Transport domain unlocking sets the uptake rate of an aspartate transporter

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Glutamate transporters terminate neurotransmission by clearing synaptically released glutamate from the extracellular space, allowing repeated rounds of signalling and preventing glutamate-mediated excitotoxicity. Crystallographic studies of a glutamate transporter homologue from the archaean Pyrococcus horikoshii, GltPh, showed that distinct transport domains translocate substrates into the cytoplasm by moving across the membrane within a central trimerization scaffold. Here we report direct observations of these ‘elevator-like’ transport domain motions in the context of reconstituted proteoliposomes and physiological ion gradients using single-molecule fluorescence resonance energy transfer (smFRET) imaging. We show that GltPh bearing two mutations introduced to impart characteristic intracellular (inward) and extracellular (outward) substrate binding sites (Fig. 1a). Comparison of GltPh structures captured in distinct conformations suggests that within the trimerization scaffold, individual transport domains undergo relocations approximately 15 Å perpendicular to the membrane, providing substrate and ion access to the extracellular (outward) and intracellular (inward) solutions. Crystal structures of GltPh revealed that the homotrimeric protein is composed of a rigid, central trimerization scaffold that supports three peripheral transport domains containing the substrate binding sites (Fig. 1a). Comparison of GltPh structures captured in distinct conformations suggests that within the trimerization scaffold, individual transport domains undergo relocations approximately 15 Å perpendicular to the membrane, providing substrate and ion access to the extracellular (outward) and intracellular (inward) solutions (Extended Data Fig. 1a).

Single-molecule imaging of GltPh provided direct evidence for bidirectional elevator-like transport domain motions9,10. Consonant with findings obtained using double electron-electron spin resonance (DEER) spectroscopy11,12, these measurements also showed that individual GltPh transport domains transition spontaneously between outward- and inward-facing conformations both when free of cargo (apo) and when bound to substrates. These transport domain motions exhibited heterogeneous dynamic behaviours, alternating between periods of rapid transitions and periods of quiescence, where the protein rests in either outward- or inward-facing states. In contrast to observations in structurally unrelated neurotransmitter sodium symporters13, substrate binding decreased transport domain dynamics in GltPh, by favouring the quiescent periods such that the frequency of domain motions converged to the substrate uptake rate6. These findings led to the hypothesis that GltPh configurations observed in crystal structures6,4, showing tight lock-and-key interactions between transport and trimerization domains, represent quiescent locked states with high substrate affinity, whereas the short-lived states sampled during dynamic periods are structurally distinct and likely have intrinsically lower substrate affinity (Extended Data Fig. 1b). This model posits that transport domain motions require a rate-limiting, structural unlocking process that changes the interface between the transport and trimerization domains, probably enabling solvent penetration into that interface9,14.

To assess the relationship between GltPh function, dynamics and structure, we employed smFRET imaging in the context of reconstituted proteoliposomes with physiological ion gradients. We compared wild-type GltPh to a gain-of-function, humanized (H) mutant R276S/M395R (H276,395-GltPh), which through unknown mechanisms exhibits a faster rate of substrate uptake15. The smFRET experiments revealed that the mutations destabilized quiescent locked states. The resulting increase in dynamics paralleled a decreased affinity for substrate and an increased transport rate. Crystallographic analyses supported this observation, showing that the transport domains of H276,395-GltPh can adopt inward-facing conformations in which the transport domain-trimerization scaffold interface is strikingly more open than previously observed. Computational modelling further suggested that increased solvation by lipid or detergent hydrophobic tails in this interface probably facilitates the formation of such conformations. These observations provide a structural rationale for functional distinctions between GltPh and the human EAATs, and establish a kinetic framework for understanding how regulation can be achieved.

Experimental design

GltPh is a structural homologue of EAATs (~35% sequence identity) that preferentially transports aspartate over glutamate, with higher substrate binding affinity and slower uptake rate. It has been suggested...

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that these distinctions may stem, in part, from the differential location of a conserved arginine residue\(^ {16} \) that is proximal both to the substrate-binding site and the transport domain–trimerization scaffold interface. Although the location of this arginine can differ in the primary sequences of glutamate transporter homologues, its position is conserved in most family members (Fig. 1a, b, and Extended Data Fig. 1c). In human EAAT1, moving this arginine from transmembrane segment (TM) 8 to helical hairpin (HP) 1 (where it is located in Glt Ph), strikingly increases substrate affinity and decreases uptake rate\(^ {15} \). Reciprocal mutagenesis of GltPh, whereby the arginine is moved from HP1 to TM8 (R276S/M395R), reduces aspartate affinity and increases the transport rate\(^ {15} \).

We took advantage of this gain-of-function mutant to probe correlations between uptake rate and transport domain dynamics. For smFRET measurements, reconstitution procedures were established to yield maximally one GltPh trimer per vesicle. Proteoliposomes were immobilized via biotinylated, fluorescently labelled Glt Ph within passivated quartz microfluidics chambers activated with a biotin–streptavidin bridge (Fig. 1d). Using this strategy, only those proteoliposomes containing Glt Ph oriented with the extracellular side facing the vesicle exterior were immobilized and imaged\(^ {20} \). Imaging experiments were initiated in the absence of substrates under isoelectric conditions and chemical gradients were established by rapidly exchanging the detergent micelles that afford higher signal-to-noise ratios and increased sample size.

**Transport rate and dynamics are correlated**

In both the absence and presence of gradients, wild-type Glt Ph, in proteoliposomes showed spontaneous transitions between low-, intermediate- and high-FRET efficiency states centred at \( \sim 0.4, \sim 0.6 \) and \( \sim 0.9 \), respectively (Fig. 1e, f and Extended Data Fig. 3). In detergent solutions, these FRET states were assigned to specific Glt Ph configurations: the low-FRET state reflects symmetric outward-facing and asymmetrically outward- and inward-facing configurations; intermediate- and high-FRET states reflect, respectively, asymmetrically inward- and outward-facing and simultaneously inward-facing proteoliposomes (Extended Data Fig. 2). In line with previous investigations\(^ {11,12} \), population FRET data from hundreds of individual proteins in the absence of gradients show that the transporter occupies the outward-facing, low-FRET state about half of the time in both detergent (46%) and lipid vesicles (54%) (Fig. 2a, Extended Data Fig. 3b and Extended Data Table 1a, b).
Transitions between low- and higher-FRET states reflect elevator-like movements of the individual transport domains between outward- and inward-facing configurations, respectively. In proteoliposomes, such transitions occurred at a rate of \( \sim 0.2 \, \text{s}^{-1} \), roughly twofold less frequently than in detergent (Fig. 2a and Extended Data Fig. 3c). Paralleling the effects of substrate binding to wild-type GltPh in detergent (Fig. 2b), a modest population shift towards the outward-facing, low-FRET state occurred under active transport conditions achieved by addition of Na\(^+\) and aspartate (Extended Data Fig. 3b and Extended Data Table 1a, b). Na\(^+\) and aspartate also reduced transport domain dynamics by tenfold to \( \sim 0.02 \, \text{s}^{-1} \) (Fig. 1f and Extended Data Fig. 3c). Thus, in the presence of chemical gradients, the frequency of transitions from outward- to inward-facing state \( (\sim 0.01 \, \text{s}^{-1}) \) mirrored the rate of radioactive substrate uptake \( (\sim 0.007 \, \text{s}^{-1}) \) (Fig. 1c).

Notably, the \( H_{276,395}\)-GltPh mutant only exhibited transitions between low- \( (0.4) \) and a single, higher- \( (0.65) \) FRET state in both proteoliposomes and detergent (Figs 1f and 2c and Extended Data Fig. 3). Similar to the wild-type protein in the absence of chemical gradients, the low-FRET state was occupied 60% of the time in detergent micelles (Extended Data Table 1a, b). The observed FRET transition frequency for \( H_{276,395}\)-GltPh was also two times slower in proteoliposomes \( (\sim 0.13 \, \text{s}^{-1}) \) compared to detergent \( (\sim 0.22 \, \text{s}^{-1}) \) (Fig. 2d and Extended Data Fig. 3c).

However, in stark contrast to the wild-type protein, the transition frequency in \( H_{276,395}\)-GltPh decreased by less than twofold to \( \sim 0.1 \, \text{s}^{-1} \) when transport-supporting chemical gradients were established (Fig. 1e, f and Extended Data Fig. 3c). Here again, the frequency of transitions from the outward- to inward-facing FRET state \( (\sim 0.05 \, \text{s}^{-1}) \) converged to the measured rate of substrate uptake \( (\sim 0.03 \, \text{s}^{-1}) \) (Fig. 1c). The quantitative correspondence observed between the rates of smFRET transitions and uptake for the wild-type and \( H_{276,395}\)-GltPh mutants provides compelling evidence that elevator-like motions of transport domains mediate solute uptake and are critical steps of the transport cycle. This finding was independent of the proteoliposome immobilization strategy used and valinomycin-mediated electrical potentials (Extended Data Fig. 4a–d).

\( H_{276,395}\)-GltPh visits distinct inward-facing states

In contrast to wild-type GltPh, which samples intermediate- \( (0.6) \) and high- \( (0.9) \) FRET states, \( H_{276,395}\)-GltPh samples only a single higher-FRET \( (0.65) \) configuration (Extended Data Fig. 3). No excursions into the 0.9 FRET state were observed even when data were collected at sixfold higher time resolution \( (15 \, \text{ms}) \) (Extended Data Fig. 4e, f). The absence of the 0.9 FRET state would be expected if only one proteomer within the \( H_{276,395}\)-GltPh trimer transitioned into inward-facing configuration at a time, while the formation of symmetric inward-facing states was disallowed. This model is, however, inconsistent with data showing that individual transport domains function independently.

An alternative hypothesis is that the inward/outward and inward/inward configurations in \( H_{276,395}\)-GltPh exhibit altered, overlapping FRET values. If this model is correct, then the gain-of-function mutations in \( H_{276,395}\)-GltPh have altered the nature of the elevator-like transport domain motions and the structure of the inward-facing state.

The energy landscape of \( H_{276,395} \)–GltPh is altered

\( \text{Na}^+ \) and aspartate significantly stabilized the higher-FRET state of \( H_{276,395}\)-GltPh in detergent micelles (Fig. 2c and Extended Data Table 1a, b). In detergent, \( \text{Na}^+ \) and aspartate have access to both the extracellular and cytoplasmic sides of the protein. Assuming that a binding equilibrium is established in each conformation, these observations suggest that substrates bind more tightly to the inward-facing \( H_{276,395}\)-GltPh conformation. Such a response was not observed for the wild-type GltPh, where substrate affinities of the inward- and outward-facing conformations are nearly the same and ligands stabilize the latter only slightly (Fig. 2a and Extended Data Table 1). Notably, the transporter blocker DL-threo-β-benzylxoyaspartate (TBOA)\(^-\) stabilized the outward-facing low-FRET states of both wild-type and \( H_{276,395}\)-GltPh (Fig. 2 and Extended Data Fig. 5a, b). As above, this suggests that TBOA preferentially binds to the outward-facing state of both isoforms. Results consistent with these findings were obtained from ensemble DEER measurements using the protein spin-labelled on the same residue (Extended Data Fig. 5c).

Interestingly, the addition of \( \text{Na}^+ \) and aspartate to \( H_{276,395}\)-GltPh proteoliposomes led to an increase in the outward-facing, low-FRET...
Notably, H276,395-GltPh exhibited an ~1,000-fold increase in apparent substrate dissociation constant (Kd) compared to the wild-type protein (Fig. 3 and Extended Data Fig. 6a). This finding was corroborated by bulk measurements (Extended Data Fig. 6b, c). Hence, the H276,395-GltPh mutations affect both transport domain dynamics and substrate affinity, even though neither of the mutated residues coordinates aspartate directly in the existing crystal structures. These observations support the hypothesis that substrate binding and transport domain dynamics are physically coupled.

Locked states are destabilized in H276,395-GltPh

The coexistence of quiescent and dynamic periods evidenced both in the absence and presence of ligands is a hallmark kinetic feature of wild-type GltPh. Binding of Na⁺ and aspartate increases the prevalence of quiescent periods and thus the average FRET state lifetimes (Fig. 4a, b). Strikingly, no evidence was found for quiescent periods in the H276,395-GltPh mutant (Fig. 4c) and rapid transport domain dynamics persisted even in the presence of saturating substrate concentrations (Fig. 4d and Extended Data Table 1c). These dynamic processes were efficiently blocked by TBOA (Fig. 2b, d), consistent with its putative role in substrate transport. In H276,395-GltPh, substrate binding increased the lifetime of the high-FRET state (~sevenfold), with no detectable impact on the low-FRET state lifetime (Fig. 4d). In both the absence and presence of ligands, the low- and higher-FRET state lifetimes were unimodal (Fig. 4c, d and Extended Data Table 1c). These findings suggest that in H276,395-GltPh, the isomerization steps leading to locked configurations of the wild-type protein are strikingly altered or inaccessible under the conditions examined, although an allosteric coupling between substrate binding and stabilization of the domain interface still exists.

Structure of the inward-facing H276,395-GltPh

To probe the underpinnings of the altered properties of H276,395-GltPh, we determined a crystal structure of the protein bound to Na⁺ ions and aspartate at a moderate resolution of ~4.5 Å (Extended Data Fig. 7). As expected from smFRET experiments (Fig. 2c), the structural model clearly showed that all protomers in the trimer spontaneously adopted

Figure 4 | Unimodal dynamic behaviour of H276,395-GltPh. a–d, Dwell time distributions (a, c) and average dwell times (b, d) for the low- (low, solid lines) and intermediate- and high-FRET states (int + high, dashed lines) obtained for the wild type (a, b) and H276,395-GltPh (c, d) in detergent. The distributions for apo (blue) and Na⁺/aspartate-bound proteins (red) were fitted to a probability density function. The fitted time constants are in Extended Data Table 1c. Average dwells are plotted as functions of Na⁺ concentration in the presence of 10 and 100 μM aspartate for wild type and H276,395-GltPh, respectively. Solid lines are fits to Hill equation with Kd = 15 mM and n = 3.2 for wild type and Kd = 19 mM and n = 3.2 for H276,395-GltPh. The data points shown are averages and standard errors from three independent biological replicates.
inward-facing configurations. The model also revealed that the transport domain’s orientations differed from those previously captured in GltPh structures, both with and without stabilizing crosslinks\(^5\). Moreover, the trimer was asymmetric, with the transport domain of protomer A occupying a position distinct from the other two.

In protomer A, the transport domain shifted further inward by 2 Å and rotates by 7° around an axis roughly perpendicular to the membrane plane with respect to the wild type (Fig. 5a). This rearrangement is accommodated by a concerted movement of helices in the scaffold domain, comprising TM1 and peripheral portions of TM2 and TM5 (Extended Data Fig. 7c), whose flexible nature was already noted\(^1\). This conformation resembles the inward-facing, locked state of the wild type\(^4\) in the close packing observed between the transport domain and the trimerization scaffold (Extended Data Fig. 7d). Molecular dynamics simulations revealed that whereas Arg 276 in the wild type forms hydrogen bonds with Asp 394 and bulk water molecules, the corresponding Arg 395 in H\(_{276,395}\)-GltPh faces the hydrophobic core of the bilayer.

The resulting membrane remodelling is driven by the hydrophobic matching force\(^25,26\), and is established by interactions of the Arg 395 with H276,395-GltPh, which can destabilize the inward-facing, locked conformation and increase water accessibility to the substrate-binding site and to the domain interface (Fig. 5b).

In protomers B and C, the transport domains undergo identical and more striking changes (Fig. 5a), each swinging away from the trimerization scaffold by about 12° compared to locked protomer A. Consequently, a large crevice opens between HP2 and the scaffold, reducing the interface between the transport and scaffold domains from \(~1,300\) Å\(^2\) to \(~900\) Å\(^2\) and allowing access to water, detergent or lipid molecules (Fig. 5c). This unusual, apparently unkotted, conformation was observed in two protomers occupying distinct crystal packing environments and therefore seems to be determined by the properties of the protein itself and not by crystal contacts. The crevice it generates is largely hydrophobic, and closes rapidly in molecular dynamics simulations when solvated only by water (Extended Data Fig. 9a–c). In contrast, the open interface between transport and trimerization domains is stable with lipids positioned in this space (Extended Data Fig. 9d–g), suggesting that solvation by lipid or detergent molecules is necessary. Notably, this crevice may allow HP2, whose gating role in the outward-facing state is well-established\(^3,14\), to open when the transport domain is inward facing (Fig. 5c). If so, the substrate release might be facilitated in the unlocked conformation, a notion consistent with the markedly reduced substrate affinity of this mutant.

**Discussion**

Conformational transitions between outward- and inward-facing states are key events in transport cycles of secondary active transporters\(^27,28\). In glutamate transporters and possibly other families\(^29–31\), such transitions involve elevator-like movements of the substrate-binding domains supported by relatively rigid scaffold domains. The frequency of such transitions in Gltp9, in lipid bilayers and in the presence of physiological ionic gradients parallels the turnover rate of substrate uptake. This relationship also holds in a gain-of-function mutant H\(_{276,395}\)-GltPh that exhibits a 1,000-fold decreased substrate affinity and a fourfold faster uptake rate. Collectively, our observations establish a direct correlation between the transport domain movements and substrate transport, and suggest an inverse relationship between substrate affinity and transport domain motions. The H\(_{276,395}\)-GltPh mutant is special in this regard, as other point mutations impact dynamics only and do not potentiate transport\(^6\).

The observed dynamic signatures strongly suggest that the rate-limiting step in this process is the unlocking of the transport domain from the trimerization scaffold (Fig. 6a). Although both the wild-type and the H\(_{276,395}\)-GltPh proteins exhibit similar transport domain structures and translocate similarly positioned charged groups (including Arg 276 in the wild type and Arg 395 in the mutant), locked states are relatively unstable in the H\(_{276,395}\)-GltPh mutant, leading to overall faster dynamics and uptake.

The locked and unlocked configurations of wild-type Gltp9, corresponding to quiescent and dynamic periods, respectively, coexist and interconvert spontaneously, which suggests that outward- and inward-facing states of Gltp9—and by extension EAA Ts—should be viewed as structurally heterogeneous ensembles. Increased quiescent period durations in the presence of substrate further suggest that ligand binding is allosterically coupled to the formation of locked states\(^7\). Based on these insights, we propose a simplified kinetic framework for the transport cycle that recapitulates the most salient experimentally observed features (Fig. 6b, Extended Data Fig. 10). The specific relationship of crystallographic snapshots of Gltp9 and related proteins to the topological features of this framework will need to be examined carefully.

The structure of H\(_{276,395}\)-GltPh (Fig. 6c) captures an unlocked configuration that appears relevant to the proposed transport cycle and uniquely suitable for ligand binding and release. Although the molecular basis of how the mutations in H\(_{276,395}\)-GltPh affect the locked–unlocked isomerization requires further investigation, molecular dynamics simulations suggest that protein–lipid interactions are pivotal (Extended Data Fig. 9). The proposed role for the lipid hydrophobic tails in facilitating domain unlocking complements previous hypotheses that transient interface hydration facilitates transport domain translocation\(^1,14\).
That two closely related GLT1p isoforms exhibit distinct kinetic and structural signatures forewarns the possibility that human EAATs differ substantially from GLT1p, especially in their dynamic properties. Probing EAATs directly is therefore essential, particularly since the extent to which they might be diverted to kinetically stable, potentially off-pathway states may represent a regulatory modality.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Sample manipulations. No statistical methods were used to predetermine sample size. DNA manipulations, protein expression, purification and labelling. Single cysteine mutations were introduced by site-directed mutagenesis (Stratagene) of a cysteine-less GluPh background, in which seven non-conserved residues had been replaced with histidines resulting in improved expression levels (termed GluPh here for brevity). Constructs were verified by DNA sequencing and transformed into E. coli DH10-B cells (Invitrogen). Proteins were expressed as C-terminal (His)4 fusions as described previously. Briefly, isolated cell membranes were re-suspended in buffer A, containing 20 mM HEPES/NaOH, pH 7.4, 200 mM NaCl, 0.1 mM l-ascorbate, 0.1 mM Tris(2-carboxyethyl)phosphine (TCEP). Membranes were solubilized in the presence of 40 mM n-dodecyl β-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)4-tag was cleaved by thrombin and proteins were further purified by size-exclusion chromatography (SEC). For smFRET experiments, protein samples at 40 μM were labelled with a mixture of maleimide-activated Cy3 and Cy5 dyes that exhibit enhanced photo-stability, as well as biotin–PEG21, at concentrations of 50, 100 and 25 μM, respectively, for 30 min at room temperature. Labelled proteins were purified away from the excess reagents by SEC. Their purity and specificity of labelling were assessed by SDS–PAGE, which was followed by fluorescence imaging and Coomassie staining.

Protein reconstitution into liposomes for smFRET analysis and transport assays. Labelled and unlabelled GluPh variants were reconstituted into liposomes as previously described. Briefly, liposomes, prepared from 3:1 (w/w) mixture of E. coli total lipid extract and egg yolk phosphatidylcholine (Avanti Polar Lipids), in a buffer containing 20 mM Tris/HEPES, pH 7.4 and 100 mM KCl, were destabilized by addition of Triton X-100 at a detergent to lipid ratio of 0.5:1 (w/w). For reconstitution, proteins were added to lipids at final protein to lipid ratio of 1:1,000 (w/w) and incubated for 30 min at room temperature. Detergents were removed by repeated incubations with Ribolins as described. For smFRET and radioactive substrate uptake experiments, the same proteoliposomes were extruded through 0.22 μm filters. This reconstitution strategy yields at most 1 and 16 GluPh trimers per vesicle, respectively. Radioactive substrate uptake was measured as previously described. Briefly, proteoliposomes were diluted into reaction buffer containing 20 mM Tris/HEPES, pH 7.4, 100 mM NaCl and 0.3 μM [3H]-l-aspartate at room temperature. Aliquots were removed at appropriate times, diluted in ice-cold quenching buffer (20 mM Tris/HEPES, pH 7.4, 100 mM LiCl) and filtered through 0.22 μm filters (Millipore). Protein concentration was estimated by the absorbance at 280 nm after correcting for the fluorophore contributions to the value. The amount of substrate uptake was normalized per mole of GluPh, monomers.

smFRET experiments. All experiments were performed using a home-built, prism-based total internal reflection fluorescence microscope constructed around a Nikon TE2000 Eclipse inverted microscope body using streptavidin-coated, passivated microfluidic imaging chambers. Except when stated otherwise, labelled proteins (either detergent solubilized or liposome-reconstituted) were surface-immobilized via a biotin–streptavidin bridge. Except when stated otherwise, imaging experiments were performed in a buffer containing: 20 mM HEPES/Tris (pH 7.4), 5 mM BME, an enzymatic oxygen scavenger system comprising 1 U ml−1 glucose oxidase (Sigma), 8 U ml−1 catalase (Sigma) and 0.1% glucose. In addition, apo-GluPh experiments included 200 mM KCl, Na+−Asp-bound experiments included 200 mM NaCl and 0.1 mM aspartate and Na+−TBOA-bound experiments included 200 mM NaCl and 10 mM TBOA. For experiments in detergent micelles, the buffers were also supplemented with 1 mM DDM. For imaging under transport conditions, the experiments were initiated in the absence of substrates (apo condition) on both sides of the membrane and chemical gradients were established by rapidly exchanging the proteoliposomes into an uptake buffer containing 100 mM NaCl and 100 μM aspartate. All data were collected at an imaging rate of 10 s−1 (100 ms integration time), except when otherwise stated. Fluorescence trajectories were selected for analysis using custom-made software implemented in Matlab (Mathworks) according to the following criteria: a single catastrophic photobleaching event; over 8:1 signal-to-background noise ratio; a FRET lifetime of at least 5 s. FRET trajectories were calculated from the acquired intensities, I355 and I561 using the formula FRET = I355/I561 + I2ζ355/2. Population contour plots were constructed by superimposing the FRET data from individual traces. Histograms of these population data were fit to Gaussian functions in Origin (OriginLab). The relative populations and dwell time distributions of each FRET state, as well as the transition frequencies between them, were obtained by idealizing the smFRET traces using QuB. Transitions density plots and the dwell time survival plots were plotted and fitted as described previously. The logarithmic histograms of the dwell times were fitted to transformed probability density functions. Over 300 molecules are included in each smFRET experiment to ensure that the experimental margin of error in the mean value of each distinct FRET state across the three experiments is less than 5%.

Crystallography. The R276S/M395R GluPh mutant was purified by SEC in buffer containing 10 mM Tris/HEPES, pH 7.4, 100 mM NaCl and 7 mM n-decyl-β-D-maltopyranoside (DDM). Protein solution at 3.5 mg ml−1 was mixed at 1:1 (v/v) ratio with the reservoir solution, containing 50 mM sodium acetate, pH 5.6–6.8, 18–20% PEG 400 and 100–150 mM magnesium acetate, and crystallized at 4°C by hanging-drop vapour diffusion. Crystals were cryoprotected in reservoir solution. Diffraction data were collected at National Synchrotron Light Source beamline X29. Diffraction data were indexed, integrated and scaled using the HKL2000 package. Anisotropy correction was applied as described previously. Further analyses were performed using CCP4 algorithms. Initial phases were determined by molecular replacement in Phaser using transport and trimeric scaffold domains as separate search models. The model was optimized by rounds of manual rebuilding in Coot and refinement in Refmac5 with TLS. During refinement, strict non-crystallographic threefold symmetry constraints were applied to the three transport domains and to regions of the scaffold domain that are involved in trimerization interactions. In addition, strict twofold symmetry constrains were applied to the entire B and C protomers, which exhibited identical positions of the transport domain. For the outward- and inward-facing states, published coordinates were used with accession numbers 2NWX (ref. 3) and 3KBC (ref. 4), respectively. For the open conformation of HP2 the accession number of the coordinates is 4OYF (ref. 6). All structural refinement calculations were generated using CCP4.

DEER measurements and data analysis. Measurements were performed at 60 K using a 17.3 GHz home-built Ku-band pulse spectrometer. A standard four-pulse DEER sequence with π/2−π−π pulse widths of 16 ns, 32 ns and 32 ns, respectively, and a 32 ns π pump pulse was used routinely. The frequency separation between detection and pump pulses was 70 MHz. The detection pulses were positioned at the low-field edge of the nitroxide spectrum. The homogeneous background was removed from the raw time-domain signals and the distances were reconstructed from the baseline-corrected and normalized signals by using Tikhonov regularization method and refined by maximum entropy method.

Molecular modelling. Molecular dynamics simulations using the Charmm27 force field (FF) and updated lipid FF were prepared as described previously and run using the NAMD 2.9 software at 300K with PME electrostatics and standard parameters for the Charmm FF. Atomic coordinates for the inward-facing wild-type GluPh were taken from PDB entry 3KBC (ref. 4) Simulations with the Gromos 54A7 FF were prepared using the LABAMDA / InflaGEOMO membrane-embedding protocol and run with the Gromacs 4.6.1 (ref. 50) simulation package with reaction-field electrostatics and standard cutoffs for the Gromos FF. All simulations included pure POPC membranes, except Charmm27 trajectory 3 (Extended Data Fig. 9), which contained a mixture of 18% POPC, 52% POPF, and 30% POPG (prepared with Charmm-GUI web tool), more similar to the composition of the liposomes used in experiments. In selected simulations (Extended Data Fig. 5A) Cx atoms were subjected to harmonic restraint potentials centred on positions from the X-ray structure with a harmonic constant of 0.1 kcal mol−1 Å−2 (NAMD) or 0.24 kcal mol−1 Å−2 (Gemvac). Docking of detergent and POPC lipid molecules was performed with Autodock Vina within the Chimera 1.8 visualization software. Lipid insertion in Charmm27 Trajectory 3 was performed as follows: (i) a frame from the molecular dynamics trajectory after 48ns of simulation time was selected; (ii) several lipid molecules restricted to various regions of the interfaces in protomers A and C were docked, ignoring the water; (iii) docking poses among the highest ranked from all docking runs were combined, such that lipid molecules fill the available hydrophobic pockets without clashing with each other, and overlapping water molecules were discarded; (iv) local minimization was performed with the Charmm27 force field, including solvent and side chains within 5 Å of inserted lipids; (v) the molecular dynamics simulation was restarted at 300 K. Data processing and plots were performed in Matlab (Mathworks).

Kinetik simulations of smFRET data. For the simulations, we assumed that protomer motions are independent. The model presented in Fig. 6 was employed to simulate the motions of individual protomers between outward- and inward-facing orientations in QuB. The time-dependent configurations of two protomers were then assigned to FRET states as described (Extended Data Fig. 3). FRET traces were generated at 100 ms time-resolution in Matlab (Mathworks) using a Gaussian distribution of FRET efficiency values and widths derived from our experimental data. Initial estimates of the kinetic parameters were based on exponential fits of the experimental dwell time distributions (Extended Data Table 1c). The parameters were then manually optimized to recapitulate the experimental observables: population FRET histograms, TDPs and the dwell-time histograms (Extended Data Fig. 10).

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Extended Data Figure 1 | Elevator model of transport and spatial conservation of a positively charged residue in glutamate transporter family. a, GltPh protomers in the outward- (left) and inward-facing (right) conformation are shown in surface representation and viewed in membrane plane. Dashed lines represent an approximate position of the membrane hydrocarbon layer. In the inward-facing state, the transport domain (blue) is moved by ~15 Å across the bilayer relative to the trimerization domain (beige). b, Schematic representation of dynamic mode-switching between stable and transient conformations. c, A single GltPh protomer is shown in cartoon representation. Cyan balls emphasize the amino acid positions at which potentially positively charged residues occur in glutamate transporter homologues. d, Occurrence frequencies of these residues at the marked positions (GltPh numbering). To obtain the frequencies, sequences were harvested from the PFAM database (accession code PF00375). Sequences were parsed to exclude those with over 70% identity and aligned using Clustal Omega. 

| Position | Frequency (%) |
|----------|---------------|
| 276      | 24            |
| 356      | 1             |
| 357      | 6             |
| 391      | 40            |
| 395      | 9             |
| 398      | 1             |
| No R/K/H | 20            |
Extended Data Figure 2 | Assignment of FRET efficiency states. a, Shown are the crystal structures of GltPh trimers in symmetrical outward (OF)- and inward (IF)-facing states and a model of an asymmetric configuration with two outward- and one inward-facing protomers. The structures are shown in surface representation and coloured as in Extended Data Fig. 1. Black lines connect Ca atoms of residue 378, and the corresponding distances are indicated above the structures. b, Expected FRET efficiency levels for these distances for all possible configurations of subunit pairs: outward/outward (OF/OF), outward/inward (OF/IF), inward/outward (IF/OF) and inward/inward (IF/IF). c, Intramolecularly stabilized 4S(COT)-maleimide Cy3 (n = 1) and Cy5 (n = 2) fluorophores used in this study synthesized as described previously with the addition of two sulfonate groups for increased solubility.
Extended Data Figure 3 | Conformational state distributions of wild-type and H276,395-GltPh in proteoliposomes.  

(a) Examples of smFRET recordings. Top panels show raw fluorescent signals originating from donor (green) and acceptor (red) dyes. Bottom panels show changes of FRET efficiency calculated from raw data (blue). Red solid lines through the data are idealizations obtained using QuB software.  

(b) Contour plots and one-dimensional population histograms in the absence and presence of Na\(^+\) and aspartate in the external liposome buffers. Buffer compositions inside and outside of the vesicles are shown above the panels. Wild-type and H\(_{276,395}\)-GltPh histograms are fitted to three and two Gaussian functions, respectively.  

(c) Transitions density (TD) plots for the wild type (left) and H\(_{276,395}\)-GltPh (right) in proteoliposomes in the absence of Na\(^+\) and aspartate in the external buffer.  

(d) Means and widths (in brackets) of FRET efficiency distributions derived from Gaussian fits to proteoliposome data in comparison to detergent data.
**Extended Data Figure 4 | Single-molecule dynamics using different liposome-attachment strategies and with higher time-resolution.**
a–d, Dynamic properties of H$_{276,395}$-GltPh under transport conditions using a different surface-immobilization strategy and in the presence of electrical potential. 

- **a**, Surface-immobilization strategy for proteoliposomes using His-tagged lipids.
- **b**, Transition frequencies for wild-type (top) and H$_{276,395}$-GltPh (bottom) trimers reconstituted into his-tagged liposomes that were site-specifically labelled in just two protomers with intramolecularly photostabilized Cy3 and Cy5 fluorophores.
- **c**, A negative inside voltage potential was established in proteoliposomes by adding valinomycin to the uptake buffer.
- **d**, Transition frequencies for wild-type (top) and H$_{276,395}$-GltPh (bottom) in the presence of valinomycin. Each experiment shown includes statistics based on >250 individual molecules. The standard error in transition frequency measurements is approximately 0.015 s$^{-1}$.

**e, f**, Dynamic properties of H$_{276,395}$-GltPh probed at 15 ms time resolution. Contour plots and one-dimensional population FRET efficiency histograms (**e**) observed for the humanized mutant in detergent solution in the absence (left) and presence (right) of 100 mM NaCl and 100 μM aspartate. Examples of single-molecule trajectories are shown in **f**.
Extended Data Figure 5 | Population changes in response to ligand binding.

**a, b**, TBOA binding to H276,395-GltPh measured in smFRET experiments. Contour plots and population FRET efficiency histograms in the presence of increasing concentrations of TBOA (**a**). Changes in low- (red) and high- (blue) FRET state populations as a function of TBOA concentration (**b**). Solid lines through the data correspond to the Hill equation $y = y_{\min} + (y_{\max} - y_{\min}) \frac{x^n}{x^n + K_d^n}$ with $K_d = 2.4$ mM and $n = 1$. The data points shown are averages and standard errors from three independent biological replicates.

**c**, Experimental time domain DEER data (left) and reconstructed distance distributions (right) for H276,395-GltPh (shown in colours) and wild-type transporter (black) spin-labelled on residue Cys378 in detergent solution. The data were collected in the absence of ligands (top), in the presence of 100 mM Na$^+$ and 350 μM aspartate (middle) and in the presence of 100 mM Na$^+$ and 480 μM TBOA (bottom). The red arrows above the distance distributions mark distances between residues 378 extracted from crystal structures of the symmetric outward- (OF/OF) and inward- (IF/IF) facing states. The data for the wild-type transporter were adapted from a published study. The data show that in the apo transporter, outward- and inward-facing states are similarly populated. Binding of Na$^+$ ions and aspartate favours the inward-facing state, whereas binding of TBOA favours the outward-facing state.
Extended Data Figure 6 | Aspartate binding experiments. a, FRET efficiency population contour plots determined for H276,395-GltPh in detergent micelles in the presence of 100 μM aspartate and increasing concentrations of Na\(^{+}\) ions (indicated above the panels). b, c, Representative aspartate binding isotherms derived from ITC experiments for the wild-type GltPh (b) and H276,395-GltPh (c) in the presence of 10 mM Na\(^{+}\) and 100 mM Na\(^{+}\), respectively. The binding of aspartate to H276,395-GltPh in the presence of 10 mM Na\(^{+}\) is too weak to measure (inset). Binding experiments were performed using small-volume Nano ITC (TA Instruments). Upper panels show raw data. The cell contained 30 μM (WT-GltPh) and 40 μM (H276,395-GltPh) protein buffer containing 20 mM HEPES/Tris, pH 7.4 and 0.1 mM DDM and indicated concentrations of NaCl. The syringe contained Asp at 200 μM concentration in the same buffer; every injection contained 5 μl. Data were processed and analysed using manufacturer’s software (lower panels). Solid lines through the data are fits to independent binding sites model with the following $K_d$, enthalpy ($\Delta H$), and apparent number of binding sites ($n$): 380 nM, 15 kcal per mol and 0.65 for the wild-type transporter, and 285 nM, 16 kcal per mol and 0.68 for H276,395-GltPh.
Extended Data Figure 7 | Data collection and refinement for Na\(^+\) and aspartate bound H\(_{276,395}\)-GltPh. a, Table showing data collection and refinement statistics. Scaling and refinement statistics were obtained after anisotropy correction by ellipsoidal truncation using high-resolution cutoffs of 4.9 Å along the a and b axis, and of 4.2 Å along the c axis. b, Stereoview of the 2\(F_o\)–\(F_c\) electron density map for H\(_{276,395}\)-GltPh contoured at 1.5\(\sigma\) around residue Arg 395 in unlocked protomer C. Protein backbone (maroon) is shown in cartoon representations and side chains are shown as lines and colored by atom type. c, Superimposed scaffold domains of the inward-facing wild type and H\(_{276,395}\)-GltPh are shown in cartoon representation. The labile portions are coloured cyan (wild type) and magenta (mutant). Helices bend at conserved Pro 60 and Pro 206 residues (spheres). d, Locked (left) and unlocked (right) mutant protomers viewed from the cytoplasm and shown in surface representation.
Extended Data Figure 8 | Arg395 adapts to its environment. a, The arginine side chain (Arg 276 in the wild type; Arg 395 in H276,395-GltPh) is seen in molecular dynamics simulations to engage in hydrogen-bonding interactions. The extent of the hydrogen bonds formation is shown as a function of simulation time in Charmm Trajectory 3 (see Extended Data Fig. 10). The main interactions of the arginine in both mutant and wild type are with water molecules, but the locations of the waters are very different. In H276,395-GltPh, the Arg 395 side chain is located 5 to 9 Å below the level of the membrane surface, so that the water molecules are those penetrating the membrane–protein interface due to remodelling of the membrane. In the wild type, the water molecules interacting with Arg 276 are in the space created inside the protein. b, The minimum distance from wild-type Met395 (top) or mutant Arg 395 (bottom) side chains to any lipid phosphate group (left) or any water molecule (right) in Charmm Trajectory 3. In H276,395-GltPh, after the initial equilibration phase, lipid phosphate groups interact with Arg 395 either directly (5 Å distance) or through water (7.5 Å distance). In the wild type, lipid head groups remain far from the hydrophobic Met 395 side chain. Water interacts constantly with Arg 395, but only occasionally with Met 395 (in protomer B, a water molecule approaches Met 395 from the inside of the protein, at the interface between transport and trimerization domains). c, The same set of distances as in b for the mutant, from a different trajectory (G54a7 Trajectory 2) obtained independently, using a different force field. The same trends are observed as in b, showing proximity to the polar environment. d, Membrane bending (blue indicates thinning, red indicates thickening) close to Arg395 (green) which exposes its side chain to a polar environment comprised of water molecules and lipid head groups. e, Root mean square deviation (r.m.s.d.) of the Arg 395 side chain with respect to the crystal structure after alignment on the trimerization domain, calculated from Charmm Trajectory 3 and G54a7 Trajectory 2. The side chain initially samples different conformations before settling into the membrane-exposed position shown in panel d.
Extended Data Figure 9 | Lipids or detergent molecules stabilize the unlocked conformation of H\textsubscript{276,395}\textsuperscript{GltPh}. a–e, Centre-of-mass distance between the transport and scaffold domains of protomers A, B, and C of H\textsubscript{276,395}\textsuperscript{GltPh} as a function of molecular dynamics simulation time. The data are from five independent simulations initiated with position restraints on the C\textsubscript{\alpha} atoms (later released at different time points) and with the domain interface solvated with water. The vertical green lines indicate the moment in the corresponding trajectory when position restraints were turned off. Panels a and b show two repeats of the same starting structure simulated with the Charmm force field\textsuperscript{45} and panel c with Gromos force field\textsuperscript{48}. The transport domains in protomers B and C collapse onto the trimerization domain rapidly and lose their ligands in some cases (red arrows). d, A simulation, in which lipid tails partially insert into the interface spontaneously; the unlocked structure is stable much longer (note the different time scales on the time axis), and the collapse is only partial. e, The trajectory of a NAMD simulation (Charmm force field) in which lipid molecules were docked into the interface of protomers B and C at the time marked by the red arrow (3 lipids per protomer). The lipids remained in the docked region for the entire duration of the simulation and stabilized the position of the transport domain. f, g, The best scored docking poses for a detergent molecule and a POPC lipid, respectively, docked at the interface of protomer C.
Extended Data Figure 10 | Simulated smFRET data recapitulate experimental observations. a–d, Simulated FRET efficiency population contour plots (left side of each panel) and cumulative population histograms (right side) for wild-type GltPh (a) and H276,395-GltPh (b), and the corresponding transition density plots (c and d), (see Fig. 2 for corresponding experimental data). As noted before, there are fewer transitions observed between the low- and high-FRET states in the wild-type transporter than would be expected from the model. This may either be because the model does not recapitulate the noise correctly or it may reflect previously uncharacterized communication between the protomers that warrants further investigation. e, f, Dwell time distributions for the low- (left panel) and intermediate- and high-FRET states (right panels) obtained for wild-type GltPh (e) and H276,395-GltPh (f) (see Fig. 4 for corresponding experimental data).
Extended Data Table 1 | FRET state assignments and populations; time constants for the slow and fast components

**a. FRET State Population Distributions in proteoliposomes**

| WT Glt\(_{ph}\) | FRET | Subunit configuration | Apo, \(P(\text{out}=0.55,\text{Transport})\), % | \(P(\text{out}=0.65,\text{Transport})\), % |
|---|---|---|---|---|
| Low | OF/OF+OF/IF | 54 | 55 | 63 | 65 |
| Intermediate | IF/OF | 27 | 25 | 22 | 22 |
| High | IF/IF | 19 | 20 | 15 | 13 |

| R276S/M395R Glt\(_{ph}\) | FRET | Subunit configuration | Apo, \(P(\text{out}=0.45,\text{Bound})\), % | \(P(\text{out}=0.5,\text{Bound})\), % |
|---|---|---|---|---|
| Lower | OF/OF+OF/IF | 46 | 45 | 49 | 50 |
| Higher | IF/IF | 29 | 30 | 24 | 25 |

**b. FRET State Population Distributions in detergent micelles**

| WT Glt\(_{ph}\) | FRET | Subunit configuration | Apo, \(P(\text{out}=0.45,\text{Bound})\), % | \(P(\text{out}=0.5,\text{Bound})\), % |
|---|---|---|---|---|
| Low | OF/OF+OF/IF | 46 | 45 | 49 | 50 |
| Intermediate | IF/IF | 29 | 30 | 24 | 25 |
| High | IF/IF | 38 | 60 | 30 | 70 |

**c. Time constant for stable (slow) and transient (fast) FRET States in detergent micelles**

| WT | Low FRET | Intermediate / High FRET |
|---|---|---|
| Apo | \(t_{\text{bind}}, \text{s}\) | \(t_{\text{slow}}, \text{s}\) | \(t_{\text{fast}}, \text{s}\) | \(t_{\text{slow}}, \text{s}\) |
| Na\(^{+}\), aspartate | \(\sim 0.6\) | \(\sim 6\) | \(\sim 0.6\) | \(\sim 5\) |
| R276S/M395R | Low FRET | Higher FRET |
| Apo | \(t, \text{s}\) | \(t, \text{s}\) |
| Na\(^{+}\), aspartate | \(\sim 1.5\) | \(\sim 1.1\) |

\(a, b\). Shown are the assignments of FRET states to configurations of labelled subunit pairs and corresponding observed populations, rounded to integer numbers. Also shown are the calculated populations considering the probability of a protomer to be in the outward facing state \(P(\text{out})\) and assuming independent protomers in the trimer. \(c\). Time constants for the wild-type transporter, \(c\), of the slow and fast components were derived from fitting the survival data compiled from the measured dwell times to double exponential function. The time constants for the H276,395-Glt\(_{ph}\) mutant were obtained by fitting the survival data to a single exponential function. Shown are averages from three independent experiments. The standard errors are within 5%. Dwell times longer than 10 s are significantly underestimated because photobleaching, which occurs with time constant of \(40\) s, is limiting the observation window.