P. Borbat, C. Ginter, J. H. Freed, O. Boudker, Conformational ensemble of the sodium-coupled aspartate transporter. Nat. Struct. Mol. Biol. 20, 275-281 (2013).
27. I. Hanelt, D. Wunnicke, E. Bordin, M. Hastert, D. J. Slotboom, Conformational heterogeneity of the aspartate transporter GltPh. Nat. Struct. Mol. Biol. 20, 210-214 (2013).
28. D. Drew, O. Boudker, Binding thermodynamics of a glutamate transporter homolog. Nat. Struct. Mol. Biol. 20, 634-640 (2013).
29. M. Groeneveld et al., Aspartate coupling stoichiometry in the glutamate transporter homologue. Biochemistry 54, 6374-6387 (2015).
30. H. Xu et al., Position of the third Na$^+$ site on the aspartate transporter GltPh and the human glutamate transporter, EAAT1. PLoS One 7, e33058 (2012).
31. Z. Huang et al., Identification of the third Na$^+$ site and the sequence of extracellular binding events in the glutamate transporter. Biophys. J. 99, 1416-1425 (2010).
32. K. D. Reddy, D. Ciko, A. J. Scopelliti, O. Boudker, Heterogenous substrate binding in a glutamate transporter homologue. bioRxiv [Preprint] (2021). https://doi.org/10.1101/2021.07.08.045167 (Accessed 14 April 2022).
33. G. Verdon et al., Structure of the third Na$^+$ site and the sequence of extracellular binding events in the glutamate transporter. Biophys. J. 99, 1416-1425 (2010).
34. C. J. Reddy, D. Ciko, A. J. Scopelliti, O. Boudker, Heterogenous substrate binding in a glutamate transporter homologue. bioRxiv [Preprint] (2021). https://doi.org/10.1101/2021.07.08.045167 (Accessed 14 April 2022).
35. D. D. Young et al., An evolved aminooacetyl-tRNA synthetase with atypical polysubstrate specificity. Biochemistry 50, 1894-1908 (2011).
36. O. Boudker, S. Oh, Isothermal titration calorimetry of ion-coupled membrane transporters. Methods 76, 171-182 (2015).