Transport dynamics in a glutamate transporter homologue

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Glutamate transporters are integral membrane proteins that catalyze neurotransmitter uptake from the synaptic cleft into the cytoplasm of glial cells and neurons2. Their mechanism of action involves transitions between extracellular (outward)-facing and intracellular (inward)-facing conformations, whereby substrate binding sites become accessible to either side of the membrane3. This process has been proposed to entail transmembrane movements of three discrete transport domains within a trimeric scaffold4. Using single-molecule fluorescence resonance energy transfer (smFRET) imaging5, we have directly observed large-scale transport domain movements in a bacterial homologue of glutamate transporters. We find that individual transport domains alternate between periods of quiescence and periods of rapid transitions, reminiscent of bursting patterns first recorded in single ion channels using patch-clamp methods6–8. We propose that the switch to the dynamic mode in glutamate transporters is due to separation of the transport domain from the trimeric scaffold, which precedes domain movements across the bilayer. This spontaneous dislodging of the substrate-loaded transport domain is approximately 100-fold slower than subsequent transmembrane movements and may be rate determining in the transport cycle.

In the brain, glutamate mediates excitatory synaptic transmission, responsible for learning, memory formation and cognition1,2. Glutamate transporters are electrochemically driven pumps that maintain a low neurotransmitter background at glutamatergic synapses, allowing for repeated rounds of signalling and preventing excitotoxicity9. The sodium/aspartate symporter from Pyrococcus horikoshii, GltPh, is the only glutamate transporter homologue with known three-dimensional structures of both outward- and inward-facing states10–12. Correspondingly, this system has served as a valuable model for establishing the structural and dynamic underpinnings of the transport cycle10–12. Because GltPh originates from a hyper-thermophilic archaeon, it has a slow turnover time of ~200 s at room temperature13, indicating that the dynamic processes required for transport may fall within the time regime accessible through smFRET imaging10–12.

Crystal structures have shown that GltPh is a homotrimer in which each protomer comprises two domains: a rigid trimerization domain14, which serves as a scaffold and mediates the inter-protomer interactions, and a transport domain, which can move over 15 Å across the bilayer15. High extracellular and low intracellular chemical potentials of sodium (Na+) drive substrate binding and unbinding to the transporter, respectively16. By contrast, large-scale transport domain movements, which serve as a scaffold and mediate the inter-protomer interactions, to hinder local rearrangements accompanying inward transport domain motions14. A K290A mutation was introduced to disrupt a network of polar interactions at the interface between the transport and trimerization domains, to hinder local rearrangements accompanying inward transport domain motions14. Consistent with expectations, we observed dramatic increases and decreases in the low-FRET, outward-facing state (Fig. 1a, Supplementary Fig. 4 and Supplementary Table 1). Such a configuration is consistent with a recent crystal structure of an asymmetric GltPh trimer15. Notably, similar FRET states and FRET state occupancies were obtained when GltPh(N378C) was reconstituted into liposomes (Supplementary Methods, Supplementary Fig. 5).

To validate our FRET state assignments, we introduced mutations distal from the site of fluorophore attachment in GltPh(N378C) to either stabilize or destabilize the outward-facing state (Fig. 1a, Supplementary Fig. 6). A G221A mutation was introduced within the hinge region between the transport and trimerization domains, to hinder local rearrangements accompanying inward transport domain motions14. A K290A mutation was introduced to disrupt a network of polar interactions at the interface between the transport and trimerization domains, to hinder local rearrangements accompanying inward transport domain motions14. Consistent with expectations, we observed dramatic increases and decreases in the low-FRET, outward-facing state population for the G221A and K290A mutants, respectively (Fig. 1f, g). We conclude that transitions between FRET states in GltPh(N378C) reflect motions of the transport domains between outward- and inward-facing orientations. This construct was pursued to examine the effect of ligand binding on conformational dynamics.

In the absence and in the presence of Na+ and Asp, the FRET state values were largely similar, whereas ligands shifted the state occupancies...
in favour of the low-FRET state (Supplementary Fig. 5). Strikingly, individual smFRET traces exhibited much more frequent transitions in the apo compared to the bound transporter (Fig. 2a, b). To quantify the dynamics, we idealized the smFRET trajectories using a model containing three kinetically linked, non-zero FRET states (Methods, Supplementary Fig. 7). The quality of idealizations and the average rates of the conformational transitions were assessed by inspection of individual trajectories as well as transition density plots, which report on the frequencies of transitions between distinct FRET states.

In apo Glt Ph, a comparable number of transitions occurred between low-, intermediate- and high-FRET states with an average frequency of 0.5 s\(^{-1}\) (Fig. 2c), suggesting that the apo transporter samples outward- and inward-facing states relatively rapidly. For the substrate-loaded transporter, the frequency of transitions was reduced by more than an order of magnitude.

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**Figure 1** | FRET efficiency changes reflect relative orientations of the transport domains. 

- **a**: GltPh protomer pairs in symmetrical outward- and inward-facing states viewed within the membrane plane. Trimerization and transport domains are coloured wheat and blue, respectively. Bound Asp and Na\(^{+}\) ions are emphasized as spheres and coloured by atom type. Introduced cysteines are highlighted in cyan with inter-protomer distances above the dotted lines. Magenta arrows mark sites of mutations altering state distributions. 
- **b**: Labeling and surface-immobilization strategies. 
- **c–g**: FRET efficiency population histograms for Asp/Na\(^{+}\)-bound transporters. Introduced mutations are indicated above the panels. The number of molecules analysed (\(n\)) is shown. Population contour plots (left) are colour-coded from tan (lowest) to red (highest population) with the colour scale shown beside the graphs. The cumulative population histogram (right) displays the time-averaged values and standard deviations. The solid black lines are fits to the sums of individual Gaussian functions (red lines).

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**Figure 2** | Dynamics in the apo and substrate bound transporter.

- **a, b**: Shown are smFRET trajectories (blue), acquired for apo (a) and Na\(^{+}\)/Asp-bound (b) GltPh(N378C). Overlaid are idealizations generated in QuB (red). Arrows mark population averages for the low- (L), intermediate- (I) and high- (H) FRET efficiencies. Data in panel b were collected using 400 ms integration time. 
- **c, d**: Transition density plots for the apo (c) and Na\(^{+}\)/Asp-bound (d) transporters show that transitions occur between three distinct FRET states (L, I and H) with an average frequency of ~0.5 s\(^{-1}\) and ~0.02 s\(^{-1}\), respectively. Initial and final FRET values for each transition are accumulated into two-dimensional histograms. Colour scale is from tan (lowest frequency) to red (highest frequency).
order of magnitude (0.02 s\(^{-1}\)). Under these conditions, we primarily observed transitions between low- and intermediate-FRET states (Fig. 2d), consistent with transport domains moving inward one at a time. The paucity of direct transitions from low- to high-FRET states in the presence and in the absence of Na\(^+\) and Asp is in line with previous functional studies\(^{12,21}\), suggesting that individual protomers undergo conformational transitions, and thus function independently of each other.

To assess the concentration dependence of the ligand-induced effects, we performed two titrations on GlpN378C: a Na\(^+\) titration in the presence of 10 \(\mu\)M Asp and an Asp titration in the presence of 2 mM Na\(^+\). At the population level, we observed a gradual stabilization of the low-FRET, outward-facing GlpN configuration as concentrations of both ligands increased (Fig. 3). Consistent with previous bulk experiments\(^{16}\), these binding isotherms yielded apparent dissociation constants (\(K_D\)) of approximately 2 mM for Na\(^+\) and 7 \(\mu\)M for Asp (Supplementary Methods). The titration data also showed a gradual decrease in the average transition frequency. At intermediate Asp concentrations, smFRET trajectories showed molecules reversibly switching between periods of quiescence and periods of rapid transitions, probably due to apo protomers (Fig. 4a, top panel). Increased substrate concentrations shortened the durations of the dynamic periods and increased the lifetimes of the stable states (Fig. 4a, lower panels; Fig. 4b). The midpoints of these changes (~10 \(\mu\)M) were consistent with the \(K_D\) of Asp binding obtained from the population data. From the apparent lifetimes of the dynamic and non-dynamic modes, we estimate the ligand binding and dissociation rates of \(k_{\text{on}}\) \(\approx 10^3\) M\(^{-1}\) s\(^{-1}\) and \(k_{\text{off}}\) \(\approx 0.1\) s\(^{-1}\), respectively, at 2 mM Na\(^+\) (Supplementary Methods). A substrate residence time of 10 s is consistent with previous estimates\(^{15}\).

Remarkably, dynamic and non-dynamic periods were also observed in the absence of both Na\(^+\) and Asp. Visual inspection of smFRET trajectories revealed molecules in which (1) rapid dynamics persisted throughout the entire observation periods; (2) stable FRET states predominated; and (3) dynamic and non-dynamic periods were reversibly visited (Supplementary Fig. 8). Consequently, biphasic FRET state lifetimes were observed (Supplementary Fig. 9), wherein the longer dwell times reflected the durations of non-dynamic periods and the shorter dwell times reflected the lifetimes of the FRET states during rapid dynamics. On average, dynamic and non-dynamic periods were approximately equally represented (Fig. 4b). The addition of Na\(^+\) and Asp shortened the dynamic periods. However, periods of dynamics persisted, even as the substrate binding sites became saturated (Fig. 4b). In the presence of 200 mM Na\(^+\) (a Na\(^+\) concentration at which the \(K_D\) for Asp is \(\approx 1\) nM (ref. 10)) and 100 \(\mu\)M Asp, when we expect the fraction of the unbound transporters to be 1 in 10\(^5\), we still observed brief dynamic periods. Such bursts were typified by one or two transient excursions to an inward-facing state with an average dwell time of 1 s (Fig. 4c and Supplementary Fig. 8), before returning to outward-facing conformations (Fig. 4d). ‘Flickers’ of this kind occurred every ~200 s and constituted about half of all transitions observed out of the outward-facing, low-FRET state. The remainder of the transitions out of the low-FRET state lead to long-lasting intermediate- or high-FRET states (Supplementary Fig. 8). To confirm that such flickers were due to the rapid transitions of the substrate-loaded transport domains, we performed identical experiments in the presence of saturating concentrations of d,l-threo-\(\beta\)-benzyloxyaspartate (TBOA)\(^{22}\), which binds to the substrate-binding site in a manner that blocks transport\(^{16}\). Compared to Asp, TBOA reduced flickers by >80% (Fig. 4e), suggesting that these excursions reflect translocation of Asp across the bilayer. Notably, similar dynamic signatures were also observed for GlpN, within the context of proteoliposomes (Supplementary Fig. 8).

We conclude that dynamic heterogeneity, which manifests kinetically as periods of quiescence punctuated by periods of rapid transitions, is an intrinsic property of GlpN, arising from spectroscopically hidden isomerization of the protein\(^{23}\). We posit that quiescent periods of substrate-bound GlpN reflect stable conformations that closely resemble crystal structures of the outward- and inward-facing states\(^{5,9}\). In these states, the transporter and trimerization domains are closely packed. Dynamic periods reflect transmembrane movements of individual transport domains. These movements are enabled by a critical protein isomerization, which involves dislodging of the transport domain from the trimeric scaffold (Supplementary Fig. 10a). Such an ‘unlocked’ configuration has been captured crystallographically\(^{15,17}\) showing disrupted packing and polar interactions at the domain interface. Consistent with this model, the interfacial K290A mutation (Supplementary Fig. 6) dramatically increases the population of molecules in the dynamic mode (Supplementary Fig. 10b–e). The existence of unlocked intermediates with potentially increased hydration at the domain interface\(^{24}\) may help explain the well known capacity of glutamate transporters and GlpN to catalyse bidirectional anion fluxes\(^{25,26}\). Our data also show that binding of Na\(^+\) and Asp significantly increases the lifetimes of stable outward- and inward-facing states, suggesting that there is an allosteric coupling between the substrate-binding site and the domain interface.

Mammalian glutamate transporters are architecturally similar to GlpN, and their transport cycle is anticipated to proceed through similar intermediates. Current kinetic models for the mammalian transporters suggest that substrate translocation is relatively fast\(^{27,28}\). However, in GlpN, we find that rapid translocation is preceded by a slow isomerization step that occurs on a timescale comparable to the transporter turnover rate ~0.005 s\(^{-1}\) (every 200 s). Therefore, under our experimental conditions, isomerization of the substrate-loaded GlpN may constitute a key, rate-limiting step in the transport cycle, whereas return of the unloaded transport domain to an outward-facing state is relatively fast (~0.5 s\(^{-1}\)). The apparent differences between GlpN and the mammalian transporters may, at least in part, be because only ensemble properties have thus far been obtained in the latter system. Here, the application of the glutamate substrate to cell membranes expressing the mammalian transporters induced transient electric currents decaying to

**Figure 3** | Na\(^+\) ions and Asp favour the outward facing state. Populations of low- (top, green), intermediate- (middle, red) and high- (bottom, blue) FRET states as a function of Na\(^+\) ions added in the presence of 10 \(\mu\)M Asp (a) and Asp added in the presence of 2 mM Na\(^+\) (b). The titrations yielded dissociation constants (\(K_D\)) of 1.6 ± 0.3 mM and 6.5 ± 2.5 \(\mu\)M and Hill coefficients of 1.3 ± 0.3 and 0.9 ± 0.3, respectively. Shown are averages and standard deviations from at least three independent data sets (each containing at least 250 molecules). A few error bars were too small to be clearly visible. Solid lines through the data points are the results of global fitting of the data to the Hill equation.
Protein samples at 40 μM Asp and 100 μM Asp. Expanded views of the flicker events (shaded in pink) are shown below the trace. d, Survival plot of the observed flickers. Solid line is a fit to a single exponential decay. e, Transition density plots for flicker events in saturating Asp (left) or TBOA (right). Average transition frequencies are 0.02 s⁻¹ and <0.005 s⁻¹, respectively. Data in panels d and e were collected with 400 ms integration time.

100 and 25 μM for 30 min at room temperature. Labelled proteins were purified away from the excess reagents by size exclusion chromatography, and their purity and specificity of labelling were assessed by SDS–PAGE followed by fluorescent imaging and Coomassie staining. The labelled proteins were reconstituted into liposomes and their Asp uptake activity measured as previously described⁶⁻⁷. For smFRET experiments, labelled proteins were surface-immobilized through the biotin-streptavidin bridge within passivated, streptavidin-derivatized microfluidic devices as previously described⁸ and imaged using a home-built total internal reflection fluorescence microscope under oxygen-scavenging conditions⁹. Determination of FRET efficiencies and selection of traces were performed using automated analysis software developed in the laboratory⁹.

Full Methods and any associated references are available in the online version of the paper.

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METHODOLOGY SUMMARY

GltPh mutants were generated within a GltPh variant lacking cysteines in which seven unconserved residues have been replaced with histidines resulting in improved chromatography⁹. Protein samples at 40 μM were labelled with a mixture of malémide-activated Cy3, Cy5 and biotin–(PEG)₁₁ at respective concentrations of 50, and 100 μM Asp. Expanded views of the flicker events (shaded in pink) are shown below the trace. d, Survival plot of the observed flickers. Solid line is a fit to a single exponential decay. e, Transition density plots for flicker events in saturating Asp (left) or TBOA (right). Average transition frequencies are 0.02 s⁻¹ and <0.005 s⁻¹, respectively. Data in panels d and e were collected with 400 ms integration time.

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Author Contributions N.A. purified GltPh mutants, carried out the experiments and analysed the data. R.B.A. prepared reagents for smFRET experiments. N.A., O.B. and S.C.B. together designed, analysed and interpreted the experiments and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.C.B. (scb2005@med.cornell.edu) or O.B. (olb2003@med.cornell.edu).
METHODS

DNA manipulations, protein expression, purification and labelling. Single cysteine mutations were introduced within a GltPh variant lacking cysteines, in which seven unconserved residues have been replaced with histidines resulting in improved expression levels (termed GltPh hereafter for brevity)10, using a QuickChange kit (Stratagene). Constructs were verified by DNA sequencing and transformed into E. coli DH10-B cells (Invitrogen). Proteins were expressed as C-terminal (His)8 fusions as described previously21,22. Briefly, isolated cell membranes were resuspended in buffer A containing 20 mM HEPES/NaOH, pH 7.4, 200 mM NaCl, 0.1 mM l-aspartate, 0.1 mM Tris (2-carboxyethyl)phosphine (TCEP). Membranes were solubilized in the presence of 40 mM n-dodecylβ-D-maltopyranoside (DDM) for 1 h at 4°C. Solubilized transporters were purified by metal-affinity chromatography in buffer A supplemented with 1 mM DDM and eluted in 250 mM imidazole. The (His)8-tag was cleaved by thrombin and proteins were further purified by size exclusion chromatography in buffer A supplemented with 1 mM DDM. GltPh was labelled at a concentration of 40 μM in buffer A with a mixture of maleimide-activated Cy3, Cy5 and biotin-(PEG)4 at 50, 100 and 25 μM final concentrations, respectively (molar ratio of ~1:2:0.5). Labelled proteins were quenched with 10 mM 2-mercaptoethanol and subsequently purified away from excess reagents by size exclusion chromatography. The extent of labelling was determined by measuring absorbance at 552 nm and 650 nm for Cy3 and Cy5, respectively.

Protein reconstitution into liposomes and transport assays. Labelled and unlabelled GltPh variants were reconstituted into liposomes and assayed as previously described10,12. Briefly, liposomes, prepared from 3:1 (w/w) mixture of E. coli total lipid extract and egg yolk phosphotidylcholine (Avanti Polar Lipids) in a buffer containing 20 mM Tris/HEPES, pH 7.4 and 200 mM KCl, were destabilized by addition of Triton X-100 at a detergent to lipid ratio of 0.5:1 (w/w). For transport assays, proteins were added at final protein to lipid ratio of 1:400 (w/w) and addition of Triton X-100 at a detergent to lipid ratio of 0.5:1 (w/w). For transport assays, the passivated microfluidic imaging chambers were prepared and the microfluidic channel was prepared by incubating with a buffer solution containing 10 mM Tris/acetate, pH 7.5 and 50 mM KCl, 1 μM BSA, 1 μM 25 nucleotide DNA duplex, 0.8 μM streptavidin (Invitrogen) and 0.1% (v/v) glycerol for 5 min. The channel was then rinsed thoroughly in buffer A with 1 mM DDM. All imaging experiments were performed in buffer A (unless otherwise stated), supplemented with 1 mM DDM, 2 mM cyclooctatetraene (Sigma), 5 mM 2-mercaptoethanol, an enzymatic oxygen scavenger system comprising 1 U per ml glucose oxidase (Sigma), 8 U per ml catalase (Sigma) and 0.1% glucose3. If not otherwise specified, all data were collected at an imaging rate of 10 s⁻¹ (100 ms integration time).

Analysis of smFRET data. Fluorescence trajectories were selected for analysis using custom-made MATLAB- (MathWorks) encoded software22,23 according to the following criteria: a single catastrophic photobleaching event; >8:1 signal-to-background noise ratio; a FRET lifetime of at least 15 frames. FRET trajectories were calculated from the acquired intensities, I_{Cy3} and I_{Cy5}, using the formula FRET = I_{Cy5}/(I_{Cy3} + I_{Cy5}). Population contour plots were constructed by superimposing the FRET data from individual traces. Histograms of these population data were fitted to the sum of three Gaussian functions for all mutants, except K55C, for which only a single Gaussian function was used. The Gaussian means, widths and amplitudes were optimized in Origin (OriginLab). The relative populations, the dwell times of each FRET state and the transition frequencies between states were obtained by idealizing the smFRET traces using Qub22,23 (Supplementary Methods). Transition density plots were generated as previously described24. The global fits of the titration curves to Hill equations were performed in Prism24 (Supplementary Methods). The dwell time survival plots were fitted to exponential decay functions and the logarithmic histograms of the dwell times were fitted to transformed probability density functions24, respectively, using Origin (Supplementary Methods).

Preparation of structural figures. All structural renderings were generated using PyMOL (version 1.5.0.4, Schrödinger) and coordinates deposited in Protein Data Bank: accession number 2NWX (ref. 10) for the outward-facing state and 3KBC (ref. 3) for the inward-facing state.

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