Both reentrant loops of the sodium-coupled glutamate transporters contain molecular determinants of cation selectivity

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In the brain, glutamate transporters terminate excitatory neurotransmission by removing this neurotransmitter from the synapse via cotransport with three sodium ions into the surrounding cells. Structural studies have identified the binding sites of the three sodium ions in glutamate transporters. The residue side-chains directly interact with the sodium ions at the Na1 and Na3 sites and are fully conserved from archaeal to eukaryotic glutamate transporters. The Na2 site is formed by three main-chain oxygens on the extracellular reentrant hairpin loop HP2 and one on transmembrane helix 7. A glycine residue on HP2 is located closely to the three main-chain oxygens in all glutamate transporters, except for the astroglial transporter GLT-1, which has a serine residue at that position. Unlike for WT GLT-1, substitution of the serine residue to glycine enables sustained glutamate transport also when sodium is replaced by lithium. Here, using functional and simulation studies, we studied the role of this serine/glycine switch on cation selectivity of substrate transport. Our results indicate that the side-chain oxygen of the serine residues can form a hydrogen bond with a main-chain oxygen on transmembrane helix 7. This leads to an expansion of the Na2 site such that water can participate in sodium coordination at Na2. Furthermore, we found other molecular determinants of cation selectivity on the nearby HP1 loop. We conclude that subtle changes in the composition of the two reentrant hairpin loops determine the cation specificity of acidic amino acid transport by glutamate transporters.

In the brain, signaling by the excitatory neurotransmitter glutamate is terminated by transport from the cleft into the cells surrounding the synapse. Transport of glutamate is an electrogenic process (1, 2) whereby one neurotransmitter molecule is co-transported with three sodium ions and a proton (3, 4), followed by the counter-transport of one potassium ion (5–7). Several crystal structures are available for two similar archaeal homologues (GltPh from Pyrococcus horikoshii and GltTm from Thermococcus kodakarensis) of the mammalian transporters (8–13). These structures reveal a trimeric assembly and showed a permeation pathway through every protomer, indicating that the protomer is the functional unit. This has been also confirmed for the eukaryotic glutamate transporters (14–17). The protomer structure contains eight transmembrane helices (TMs)3 and two oppositely oriented reentrant loops, one between TMs 6 and 7 (HP1) and the second between TMs 7 and 8 (HP2) (8). The transport domain includes HP1 and HP2 and TMs 3, 6, 7, and 8, whereas the trimerization domain consists of TMs 1, 2, 4, and 5.

Many amino acid residues important for the interaction with sodium (18, 19), potassium (5, 20), and glutamate (21, 22) in eukaryotic transporters are conserved in the archaeal homologues, where these residues are oriented toward the binding pocket. Very recently, direct support for the transferability of information between the bacterial and the human transporters has been obtained through similarity to the crystal structures of a thermo-stabilized form of the human glutamate transporter EAAT1 (23). Substrate translocation appears to take place by an “elevator-like” mechanism (10, 24) whereby the transport domain moves relative to the trimerization domain (25) by ~15 Å. As a result, the substrate-binding site is alternatingly exposed to the extracellular and intracellular sides, enabling substrate translocation (8–10).

In GltPh, the replacement of sodium ions by thallium ions allowed the use of their anomalous signal to identify the potential locations of two of the three sodium-binding sites (Na1 and Na2) (9). Functional and simulation studies indicated possible locations of Na3, the third sodium-binding site (26–29). A recently solved GltTm structure confirmed the predicted location of the third sodium-binding site (13) (Fig. 1, A and B). The Na2 site is formed by three main-chain oxygens of the HP2 loop and another one from TM7a. Functional and structural studies indicate that the role of sodium binding to the Na2 site is to stabilize the HP2 loop in the conformation that closes the binding pocket (9, 30) of the substrate-loaded transporter. In close

The abbreviations used are: TM, transmembrane; MD, molecular dynamics; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.
proximity of the HP2 main-chain oxygens, a glycine residue is conserved in the glutamate transporters, except for the glial glutamate transporter GLT-1, where a serine residue occupies this position (Ser-440, which is equivalent to Gly-354 of GltPh). Remarkably, replacement of this serine residue by glycine conferred the ability of lithium to drive transport (18, 31), rendering lithium almost as effective to support net influx of acidic amino acids with $K_m$ and $V_{max}$ values (18) similar to those for sodium in WT GLT-1.

In contrast to ion channels, our knowledge on the structural basis of ion selectivity in ion-coupled transporters is very limited. Thus, analysis of the structural basis of cation selectivity by mutants of residue 440 in GLT-1 and at the equivalent positions in other glutamate transporters offers an opportunity to obtain information on molecular determinants of cation selectivity in the glutamate transporter family. Therefore, we have studied the role of this serine/glycine switch and other mutants at nearby positions in cation specificity, using functional and simulation studies.

**Results**

**Sequence conservation of the position on the HP2 loop corresponding to Ser-440 of GLT-1**

A BLAST search was performed using the sequences of GltPh, GLT-1, and the neuronal glutamate transporter EAAC1. Up to 500 sequences were selected with a sequence identity of 60% or higher. These sequences were aligned removing incomplete sequences. All sequences of GltPh and EAAC1 homologues have a glycine in the HP2 loop, whereas all GLT-1 sequences carry a serine residue in the corresponding position. A Consurf analysis and a mapping of the residue conservation score (32) on to the 3D structure of GltPh-WT (PDB ID 2NWX) (Fig. 1C) revealed that the substrate-binding site and the binding sites of the three sodium as well as the HP2 loop are highly conserved. Specifically, using the analysis of 500 orthologous sequences of GltPh, GLT-1, and EAAC1, we found that the residue in the respective position Gly-354/Ser-440/Gly-410 is 100% conserved. The data also showed that the HP2 loop between the Na2 site and position Gly-354/Ser-440/Gly-410 is very much conserved, supporting the notion of the functional relevance of the studied region.

**Cation selectivity of Ser-440 GLT-1 mutants**

To determine whether the selectivity in GLT-1 of sodium over lithium is determined by the polarity and/or the size of the amino acid residue at position 440, Ser-440 was mutated to a variety of hydrophilic and hydrophobic amino acids. Uptake of D-$[^3]$H]aspartate by S440G was around 25% of wildtype (WT) GLT-1 (Figs. 2A and 3A, Table 1). In agreement with our earlier observations (18), we find that the selectivity for sodium over lithium to sustain transport was dramatically reduced by the mutation. We found that transport by GLT-1-WT in the presence of lithium was only 2–3% compared with the presence of sodium. In contrast, lithium was least as effective as sodium to support uptake of D-$[^3]$H]aspartate by the S440G mutant (Fig. 2B, Table 1). Enlargement of the side-chain at position 440 by replacing Ser-440 with the larger hydrophobic isoleucine or leucine resulted in non-detectable transport activity (Fig. 3A, Table 1), prohibiting the measurement of sodium selectivity. This result could be the consequence of an intrinsic effect of the mutation on the transport activity, for instance, by a perturbing the Na2 site.

Alternatively, the mutation could alter the steady-state levels of the mutant transporter at the plasma membrane as a consequence of impaired trafficking. Therefore, we determined the expression levels of the WT and all Ser-440 mutant transporters at the plasma membrane by biotinylation of external lysine residues, using the membrane-impermeant Sulfo-NHS-SS-Biotin. After lysis of the cells, the biotinylated proteins were isolated using streptavidin-agarose beads followed by SDS-PAGE and Western blotting with an antibody that is specific for GLT-1 (see “Experimental procedures”). Specificity is confirmed with cells expressing only vector (SK, Fig. 4), which showed the absence of any band. The upper and lower parts of the broad band represent the fully N-glycosylated transporters and the immature transporters, respectively (33), and faster running bands are probably due to some proteolysis during the biotinylation procedure. The S440L and S440I mutants exhibited plasma membrane expression of 30–40% of that of GLT-1–WT (Fig. 4, Table 2). However, because these mutants were devoid of any transport activity (Fig. 3A), we conclude that they have an intrinsic transport defect. The surface expression of the S440G mutant was also lower than that of GLT-
When normalized for the expression levels, the activity of S440G was more than 40% of that of WT GLT-1 (Fig. 4, Table 2). The mutant where Ser-440 was replaced by threonine, which maintains the side-chain hydroxyl group (S440T), exhibited a slightly higher activity than S440G (Fig. 2A, Table 1). After correcting for surface expression, transport activity of S440T was over 80% compared with GLT-1–WT (Fig. 4, Table 2). The sodium/lithium selectivity of sustaining transport by this conservative replacement was similar or maybe even higher than GLT-1–WT (Fig. 2B, Table 1). Transport of D-[3H]aspartate by the hydrophilic replacement mutant S440N was around 20% compared with GLT-1–WT, also after normalizing for the surface expression (Figs. 2A and 4, Tables 1 and 2). The sodium selectivity of transport of D-[3H]aspartate remained similar to WT (Fig. 2B, Table 1). The activity of S440D was very low (Fig. 2A), mainly due to its low expression at the plasma membrane (Fig. 4, Table 2). Nevertheless, our transport assay is sensitive enough to monitor the sodium selectivity of transport, which was also similar to GLT-1–WT (Fig. 2B, Table 1).

Substitution of Ser-440 by alanine resulted in transport levels that were between GLT-1–WT and S440G (Fig. 3A, Table 1). Interestingly, the sodium selectivity of this conservative mutant was also strongly reduced, although not as much as in S440G (Fig. 3B, Table 1). Further enlargement of the side-chain of Ser-440 with the hydrophobic amino acid valine resulted in low yet detectable levels of transport (Fig. 3A, Table 1), whereas its sodium selectivity remained similar to GLT-1–WT (Fig. 3B, Table 1). Also, the plasma membrane expression level of the low activity S440V mutant was similar to that of GLT-1–WT as judged by surface biotinylation (Fig. 4, Table 2). Thus, the low level of transport by this mutant is caused by a functional defect of the transporter.
### Table 1
Summary of the transport activity and cation selectivity of Ser/Gly switch mutants of GLT-1, GltPh, and EAAC1

The transport activity is given as percentage of the wildtype and cation selectivity is reported as the ratio between the lithium driven uptake and the mean sodium driven D-[3H]aspartate uptake. Shown is the mean ± S.D. (summarized from Figs. 2, 3, 5, 8, and 10).

| Mutant | D-[3H]Asp uptake (WT) | Li/Na S.D. (summarized from Figs. 2, 3, 5, 8, and 10) |
|--------|-----------------------|--------------------------------------------------|
| GLT-1  |                       |                                                  |
| WT     | 100                   | 2.8 ± 1.0                                        |
| S440G  | 25.0 ± 3.9            | 117.2 ± 7.9                                      |
| S440A  | 50.4 ± 4.0            | 20.7 ± 3.9                                       |
| S440D  | 3.3 ± 0.8             | 0.3 ± 0.6                                        |
| S441I  | 0.4 ± 0.5             |                                                   |
| S440L  | 0.6 ± 0.8             |                                                   |
| S440T  | 34.8 ± 1.8            | 0.2 ± 0.3                                        |
| S440V  | 3.0 ± 0.8             | 0.3 ± 0.7                                        |
| S440N  | 22.3 ± 3.0            | 0.3 ± 0.4                                        |
| S440L/S440G | 25.3 ± 3.2 | 35.6 ± 4.0                                      |
| GltPh  |                       |                                                  |
| WT     | 100                   | 73.4 ± 3.2                                       |
| G354S  | 79.5 ± 10.1           | 2.0 ± 1.1                                        |
| G354L  | 2.3 ± 1.0             | 6.9 ± 8.2                                        |
| EAAC1  |                       |                                                  |
| WT     | 100                   | 10.6 ± 2.9                                       |
| G410S  | 30.1 ± 4.8            | 0.5 ± 0.6                                        |

Figure 4. Cell-surface biotinylation of GLT-1 Ser-440 mutants. HeLa cells transfected with GLT-1 (WT), the indicated mutants or with the vector alone (SK), were biotinylated and processed as described under “Experimental procedures.” The indicated markers were run in the lane to the left of WT and contain Prestained Protein Marker, Broad Range (catalogue number P77065) was from New England Biolabs. The gel shown is representative of three different experiments. The intensities of all bands were summed up, because they are transporter specific (see the SK control lane of the empty vector).

### Table 2
Transport of GLT-1 mutants normalized to surface expression

Intensity was determined by normalizing the biotinylated mutant bands as seen in Fig. 4 to the that of the GLT-1–WT band using ImageJ 1.x technology. The intensity percentage of individual mutants to their mean ± S.D. sodium driven D-[3H]aspartate uptake (Figs. 2A and 3A) is shown.

| Mutant | D-[3H]Asp uptake of WT | Intensity, normalized to WT | D-[3H]Asp uptake/Intensity of WT |
|--------|------------------------|---------------------------|---------------------------------|
| WT     | 100                    | 1                         | 100                             |
| S440G  | 25.0 ± 3.9             | 0.58 ± 0.30               | 43.0                            |
| S440A  | 50.4 ± 4.0             | 0.55 ± 0.09               | 91.7                            |
| S440D  | 3.3 ± 0.8              | 0.45 ± 0.14               | 7.3                             |
| S440I  | 0.4 ± 0.5              | 0.38 ± 0.12               | 1.1                             |
| S440L  | 0.6 ± 0.8              | 0.29 ± 0.07               | 2.1                             |
| S440T  | 34.8 ± 1.8             | 0.40 ± 0.27               | 87.1                            |
| S440V  | 3.0 ± 0.8              | 0.82 ± 0.09               | 3.5                             |
| S440N  | 22.3 ± 3.0             | 0.10 ± 0.15               | 22.1                            |

### Experimental and computational analysis of the GltPh mutants

The crystal structures of the archaean transporter homologue GltPh indicate that HP2 contributes several interacting groups to the Na2 site (9). These groups are close to the glycine residue that occupies the equivalent position of Ser-440 of GLT-1 (position 354 in GltPh). Transport of D-[3H]aspartate and L-[3H]aspartate was dramatically increased by the G354S mutation, because the ability to transport in the sole presence of lithium was lost (Fig. 5B, Table 1). This indicates that the observations on the sodium specificity of residue Ser-440 in GLT-1 are recapitulated in GltPh. Therefore, molecular dynamics (MD) simulations were performed to investigate the molecular origin of the effects using GltPh-WT and GltPh mutants in which Gly-354 was replaced by Ser or Leu.

The structure of GltPh-WT (PDB ID 2NWX) was used in this study and the mutant structures were modeled by replacing Gly-354 with serine or leucine. Trimeric transporters were inserted in a pre-equilibrated POPC membrane and simulated for 100 ns. The systems were extensively energy minimized after insertion, followed by a slow release of the constraints on GltPh substrate, and co-transported ions in four 2.5-ns long steps (applying 1000, 100, 10, and 1 kJ/mol). All systems were simulated for 100 ns. The simulations showed that the sodium ion did not remain bound to the Na2 site in all protomers of all replications, indicating that the starting geometry was not ideal for sodium binding, as already observed earlier (34). In that study it was also found that the sodium in the Na2 site dissociated from transporter. This observation is force field independent, as we used the Amber force field in this study, whereas previously the OPLS force field was applied (35). Using different equilibration protocols did not significantly change the unbinding behavior. We repeated the simulations using the higher resolution (2.96 Å) GltPh-WT structure with the PDB ID 2NWL, which is structurally very similar to 2NWX.
Table 1

independent simulations and the three protomers of the GltPh

Table 1

Figure 6. Probability distributions of distances. A, the distance between the Ca atoms of residue 279 on HP1 and residue 354 on HP2, averaged over all simulations, quantifies HP2 gate closure. The geometry of the Na2 site and therefore of sodium binding is quantified by measuring the probability distribution of distances across the Na2 sites between carbonyl oxygens of Thr-308 on TM7 and Ile-350 (B) and Ile-352 (C) on HP2 loop for the protomers that maintained a bound sodium ion.

but was solved without co-crystallized ions. Again, a propensity of sodium unbinding from the Na2 site was observed. It is known from electrophysiological studies that two of the three sodium ions and the substrate bind fast, followed by slow binding of the third sodium ion (36). Most likely, sodium 1 and 3 and the substrate bind first and close the HP2 gate, whereas Na2 is the slow sodium-binding site. Slow binding does imply that structural adjustments are required or alternatively the binding energy is low. A previous study on GltPh (34) reported that structural adjustments are required before sodium binding can become strong. It is therefore tempting to speculate that the geometry of the Na2 side might not be optimal for sodium binding, maybe because of the conditions present in the crystals.

Unbinding of sodium from the Na2 site was especially pronounced in the GltPh-G354L mutant, which could not maintain a sodium ion coordinated in the Na2 site. HP2 gate closure was similar between GltPh-WT and the GltPh-G354S mutant. We measured closure by determining the distance between the Ca atoms of residue 279 on the HP1 loop and residue 354 on the HP2 loop (Figs. 6 and 7). The HP2 gate opened in a subpopulation of those GltPh protomers from which sodium was unbound from the Na2 site. We calculated a mean probability of observing a closed HP2 gate conformation by averaging over the four independent simulations and the three protomers of the GltPh trimer. The probability distributions of distances (Fig. 6A) between the Ca atoms of residues 279 and 354 showed that the average distance for the GltPh-G354S mutant was slightly larger than that of GltPh-WT. In contrast, the HP2 gate opened in the larger fraction of GltPh-G354L transporters, as indicated by the larger peak at 0.6 nm in Fig. 6A. The long tail of the distribution is indicative of the existence of open and dynamic conformations of the HP2 gate, which is smallest for GltPh-WT and GltPh-G354S.

The closed conformation of the HP2 gate in GltPh-G354S is stabilized by a hydrogen bond between the hydroxyl group on the side-chain of Ser-354 and the backbone carbonyl oxygen of residue Ala-307 (Fig. 7). This hydrogen bond links the HP2 gate to the backbone of TM7, thereby stabilizing the HP2 conformation. In contrast, the side-chain of GltPh-G354L does not carry a hydrogen bond donor and can therefore not stabilize the conformation of the closed HP2 gate by the same interaction. This is probably the reason for the lack of transport by GLT-1–S440L (Fig. 3A, Table 1) and the very low transport by GltPh-G354L (Fig. 5A, Table 1).

The simulations consistently showed that sodium coordination in the Na2 site differed between GltPh-WT and the GltPh-G354S mutant, as the hydration of the sodium ion in the GltPh-G354S increased as quantified by the number of waters within 0.4 nm cutoff, which increased ~2-fold. Importantly, the hydrogen bond between the side-chain hydroxyl group of GltPh-G354S and the carbonyl oxygen of Ala-307 from TM7 also changed the geometry at the Na2 site: the residues of the HP2 loop contributing to the Na2 site are at a larger distance from the backbone of TM7 in the GltPh-G354S mutant, therefore enlarging the Na2 site as compared with WT. To quantify the change in geometry of the sodium coordinating residues in the Na2 site, we measured the distance between the backbone carbonyl oxygen atoms of Thr-308 and Ile-350 (Fig. 6B) and between Thr-308 and Thr-352 (Fig. 6C). These probability distributions (of stably sodium bound Na2 sites) showed a clear difference between GltPh-WT and GltPh-G354S, revealing that the compactness of the Na2 site decreased in the GltPh-G354S mutant. The GltPh-G354L mutant positioned the HP2 loop at the Na2 site even further away, as shown in Fig. 6B.

Cation selectivity of HP1 mutants

To substantiate our findings on the role of the glycine/serine switch on cation selectivity of acidic amino acid transport, we also investigated the role of the corresponding residue on ion selectivity in the neuronal transporter EAAC1. Like GltPh, this transporter has a Gly residue at position 410, which is equivalent to residue 440 of GLT-1. We measured radioactive transport of both L- and D-[3H]aspartate, because the cation selectivity of the neuronal transporter EAAC1 depends on the transported substrate with transport of the L-isomer exhibiting a lower sodium selectivity than that of the D-isomer (37). Transport by EAAC1-G410S was 30.1 ± 4.8 and 53.2 ± 12.0% for D- and L-[3H]aspartate, respectively (n = 3) (Fig. 8A, Table 1).
ability of lithium to support transport of \( L^-{\text{[3H]}} \)aspartate in EAAC1-WT was around 33% as compared with transport sustained by sodium, whereas it was around 10% for \( D^-{\text{[3H]}} \)aspartate (Fig. 8B, Table 1). Importantly, replacement of Gly-410 by Ser virtually abolished the ability of lithium to support transport of both \( D^- \) and \( L^-{\text{[3H]}} \)aspartate (Fig. 8B, Table 1). The fact that lithium was much less effective to stimulate \( D^-{\text{[3H]}} \)aspartate transport in EAAC1-WT than in GLT-1–S440G (Figs. 2B, 3B, and 8B, Table 1) suggests that there must be determinants in addition to the glycine-serine switch that contribute to the cation selectivity.

On the HP1 loop, several amino acid residues differ between EAAC1 and GLT-1, including (EAAC1 numbering) Ile-330, Ser-331, and Ser-334, which are Thr, Ala, and Ala, respectively, in GLT-1. The close proximity between the HP1 and HP2 loops suggested that these residues could also contribute to the cation specificity of transport. Therefore, we mutated these EAAC1 residues to their GLT-1 counterparts to make EAAC1 more GLT-1–S440G like. The mutations were well-tolerated as the \( D^-{\text{[3H]}} \)aspartate transport activity of these mutants was similar to EAAC1-WT, except for EAAC1-I330T/S334A, where it increased almost 2-fold (Fig. 9A). Significantly, the ability of lithium to support \( D^-{\text{[3H]}} \)aspartate transport was markedly increased by the Ile to Thr mutation at position 330 (Fig. 9B). The effect of the S331A mutation on the sodium selectivity was much smaller, although not negligible. Importantly, the cation selectivity was further decreased in the I330T/S331A double mutant (Fig. 9B). On the other hand, introduction of the S334A mutation had very little effect, also in the I330T background. Upon introduction of the reverse mutations in GLT-1, we found that replacing Thr-360 by Ile had only a minor effect on transport activity (Fig. 10A) and on sodium selectivity (Fig. 10B). Importantly, combining the T360I mutation with the S440G mutation (Fig. 10B) to resemble the residues present in EAAC1, markedly increased the ability of lithium to support transport in S440G as compared with T360I/S440G (Fig. 10B).

**Discussion**

Because of the proximity of Ser-440 on the HP2 loop to the Na2 site identified in the archeal transporters GltPh (9) and GltTk (13) and the astroglial glutamate transporter EAAT1 (23), we considered the possibility that the side-chain hydroxyl of Ser-440 may participate in the Na2 site of GLT-1, whose crystal structure has not yet been determined. The results of our studies with Ser-440 mutants (Figs. 2 and 3) are not consistent with the idea that the hydroxyl oxygen of the serine side chain directly coordinates Na2. Replacement of Ser-440 by other hydrophilic amino acids (Thr, Asn, and Asp) did not affect the sodium selectivity (Fig. 2B), contrary to the expectation of a *bona fide* cation-binding site that has stringent geometry and side-chain chemistry requirements. However, our MD simulations suggest a different possibility.

We have used MD simulations on GltPh (Figs. 6 and 7) to investigate the role of the switch in the function of the Na2 site, simulating WT and GltPh-G354S. The MD simulations showed that the polar side-chain in GltPh-G354S may form an additional hydrogen bond to the backbone carbonyl oxygen of Ala-307 in TM7. The side-chain orientation of Ser-354 in the starting conformation of the GltPh-G354S simulations did not allow for preforming this hydrogen bond due to the backbone conformation of the HP2 loop in the crystal. Reorientation of the water exposed side-chain of Ser-354 occurred fast and the hydrogen bond to the backbone carbonyl oxygen of Ala-307 formed in the first 10 ns. Once the hydrogen bond was estab-
Cation selectivity in glutamate transporters

Figure 9. Characterization of transport activity and cation selectivity of EAAC1-WT and HP1 mutants. HeLa cells expressing EAAC1-WT, HP1 single and double mutants in the pBluescript SK(−) background were measured for sodium (A)- and lithium (B)-dependent D-[3H]aspartate uptake (plotted as % Li/Na) at room temperature for 10 min, as described under “Experimental procedures.” Individual data points are shown as triangles and the horizontal line represents the mean ± S.D. (error bars) of at least three separate experiments performed in triplicate. Stars represent significance of p < 0.0001.

Figure 10. Characterization of transport activity and cation selectivity of GLT-1 HP1 mutant T360I in GLT-1–WT and S440G background. HeLa cells expressing GLT-1–WT and the indicated mutants in the pBluescript SK(−) background were measured for sodium (A)- and lithium (B)-dependent D-[3H]aspartate uptake (plotted as % Li/Na) at room temperature for 10 min, as described under “Experimental procedures.” Individual data points are shown as triangles and the horizontal line represents the mean ± S.D. (error bars) of at least three separate experiments performed in triplicate. One star represents significance of p < 0.01 and two stars represent significance of p < 0.0001.

In the MD simulations, because we observed that Glt<sub>pr</sub>–WT and the Glt<sub>pr</sub>–G354S mutant could similarly maintain a bound sodium ion in the protoners over the set of four independent simulations. The most likely reason for this observation is that the oxygen atom of the Glt<sub>pr</sub>–G354S side chain, which is positioned close to the Na2 site, forms a water bridged interaction with sodium. Thereby the bound sodium is stabilized in the more open Na2 site through an additional interaction by a structural water molecule, which is positioned by interactions to the hydroxyl group of the G354S serine side-chain. The similarity between the results with GLT-1–WT and GLT-1–S440T (Fig. 2) supports this interpretation, because only serine and threonine have a hydroxyl group in the H9252-position of their side-chain. It is possible that the polar/charged Asn and Asp side-chains introduced in position 440 of GLT-1 could also enlarge the Na2 site, thereby explaining the lowered transport activity (Fig. 2A) and the increased sodium selectivity of the S440N and S440D mutants (Fig. 2B).

The hydrophobic substitution mutations to isoleucine and leucine (Fig. 3A) were not tolerated, because they are most likely expected to perturb the structure and/or polarity of the Na2 site. Indeed, this is supported by the simulations of the Glt<sub>pr</sub>–G354L mutant (Figs. 6 and 7C). The extremely low activity of Glt<sub>pr</sub>–G354L precludes the reliable determination of the sodium selectivity (Fig. 5B).

The smaller side-chains Ala and Gly are tolerated, but change the functionality of the Na2 site. Complete removal of the side-chain (Gly substitution) abrogated the ion selectivity in glutamate transporters, which carry a Gly at this position, such as Glt<sub>pr</sub> or EAAC1. Consistently, introduction of Ser in place of Gly in Glt<sub>pr</sub> or EAAC1 increased the sodium selectivity (see Figs. 5 and 8). It is likely that the Gly residue, introduced at position 440 of GLT-1 and present in the other acidic amino acid trans-
porters, confers additional flexibility to the HP2 loop. This could enable an improved closing-in of the HP2 loop on the binding pocket by sequestering the substrate and also on the Na2 site by accepting not only sodium (9, 30) but also lithium. The broadened cation specificity by an amino acid residue with a small side-chain is further illustrated by the observation that Ala can partially mimic the promiscuity of the cation driving transport (Fig. 8B). In the S440V mutant the side-chain is further enlarged, resulting in barely detectable but highly sodium selective transport. Thus the cation specificity of acidic amino acid transport is a function of both hydrophilicity and size of the side-chain at position 440 of GLT-1.

Therefore, these results are fully consistent with the idea that the Ser/Gly switch controls the cation selectivity of the Na2 site. The fact that lithium can drive transport by GLT-1–S440G shows that this cation can functionally replace sodium at all three binding sites and indicates that in GLT-1–WT lithium can do so at the Na1 and Na3 sites but not at the Na2 site. Taken together these data show that cation selectivity is contingent to the Na2 site, whereas the Na1 and Na3 sites are promiscuous. Importantly, the broadened cation selectivity of aspartate transport by GLT-1–S440G was partially recapitulated in the neuronal transporter EAAC1, which also has a Gly at the equivalent position (Fig. 8B). The lower transport by EAAC1-WT of l-aspartate in the presence of lithium as compared with the presence of sodium (19) is consistent with the observation that transport of l-[3H]aspartate by EAAC1 is significantly lower for lithium than sodium (Fig. 8B). The sodium selectivity was higher for d-[3H]aspartate transport than for l-[3H]aspartate transport (Fig. 8B). This is in harmony with the observations that (i) lithium is more effective in supporting transport of l-aspartate than l-glutamate or d-aspartate (37) and (ii) that cation binding influences the substrate selectivity of glutamate transporters (31).

Importantly the reverse mutation EAAC1-G410S dramatically increased the sodium selectivity (Fig. 8B), which is in full agreement with our conclusion that the Ser/Gly switch controls the sodium selectivity in GLT-1. Our observation that lithium is less efficient to support transport by EAAC1 than by the GLT-1–S440G mutant is at least in part due to the difference in molecular determinants located on the HP1 loop that is located in close proximity to HP2 (8). The main determinant is Ile-330 of EAAC1 (Fig. 9B), which is a Thr in GLT-1 (Thr-360) and in all the other acidic amino acid transporters. An additional determinant is Ser-331. Even though the S331A mutation had little effect on sodium specificity, the mutation decreased the sodium selectivity of transport by the I330T mutant (Fig. 9B). This suggests that there is an interchange between the HP1 residues influencing the conformation of this loop and thereby indirectly the geometry of the Na2 site. Consistent with the effect of the EAAC1-I330T mutation, the reverse mutation GLT-1–T360I rendered lithium less effective to support transport by S440G. It therefore appears that molecular determinants on both reentrant loops determine the cation selectivity of the glutamate transporters by subtle conformational changes. Final establishment of this idea will be hopefully achieved upon successful structure determination of GLT-1 and GLT-1–S440G.

Experimental procedures

Generation and sub-cloning of GLT-1 and EAAC1 mutants

GLT-1 (38) and the C-terminal histidine-tagged versions of rabbit EAAC1 (39, 40) in the vector pBluescript SK− (Stratagene) were used as a parent for site-directed mutagenesis (41, 42). The introduced mutations were verified by sequencing the entire insert.

Cell growth and expression

HeLa cells were cultured (43), infected with the recombinant vaccinia/T7 virus vTF7-3 (44), and transfected with the plasmid DNA harboring the WT or mutant constructs or with the plasmid vector alone (43). Transport of d- and l-[3H]aspartate was done as described (41). Briefly, HeLa cells were plated on 24-well plates and washed with transport medium containing 150 mM NaCl, 5 mM Kp, pH 7.4, 0.5 mM MgSO4, and 0.3 mM CaCl2. Each well was then incubated with 200 µl of transport medium supplemented with 0.4 µCi of the radiolabeled substrate for 10 min, followed by washing with a solution containing 150 mM choline chloride, 5 mM Kp, pH 7.4, 0.5 mM MgSO4, and 0.3 mM CaCl2, solubilization of the cells with SDS and scintillation counting.

Mutagenesis, expression, purification reconstitution, and radioactive transport

Gltph-WT, Gltph-G354S, and Gltph-G354L were expressed, purified (45), and reconstitution using Sephadex G50 spin columns and radioactive uptake were done as described (7, 38).

Cell-surface biotinylation

Labeling of WT and mutant transporters at the cell-surface, using Sulfo-NHS-SS-Biotin (Pierce), quenching the reaction, cell lysis, and isolation of the biotinylated proteins by streptavidin-agarose beads (Pierce) was done as described previously (46). After SDS-PAGE (10% gel) and transfer to nitrocellulose, the GLT-1 protein was detected with an affinity-purified antibody directed against a polyclonal antibody, which was raised against GLT-1 purified from rat brain (47), at a 1:5,000 dilution. A horseradish peroxidase-conjugated secondary antibody was used at a 1:40,000 dilution together with ECL. 1% of goat serum was present in all antibody, blocking, and washing solutions, to minimize the appearance of nonspecific bands.

Molecular dynamics simulations

The crystal structure of Gltph was inserted in a pre-equilibrated POPC membrane. Sodium 3 was positioned as identified in Gltph. The G354S and G354L mutations were created using Modeller (48) and the best of 4 models were selected using the DOPE Score (49). The lipid membrane is described by the Berger force field (50), whereas the Amber99SB-ILDN force field (51) was used for the protein. All simulations were carried out with GROMACS version 5.1.4 (52). All systems were solvated in water and neutralized. After energy minimized, a slow release protocol of stepwise reduction of position restraints (1000, 100, 10, and 1 k J mol−1 nm−2) on the Ca atoms was used for equilibration. Temperature was maintained at 310 K applying velocity rescaling and using 0.5-ps coupling time. Pressure...
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was isotropically set to 1 bar with 20.1 ps coupling time using the Parrinello–Rahman barostat (53). A short range cut-off of 0.9 nm was applied for electrostatic interactions, whereas long range interactions were treated by the particle mesh Ewald method (54). Four systems for each configuration of GltPh-WT, -G354S, and -G354L were first independently equilibrated, followed by 100-ns long unbiased simulations using a time step of 2 fs.

Statistical evaluations

The significance of differences in transport between the mutants and WT and under the indicated conditions was determined with GraphPad Prism version 7.0, using a one-way analysis of significance with a post hoc Dunnett multiple comparison test (Figs. 2, 3, 9, and 10) or a type 2, two-tailed t test (Figs. 5 and 8). All graphs were made using MATLAB R2017.

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