Molecular Determinants of Substrate Specificity in Sodium-coupled Glutamate Transporters*

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Background: Archaeal and brain transporters differ in substrate specificity.
Results: Two residues from hairpin 2 play a role in the substrate specificity of a glutamate transporter homologue.
Conclusion: Hairpin 2 plays a role in the selection and translocation of the substrate.
Significance: This work provides new insights into the molecular basis of substrate specificity of transporters.

Crystal structures of the archaean homologue Gltph have provided important insights into the molecular mechanism of transport of the excitatory neurotransmitter glutamate. Whereas mammalian glutamate transporters can translocate both glutamate and aspartate, Gltph is only one capable of aspartate transport. Most of the amino acid residues that surround the aspartate substrate in the binding pocket of Gltph are highly conserved. However, in the brain transporters, Thr-352 and Met-362 of the reentrant hairpin loop 2 are replaced by the smaller Ala and Thr, respectively. Therefore, we have studied the effects of T352A and M362T on binding and transport of aspartate and glutamate by Gltph. Substrate-dependent intrinsic fluorescence changes were monitored in transporter constructs containing the L130W mutation. Gltph-L130W/T352A exhibited an ~15-fold higher apparent affinity for l-glutamate than the wild type transporter, and the M362T mutation resulted in an increased affinity of ~40-fold. An even larger increase of the apparent affinity for l-glutamate, around 130-fold higher than that of wild type, was observed with the T352A/M362T double mutant. Radioactive uptake experiments show that Gltph-T352A not only transports aspartate but also l-glutamate. Remarkably, Gltph-M362T exhibited l-aspartate but not l-glutamate transport. The double mutant retained the ability to transport l-glutamate, but its kinetic parameters were very similar to those of Gltph-T352A alone. The differential impact of mutation on binding and transport of glutamate suggests that hairpin loop 2 not only plays a role in the selection of the substrate but also in its translocation.

The SLC1 family includes five mammalian sodium-coupled glutamate transporters (EAATs) that are key elements in the termination of glutamatergic synaptic transmission and keep the resting glutamate concentrations below neurotoxic levels. These transporters have an overall amino acid sequence identity of around 50% and include glial as well as neuronal glutamate transporters (1, 2). Glutamate uptake by the EAATs is an electrogenic process (3, 4) in which the transmitter is co-transported with three sodium ions and one proton (5, 6), followed by the counter-transport of one potassium ion (7–9).

Several crystal structures of the archaean glutamate transporter homologues Gltph and GltTk are now available (10–15). The structures reveal a homotrimer with substrate-binding sites in each of the protomers, indicating that the protomer is the functional unit. This is also the case for the eukaryotic glutamate transporters (16–18). The protomer contains eight transmembrane segments (TM) and two hairpin loops, one between TM6 and -7 (HP1) and the other between TM7 and -8 (HP2) (13). HP1, HP2, TM7, and TM8 surround the binding pocket (Fig. 1, A and B). Moreover, several of the amino acid residues of the brain transporters that have been inferred to be important in the interaction with the substrate (19–21) are facing toward the binding pocket. These and other observations indicate that the structures of the homologues represent excellent models for the brain transporters.

Two recent fluorescence studies, employing the substrate-dependent increase of the fluorescence of a tryptophan residue introduced at position 130 of Gltph (L130W) (14), demonstrated that Gltph binds amino acids by induced fit after association of two out of the three co-transported sodium ions to the empty transporter (22, 23). After the amino acid association reaction, which is under diffusional control, a conformational change, apparently resulting from the closure of the external HP2 gate (22, 24), enhances the tightness of binding. Gltph selects between different substrates by these two consecutive processes. l-Aspartate as the preferred substrate exhibits the highest association rates and HP2 closure rate (22). The closure

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3 The abbreviations used are: EAAT, excitatory amino acid transporter; TM, transmembrane domain; HP, hairpin loop.
of HP2 is followed by substrate translocation via an “elevator-like” mechanism (15, 25), where the transport domain, which includes HP1, HP2, and TM3 and -6 – 8, moves relative to the fixed trimerization domain (26) by around 15 Å. The lateral movement of the transport domain at intermediate positions results in the formation of an anion-selective conduction pathway (27) and thus permits prokaryotic (28) and eukaryotic transporters (29, 30) to operate as glutamate-gated anion channels.

Although archaeal transporters represent excellent models for multiple aspects of brain transporter function, there is an important difference between the eukaryotic and archaeal transporters. Specifically, GlpK and GltK do not recognize l-glutamate and therefore actually function as aspartate transporters (14). Because HP2 appears to be a molecular determinant of the induced fit (22), this reentrant loop likely contains residues that are involved in substrate specificity. We have therefore studied the transport properties of selected GlpK-HP2 mutants to better understand how GlpK selects against L-Glu and for L-Asp.

### Experimental Procedures

**Mutagenesis, Expression, and Purification of GlpK**—Wild type (WT)-GlpK, containing a C-terminal His8 tag and cloned into the pBAD24 vector was a gift of Eric Gouaux. The cDNA of the coding region of GlpK-WT was cloned into pBluescript KS+ using BamHI and EcoRI. Single-stranded uracil-containing DNA was prepared from this construct, and mutations were introduced as described previously (31, 32). The mutations were verified by sequencing and transferred back into the original construct in the pBAD24 vector using unique restriction enzymes. Heterologous expression of the mutants and the WT were verified by sequencing and transferred back into the original construct in the pBAD24 vector using unique restriction enzymes.

**Reconstitution and Radioactive Uptake**—This was performed as described using Sephadex G-50 spin columns (1, 9). Briefly, 20 μl of purified transporter solution, containing 0.1% n-dodecyl β-D-maltopyranoside, was mixed with liposomes (4 μmol of asolectin and 0.7 μmol of brain phospholipids) resuspended in the desired “in” medium (see below), 400 mM NaCl, and 1% cholate in a final volume of 200 μl. After a 10-min incubation on ice, the mixture was centrifuged through the spin column to verify that the results of the transport experiments were not influenced by the tag.

**Fluorescence Measurements**—Fluorescence of the L130W parent and mutant transporters in detergent micelles was measured in a QuantaMaster 4 spectrophuorometer (Photon Technology, Inc.). Prior to measurements, to remove potentially bound amino acids, the purified protein was subjected to four successive purification steps using disposable desalting columns (GE Healthcare) equilibrated with 200 mM choline chloride, 20 mM Tris, pH 7.4, 0.05% n-dodecyl β-D-maltopyranoside, followed by one additional step with measuring buffer (200 mM NaCl, 20 mM Tris, pH 7.4, 0.05% n-dodecyl β-D-maltopyranoside). Trp-130 fluorescence titrations of the transporters with amino acids were done under constant stirring at temperatures of 22–25 °C in a 1-cm cuvette. The protein was diluted to 50–250 nM in measuring buffer, and responses to L-aspartate or L-glutamate were registered after adding aliquots of concentrated stock solutions. Fluorescence emission at 320 nm upon excitation at 295 nm was determined with the excitation and emission slits set to a bandpass of 4 nm. The measured photomultiplier photon counts were corrected for the excitation intensity. To obtain the Trp-130 fluorescence intensities, the signal of the measuring buffer alone was subtracted. The fluorescence intensity data were corrected for the error resulting from a decreased concentration of the fluorophore on the addition of any solution volume. To determine binding affinities, the Hill Equation 1,

\[
\text{Fraction of bound protein} = \frac{[L]^n}{[L]^n + K_d^n} \quad (\text{Eq. 1})
\]

where L is any ligand and \(n_H\) is the Hill coefficient, was fitted to the resulting amino acid concentration dependences of the fluorescence intensity. In cases where the GlpK concentration was higher than the expected \(K_d\), the quadratic Morrison Equation 2 (33),

\[
\text{Fraction of bound protein} = \frac{K_d + [P] + [L] - \sqrt{(K_d + [P] + [L])^2 - 4[P][L]}}{2[P]} \quad (\text{Eq. 2})
\]

where P is protein, was employed.

**Statistical Evaluations**—The significance of differences in transport between the mutants and WT and under the indicated conditions was determined using a one-way analysis of variance with a post hoc Dunnett’s multiple-comparison test (Figs. 5 and 6) or a type 2 two-tailed t test (Figs. 8 and 9).
Results

Substrate Binding by HP2 Mutants—To identify molecular determinants for the difference in substrate selectivity between mammalian EAATs and GltPh, we initially searched for amino acid residues within HP2 that are in close proximity to the substrate and conserved between the mammalian EAATs yet differ in GltPh. Such residues are expected to have a smaller side chain in the EAATs than their GltPh counterpart to accommodate the larger glutamate in the substrate binding pocket. Two residues fulfill these criteria, namely Thr-352 (GltPh numbering) which is Ala in the eukaryotic transporters, and Met-362, which is slightly larger than the Thr occupying this position in the eukaryotic transporters (Fig. 1C). In the outward-facing Asp-bound structure of GltPh (Protein Data Bank code 2NWX), the methyl group of Thr-352 (GltPh numbering) is only ~3.6 and ~4.3 Å away from an oxygen atom of the β-carboxyl group and the β-carbon of the bound aspartate substrate, respectively, and could clash with bound glutamate. We also considered Met-362 (Fig. 1), because in an alternative side-chain conformation, such as that seen in the inhibitor-bound conformation of GltPh (14), this residue could potentially be very close to the bound substrate. Therefore, we studied the effects of the T352A and M362T mutations on binding and transport of aspartate and glutamate by GltPh.

Coupled binding of aspartate and Na$^+$ to GltPh can be monitored in GltPh-L130W; this tryptophan is introduced at the junction between TM4 and the loop connecting TM3 and -4. This mutation enables the measurement of substrate-dependent changes of fluorescence signals by GltPh (14). The L130W mutation does not affect functionality; the rate of L-aspartate transport by the reconstituted L130W proteoliposomes under standard transport conditions actually exhibited an even higher uptake rate than GltPh-WT, namely 60.3 ± 15.2 versus 34.9 ± 5.2 pmol/μg of protein/3 min ($n = 3$). We introduced the T352A and M362T mutations singly and together in the L130W background. GltPh-L130W and the three mutant transporters were expressed and purified. The concentration dependence of L-glutamate-induced fluorescence increase was monitored with the purified proteins in detergent micelles (Fig. 2). We chose a high sodium concentration (200 mM) to approach saturation of the sodium-binding sites that are occupied before amino acid binding (22). Fitting the Hill equation to the dose-response curves yielded a $K_D$ of 12.6 ± 0.7 μM ($n = 4$) for L-glutamate binding to GltPh-T352A/L130W. This is almost...
better than L-glutamate. In contrast, the M362T mutant disassess this, we measured L-aspartate binding to the side-chain apparent affinities through a change in sodium binding. To increase in acidic amino acid binding or an indirect change of acids are involved in substrate selectivity.

Apparent substrate affinity to GltPh-T352A/L130W is an effect that is specific to L-glutamate, although L-aspartate still binds the double mutant, the increase in substrate affinity is highly specific for L-glutamate, which is demonstrated by an L-aspartate-binding $K_D$ that is only slightly lower than that of L130W (Fig. 3 and Table 1). These results suggest that Thr-352 is an important molecular determinant of the specificity for L-aspartate over L-glutamate, whereas Met-362 is responsible for the affinity of both amino acid types to GltPhp.

Acidic Amino Acid Transport by T352A—To compare the transport properties of GltPhp-WT and GltPhp-T352A, the purified transporters were reconstituted into liposomes. Dilution of K⁺-loaded proteoliposomes into an external solution containing Na⁺ and the potassium ionophore valinomycin resulted in the imposition of an inward-directed electrochemical potential gradient for Na⁺. We observed similar initial rates and extent of L-[³H]aspartate transport by GltPhp-T352A and GltPhp-WT (Fig. 4), and the same was true for transport of D-[³H]aspartate, which is also a substrate of GltPhp (data not shown) (14). The kinetic parameters of L-[³H]aspartate transport by T352A and WT were also similar (Table 2). However, although no detectable transport of L-[³H]glutamate was observed with liposomes inlaid with GltPhp-WT, T352A was able to transport this substrate, albeit with a lower rate than that of L-[³H]aspartate (Fig. 4). The $V_{\max}$ value for glutamate is around 5-fold lower than that of aspartate and the $K_m$ value is increased by a similar factor (Table 2).

We next studied the ion dependence of L-[³H]glutamate uptake in the GltPhp mutants. Uptake of L-[³H]glutamate by T352A liposomes is strictly dependent on external sodium.

![Figure 2](image2.png)

**Table 1**

| Substrate | $K_D$ (nM) | $V_{\max}$ (pmol/mg/min) |
|-----------|------------|--------------------------|
| GlutPhp   |            |                          |
| T352A     | 4.9 ± 2.1  | 237 ± 55                 |
| T352A     | 5.2 ± 2.5  | 12.6 ± 0.7               |
| M362T     | 0.3 ± 0.1  | 4.7 ± 0.3                |
| T352A/M362T| 1.4 ± 0.2  | 1.5 ± 0.1                |

![Figure 3](image3.png)
because it was not observed when sodium was substituted by lithium or by choline (Fig. 5A). Because of the outward K+ gradient, addition of the potassium ionophore valinomycin is expected to result in the generation of an interior negative membrane potential. Repeating the experiments in the absence of valinomycin led to a pronounced reduction of uptake (Fig. 5A), which permeability that nigericin does not fully dissipate the ion gradients expected to result in the generation of an interior negative membrane potential by enabling anions to enter the proteoliposomes (28). With Tris as the internal cation and the highly permeant nitrate as the external anion, the rate of L-glutamate uptake approached that of the potassium-loaded T352A proteoliposomes (Fig. 5B). In the presence of the impermeant gluconate anion (34), the rate of L-[3H]glutamate uptake by the Tris-containing proteoliposomes was markedly reduced. Intermediate rates were observed with external chloride (Fig. 5B), which permeates the anion-conducting pathway faster than gluconate but slower than nitrate (27–29). The ionophore nigericin, which under our experimental conditions is expected to exchange internal potassium with external sodium and thereby dissipate the electrochemical sodium gradient, almost totally blocked the uptake of glutamate (Fig. 5A).

The ionic requirements for L-aspartate uptake by the GltPh-T352A liposomes (Fig. 5A) differed from those of L-glutamate uptake (Fig. 5A). In contrast to L-glutamate uptake, significant L-aspartate uptake was also seen in the presence of lithium, although sodium was more effective (Fig. 6A). Surprisingly, uptake of L-aspartate in the presence of either lithium or sodium was only partly sensitive to nigericin, even though this ionophore was expected to dissipate the electrochemical sodium gradient. In contrast, no L-aspartate uptake could be detected under other conditions that lack sodium, namely when sodium was replaced by choline (Fig. 6A). Furthermore, it should be noted that the stimulation of L-aspartate uptake by valinomycin was much smaller than that of L-glutamate (Figs. 5A and 6A). To determine whether the T352A mutation influences the characteristics of L-aspartate uptake, we also analyzed the properties of GltPh-WT. As can be seen in Fig. 6, A and B, the results for GltPh-WT were very similar, including the surprising partial inhibition by nigericin. To eliminate the possibility that nigericin does not fully dissipate the ion gradients...
during the 3 min of aspartate uptake, we did experiments with l-aspartate added to the reaction mixtures 3 min after the proteoliposomes. However, the inhibition by nigericin was still partial (data not shown).

Based on the above results, we considered the possibility that some of the measured l-aspartate uptake might reflect binding of l-aspartate rather than transport. In particular, under the conditions of the experiments depicted in Fig. 4, the number of aspartate molecules taken up was similar to the number of GltPh-T352A and WT monomers. In contrast to binding, during transport it should be possible to establish substrate/monomer ratios much larger than one. Therefore, we increased the aspartate concentration to near-saturating levels (Table 2) by the addition of 1 μM unlabeled aspartate and extended the incubation times to achieve maximal accumulation levels. The experiments depicted in Fig. 7 show that under these conditions the number of substrate molecules per transporter monomer was markedly increased. The average numbers for aspartate uptake were 4.1 ± 0.9 (n = 4) and 3.8 ± 0.9 (n = 4) for T352A and WT, respectively. In contrast to experiments without nigericin, aspartate uptake in the presence of the ionophore stayed virtually constant after 5 min under these conditions. Consequently, the nigericin-resistant l-[3H]aspartate uptake was around 10–15% as compared with 50–60% under standard transport conditions (Fig. 6) and approached that of l-[3H]glutamate under the same conditions (Fig. 7, A-C), meaning that at saturating l-aspartate concentrations most of the uptake reflects transport. With l-glutamate (Fig. 7A) an even higher substrate per monomer ratio than with l-aspartate was reached, namely 16.0 ± 0.9 (n = 6) after 2 h, and this ratio increased further at longer incubation times. The percentage of the nigericin-insensitive component of l-aspartate uptake at 30 °C was also reduced relative to that at 22 °C (data not shown), presumably because the Q10 for transport is much higher than that for binding. Thus, the nigericin-insensitive l-[3H]aspartate uptake component by GltPh-WT and -T352A, seen under standard transport conditions (Fig. 6, A and B), is likely due to binding. Such a component was not seen with l-[3H]glutamate, probably because of the lower apparent affinity of GltPh-WT for this substrate than for l-aspartate (Tables 1 and 2).

Impact of M362T on Substrate Specificity of Transport—The T352A and M362T mutants both improved l-glutamate binding relative to GltPh-WT and acted synergistically in the T352A/M362T double mutant (Fig. 2). In contrast, l-[3H]glutamate uptake was observed with GltPh-T352A (Figs. 4, 5, 7, and 8A) but not with GltPh-M362T, even though the latter was capable of l-[3H]aspartate uptake (Fig. 8B). Moreover, the M362T mutation did not affect the ability of T352A to transport l-[3H]glutamate. The GltPh-T352A/M362T double mutant exhibited l-[3H]glutamate uptake (Fig. 8C) with similar kinetic parameters as the T352A single mutant (Table 2). Because binding experiments by the mutants (Figs. 2 and 3 and Table 1) were performed in the background of the L130W mutation, we also analyzed the substrate specificity of transport by the same mutants. To ensure that the L130W mutation had no effect on the transport characteristics, we first performed a few control experiments. Similar to GltPh, l-[3H]aspartate uptake by L130W was sodium-dependent, because when sodium was replaced by choline, uptake was undetectable. Moreover, the percentage of nigericin-resistant uptake under standard transport conditions was 43.4 ± 6.1%, which is similar to that by GltPh-WT (Fig. 6). As can be seen in Fig. 9, the substrate specificity of transport was not affected by the simultaneous presence of the L130W mutation (Figs. 8 and 9).

Discussion

Although eukaryotic SLC1 transporters transport glutamate and aspartate with similar efficiencies (35), the archaeal homologue GltPh is actually an aspartate transporter that interacts only very weakly with l-glutamate (14). In several GltPh structures, the bound l-aspartate appears coordinated by the side chains of TM7 and TM8 residues (13, 14). Specifically, Thr-314 and Arg-397 interact with the β-carboxyl group of the substrate; Asn-401 interacts with the α-carboxyl group, and Asp-394 and Thr-398 interact with the α-amino group of the bound substrate. These residues are also fully conserved in the eukaryotic glutamate transporters, and therefore other side chains are expected to play a role in this difference in substrate selectivity. Here, we have identified two HP2 residues that serve as molecular determinants of substrate binding to GltPh (Figs. 2 and 3).

In the outward-occluded conformation of GltPh, the HP2 loop has closed onto the binding pocket (13). The non-transportable aspartate analogue DL-threo-β-benzoyloxyaspartate is coordinated by the same side chains, but the bulky phenyl group of the analogue prevents the closure of HP2, thereby locking the transporter in the outward-open conformation (14). Our data suggest that when position 352 of HP2 is occupied by Thr-352, the loop can occlude the bound aspartate but not glutamate, whose side chain is longer; the extra methylene group is predicted to result in a clash with Thr-352 (Fig. 1D). A
previous study concluded that the substrate (aspartate)-induced fluorescence change in L130W-GltPh is caused by HP2 closure (22). As judged from the fluorescence change upon glutamate titration with L130W-GltPh (Fig. 2), HP2 apparently also closes over glutamate in the presence of the WT side chain at Thr-352, albeit with a reduced probability, resulting in a dramatically decreased apparent affinity as compared with T352A-GltPh (Table 1). The notion of glutamate providing a steric hindrance to HP2 closure also provides an explanation for an earlier observation made prior to the determination of the steric accessibility of Thr-352 of GltPh, to sulfhydryl reagents was decreased by aspartate but not by glutamate (36). This reduction presumably reflects a steric block by bound substrate. It was further found that glutamate could bind to A348C-GLT-1, because in the presence of a large excess of glutamate, aspartate could not protect Cys-438 (36). Thus, it is likely that glutamate transport by GltPh is more efficient.

With saturating substrate concentrations, we observed aspartate and glutamate ratios per transporter that are significantly higher than one (Fig. 7), indicating that under these conditions a substantial part of the measured radioactive “uptake” represents transport rather than binding. The reason for the higher substrate/transporter ratios for glutamate than for aspartate is not clear. A possible explanation could be some slippage of sodium unaccompanied by substrate through the transporter. This slippage could be more pronounced for aspartate than for glutamate and thereby lead to less accumulation of the former. Previous studies by other groups do not hint at a binding component of the uptake of aspartate by WT (14, 28, 38), but this discrepancy is likely due to differences between the reconstitution protocols used. The idea that some of the measured L-aspartate uptake might reflect binding of L-aspartate rather than transport could perhaps explain the shape of the uptake kinetics observed in Fig. 4. It is likely that the fast and slow components represent binding and transport, respectively.

Lithium partly replaced sodium to support uptake of aspartate (Fig. 6A), but not that of glutamate (Fig. 5A). This lithium-dependent uptake was partially inhibited by nigericin just like the sodium-dependent uptake. Because nigericin is able to dissipate the driving force for transport, the nigericin-sensitive aspartate uptake probably represents transport. The dependence of the cation selectivity on the nature of the substrate is reminiscent of similar observations made with the neuronal transporter EAAC1 (39, 40). These substrate-dependent effects in GltPh and EAAC1 may be explained by a direct substrate-ion interaction, as observed in one of the proposed Na3 sites, which is formed by the side chains of conserved threonine and asparagine residues from TM7 and -8 as well as carboxyl oxygens of the acidic amino acid substrate (41). Alternatively, differential roles of Li+ could also be explained by a substrate-dependent alteration of the positioning of HP2, which would affect coordination of cation in the Na2 site; the backbone carbonyl of T352A, in particular, contributes directly to the Na2 site (Fig. 1, B and D).

The most intriguing result in our study is that although glutamate induced the tryptophan-sensitive conformational change in M362T (Table 1 and Fig. 2), the same mutant could not transport this amino acid (Figs. 8 and 9). Apparently, the smaller side chain of Thr at position 362 of HP2 also accommodates the occlusion of glutamate, and yet the mutant transporter cannot mediate one or more of the subsequent steps of the transport cycle such as the translocation by the so-called
elevator movement (15, 25). In particular, the relative positioning or internal structure of HP2 is likely to be sensitive to the bound substrate, as illustrated by a comparison of inward-facing apo and inward-facing Asp-bound structures of GltPh (10, 14), where the position of Met-362 changes by ~3 Å. Moreover, Met-362 forms contacts with TM2 in these inward-facing states, specifically with Ile-61 and Val-62, which are replaced by the relatively similar residues Leu and Ile, respectively, in EAAT1–4. Whatever the cause, the differential impact of mutation on binding and transport of glutamate suggests that HP2 plays a role not only in the selection of the substrate, but also in its translocation. It can be anticipated that additional functional studies on GltPh and other transporters with known structures will lead to further insights into the mechanism of transport.

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