Induced fit substrate binding to an archaean glutamate transporter homologue

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Excitatory amino acid transporters (EAATs) are a class of glutamate transporters that terminate glutamatergic synaptic transmission in the mammalian CNS. GltPh, an archaean EAAT homolog from Pyrococcus horikoshii, is currently the only member with a known 3D structure. Here, we studied the kinetics of substrate binding of a single tryptophan mutant (L130W) GltPh, in detergent micelles. At low millimolar [Na+]fl, the addition of L-aspartate resulted in complex time courses of W130 fluorescence changes over tens of seconds. With increasing [Na+]fl, the kinetics were dominated by a fast component (kobs,fast, KD (Na+)) = 22 ± 3 mM, nHill = 1.7 ± 0.3) with values of kobs,fast rising in a saturable manner to ≈500 s−1 (at 6 °C) with increasing [L-aspartate]. The binding kinetics of L-aspartate differed from the binding kinetics of two alternative substrates: L-cysteine and D-aspartate. L-cysteine sulfinic acid bound with higher affinity than L-aspartate but involved lower saturating rates, whereas the saturating rates after D-aspartate binding were higher. Thus, after the association of two Na+ to the empty transporter, GltPh, binds amino acids by induced fit. Cross-linking and proteolysis experiments suggest that the induced fit results from the closure of helical hairpin 2. This conformational change is faster for GltPh than for most mammalian homologues, whereas the amino acid residue binding rates are similar. Our data reveal the importance of induced fit for substrate selection in EAATs and illustrate how high-affinity binding and the efficient transport of glutamate can be accomplished simultaneously by this class of transporters.

In the CNS of vertebrates, excitatory amino acid transporters (EAATs) catalyze the energetically uphill movement of glutamate into glial and neuronal cells by coupling to the passage of sodium and potassium ions as well as protons along their respective electrochemical gradients (1). High-resolution crystal structures of a prokaryotic member of the EAAT family, the archaean aspartate transporter GltPh, in aspartate- or inhibitor-bound conformation showed the substrate binding site to be either occluded from or exposed to the extracellular solvent, depending on the position of one of the reentrant loops [helical hairpin 2 (HP2)] (2) (Fig. 1A and B). Many secondary-active transporters use an alternating access mechanism of transport (3). In its simplest form, two gates open and close alternately and thereby, allow or prevent the entry of the transported substrate(s) from both membrane sides into an aqueous conduction pathway with a central binding site (single binding center gated pore) (4). Initially, a mechanism with HP2 acting as the extracellular gate was considered sufficient to explain the coupled transport by GltPh. However, the structural identification of the inward facing state of the transporter revealed a large, piston-like movement of the substrate binding domain as an essential rearrangement underlying substrate transport (5, 6) (Fig. 1C).

Many enzymes function according to an induced fit mechanism (7): after an initial loose association of the substrate, the enzyme proceeds to a state where the substrate is bound tightly, thereby releasing additional energy for catalytic function. For transporters, the induced transition fit concept proposes that this energy is used to drive the conformational changes that are necessary for the substrate translocation (8). The transition leading to the fit state can consist of only side chain rearrangements (9), or it can comprise conformational changes of the peptide backbone (10). Because different ligands induce fit states that differ in tightness and/or differently affect the function of the protein, induced fit contributes to selectivity (11). In GltPh, HP2 closes after the association of the substrate from the external membrane side and might be involved in substrate selection by induced fit.

Here, we performed stopped-flow experiments on detergent-solubilized GltPh while using the intrinsic fluorescence intensity variation of the tryptophan mutation L130W as well as the tryosine fluorescence of the WT transporter. Our data clarify the order of the steps in the binding sequence and reveal the rates of both substrate binding and the associated conformational changes. Our findings permit a functional comparison of archaean and mammalian EAAT homologues and delineate a role for HP2 in substrate selection by induced fit in these transporters.

**Results**

**Fluorescence Spectra of L130W and WT GltPh Reflect the Binding of Na+ and Aspartate.** L130W GltPh contains a single tryptophan at the junction between the transmembrane domain (TM) 4 and the TM3–TM4 loop (Fig. 1C). W130 reports on the coupled binding of aspartate and Na+ by a fluorescence increase and allows for the quantification of binding by monitoring intrinsic protein fluorescence (2). We expressed and purified WT and L130W GltPh and measured the spectral properties of proteins in detergent micelles. The emission spectra of L130W GltPh peaked at ~318 nm (Fig. 1D), which suggests an exposition of W130 to a hydrophobic environment (12). On the addition of L-aspartate to L130W GltPh in the presence of 100 mM Na+, the emission increased without a change in spectral shape. The sole addition of 100 mM Na+ resulted in a relatively small increase that was limited to lower wavelengths, which indicates an origin other than the fluorescence increase on the coupled binding of Na+ and aspartate. The addition of aspartate in the absence of Na+ was previously reported to have no effect on fluorescence when monitored at a single wavelength (2).

Exciting WT GltPh at 295 nm results in fluorescence with a similar emission maximum to L130W GltPh. This fluorescence is most likely caused by tyrosine, because WT GltPh contains 18 tyrosine residues and no tryptophan (SI Results). The addition of

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**Na\(^+\) results in a small shift to a higher intensity that is limited to lower wavelengths. The extent of the increase becomes considerable when a shorter excitation wavelength is used (Fig. 1E). On the addition of aspartate, an additional smaller increase is detectable.**

These results show that L130W GltPh fluorescence is well-suited to report on the coupled binding of aspartate and Na\(^+\), whereas the tyrosine fluorescence of WT GltPh permits the monitoring of Na\(^+\) binding to the empty transporter.

**Rapid Substrate Addition Reveals Slow Processes That Precede Aspartate Association.** Fig. 2 shows the L130W GltPh fluorescence changes on the simultaneous rapid addition of 1-aspartate and Na\(^+\). The results are compared with those of aspartate-free protein with two substrates, we observed a complex time course of fluorescence increase on timescales ranging from milliseconds to seconds. In contrast, on the addition of L-aspartate to Na\(^+\)-preincubated protein, almost all of the fluorescence change occurred within a few milliseconds. We conclude that the Na\(^+\)-bound state is a prerequisite for fast aspartate binding. The observed slow transitions represent processes that lead to the state from which aspartate binding occurs, such as the initial Na\(^+\) binding and either preceding or consecutive events. In accordance with this conclusion, the overall rate of the fluorescence increase after the simultaneous addition of the substrates is Na\(^+\)-dependent (Fig. S1).

**Two Na\(^+\) Bind Before Aspartate.** To determine the [Na\(^+\)] dependence of relative amplitudes and the observed rates ($k_{obs,fast}$) of the fast process, we fitted sums of exponential functions to the fluorescence transients resulting from the addition of 1-aspartate after preincubation with various [Na\(^+\)] (Table S1 shows the results). When using protein preincubated with 5 mM Na\(^+\), most of the fluorescence change after 1-aspartate addition was slow, with only a small fraction of the amplitude assigned to $k_{obs,fast}$ (Fig. 2B and Table S1). Increasing [Na\(^+\)] resulted in higher relative amplitudes of $k_{obs,fast}$, whereas the fitted values of $k_{obs,fast}$ were around 400 s\(^{-1}\) and did not depend on Na\(^+\). At 400 mM Na\(^+\), the time course was entirely dominated by $k_{obs,fast}$—Fig. 2C.
shows the relative amplitudes of $k_{obs,fast}$ plotted against the [Na$^+$]. A fit of this relationship with a Hill function yields a Hill coefficient greater than one ($n_{Hill} = 1.7 \pm 0.3$, $K_D = 22 \pm 3 \text{ mM}$; $n = 3$), which is in agreement with at least two Na$^+$ binding before aspartate. Given that one Na$^+$ has to bind after aspartate (2) and the total number of transported Na$^+$ is three (13), we conclude that two Na$^+$ bind before aspartate binding.

For lower [Na$^+$], the fits with sums of exponentials did not provide an unambiguous quantitative description of the remaining, slower processes. The fit results were satisfying for individual traces but occasionally provided inconsistent rate constants on repetition of the experiment under the same experimental conditions, presumably because the signal-to-noise ratio of the data was not always sufficient to detect all underlying processes. Thus, we only used the values and the amplitudes of $k_{obs,fast}$ for additional analysis.

L130W might have affected substrate binding, and we, therefore, performed additional experiments exploiting the tyrosine emission of WT GltPh (Fig. 2D). The increase in fluorescence intensity with [Na$^+$] could be fitted with a Hill function yielding parameters that, within the experimental error, did not differ from the results obtained with rapid aspartate binding to L130W GltPh$_{\text{ WT}}$ ($n_{Hill} = 1.9 \pm 0.2$, $K_D = 25 \pm 2 \text{ mM}$; $n = 3$). In addition, the kinetics of Na$^+$ binding to WT GltPh$_{\text{ WT}}$ are comparable with the kinetics observed after mixing t-aspartate and Na$^+$ with L130W GltPh$_{\text{ WT}}$ (Fig. S2). Thus, L130W leaves the initial sodium binding largely unperturbed.

**Induced Fit Mechanism Allows for Substrate Selectivity.** Fig. 3A shows the fluorescence time course of Na$^+$-bound L130W GltPh$_{\text{ WT}}$ on addition of various concentrations of t-aspartate. The rate constant of this process, $k_{obs,fast}$, increases in a saturable manner with [t-aspartate] (Fig. 3B), which is expected for $k_{obs,fast}$ reflecting t-aspartate binding. The observed deviation from a linear [t-aspartate] dependence—predicted by a one-step association of the substrate and transporter (14)—indicates an additional unimolecular reaction that limits the overall rate at high [t-aspartate].

The temperature dependence of the aspartate association kinetics at saturating [t-aspartate] (500 µM) (Fig. 3B) provides an activation energy ($E_a$) of 68 ± 6 kJ/mol, which is equivalent to a $Q_{10}$ of 2.7 for the rate limiting step (Fig. 3C). These values correspond to an energy barrier too high to be assigned to a diffusion-controlled reaction and therefore, provide additional evidence for a conformational change associated with t-aspartate binding.

This conformational change could occur after the association of the substrate to the binding site, a scenario that is found in an induced fit mechanism (7) (Scheme 1). The alternative mechanism, where a conformational change is necessary to permit subsequent substrate association (Scheme 2), is commonly referred to as conformational selection (15):

$$\text{P} + \text{L} \xrightarrow{k_w} \frac{k_w}{k_{off}} \text{PL} \xrightarrow{k_2} \text{P}^* + \text{L} \xrightarrow{k_{off}} \text{P}^* + \text{L} \xrightarrow{k_2} \text{P}^* \text{L} \text{[Scheme 2]},}$$

where L is the ligand and P or P* denote distinct conformations of the protein.

Both mechanisms can result in saturating behavior, because the overall reaction is limited by the unimolecular reaction. One way to discriminate kinetically between Schemes 1 and 2 is to use alternative substrates (16, 17). In the case of an induced fit mechanism, the ligands are already associated to the protein and may modulate the unimolecular reaction rate (i.e., the nature of the ligand can affect $k_1$ and $k_2$, which results in distinct saturation levels for different ligands). If conformational selection was operating, the unimolecular step would occur in the absence of the ligand, and $k_1$ and $k_2$ would be independent of the properties of the ligand.

We determined $k_{obs,fast}$ for different concentrations of two additional ligands, D-aspartate and t-cysteine sulfinic acid (t-CS). L-CS is a substrate with comparable transport activity with t-aspartate (Fig. S3C) but lower affinity (2) (Fig. S3A and B). To date, it has been unclear whether GltPh$_{\text{ WT}}$ distinguishes between t- and l-aspartate (2, 18). The saturating values of $k_{obs,fast}$ provide a clear distinction between t-aspartate and the tested alternative ligands, because they were larger for D-aspartate and less than...
one-half as large for L-CS (Fig. 3B), which is in agreement with induced fit (Scheme 1).

Scheme 1 predicts fluorescence kinetics with two exponential components. Our experiments were performed under pseudo-first order conditions, with [L] in large excess over [P]. This case leads to two nonzero solutions for the observed rate constants (SI Results shows their derivation), of which only the solution representing the slower of the two components accounts for the saturating behavior of \( k_{\text{obs,fast}} \) (Fig. 3B):

\[
k_{\text{obs,fast}} = \frac{1}{2} \left[ (k_{\text{on}} [L] + k_{\text{off}} + k_{1} + k_{2}) - \sqrt{([k_{\text{on}} [L] + k_{\text{off}} - k_{1} - k_{2}]^2 + 4k_{1}k_{2})} \right].
\]  
(1)

We fitted Eq. 1 to the [amino acid] dependencies of \( k_{\text{obs,fast}} \) to determine the substrate association/dissociation rates (\( k_{\text{on}}, k_{\text{off}} \)) as well as the forward rate constant of the conformational change (\( k_{1} \)) (Table 1). These fits reliably constrain \( k_{1} \), which results in distinct values for all tested substrates as anticipated above. For the binomolecular step, we find similar association rate constants (\( k_{\text{on}} \)), whereas the \( k_{\text{off}} \) values differ among substrates. The comparison of the L- or D-aspartate and L-CS data illustrates a crucial role for the induced fit mechanism for substrate selection. L-CS exhibits a considerably smaller \( k_{\text{off}} \) value, which results in tighter initial binding. However, the higher forward rates of the subsequent conformational change promote the higher apparent constants for the remaining parameters. Although it was not possible to determine \( k_{2} \) values by fitting, these values can be estimated from the apparent binding affinities measured at 20 °C (2) according to \( k_{2} = (k_{\text{Dissoc}} / k_{\text{on}})k_{1} \), when assuming that there are no differences in the temperature dependencies for the diverse substrates. The resulting \( k_{2} \) values are smaller for L- or D-aspartate (0.1 or 0.2 s\(^{-1}\)) than for L-CS (3.7 s\(^{-1}\)). Thus, aspartate exhibits higher rates of establishing induced fit, and the lower rates back to the loosely bound state. The simulated binding time courses based on the obtained values of the rate constants show consistent agreement with experimental traces and sensitivity to parameter changes (Fig. S4), showing the accuracy of our model.

**W130 Fluorescence Changes Do Not Require Isomerization to the Inward-Facing State.** Crystallographic studies revealed that HP2 alternately exposes and occludes the substrate binding site (2), which makes the latter movement a perfect candidate for a conformational change involved in an induced fit mechanism. However, there are additional transitions within the transport cycle that might contribute to the observed fluorescence changes.

To test for a possible contribution of movements of the transport domain relative to the trimerization domain (5, 6, 19, Table 1. Best fit parameters of the \( k_{\text{obs,fast}} \) substrate dependence

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>( k_{\text{on}} ) (10(^{-3}) M(^{-1}) s(^{-1}))</th>
<th>( k_{\text{off}} ) (s(^{-1}))</th>
<th>( k_{1} ) (s(^{-1}))</th>
<th>( k_{2} )</th>
<th>( K_{D} ) (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-aspartate</td>
<td>3.0 ± 0.2</td>
<td>250 ± 40</td>
<td>506 ± 4</td>
<td>n.d.</td>
<td>8.3 ± 2.0</td>
</tr>
<tr>
<td>D-aspartate</td>
<td>2.8 ± 0.5</td>
<td>200 ± 320</td>
<td>750 ± 10</td>
<td>n.d.</td>
<td>6.2 ± 20</td>
</tr>
<tr>
<td>L-CS</td>
<td>3.9 ± 0.6</td>
<td>41 ± 19</td>
<td>194 ± 5</td>
<td>n.d.</td>
<td>1.1 ± 0.7</td>
</tr>
</tbody>
</table>

n.d., not determined.

*Intrinsic dissociation constant calculated from \( K_{D} = k_{\text{off}} / k_{\text{on}} \).

†The SE of the fit is indicated.

‡Parens contain 95% confidence intervals for the measured rate constants and 90% confidence intervals for calculated \( K_{D} \) values.

§\( k_{2} \) was fixed to 10 s\(^{-1}\) in all fits.

![Fig. 4. Structural basis for W130 fluorescence changes.](A) The sites of cross-linking mutations in cartoon representations of a single GltPh protomer in (A) the inward-facing conformation (K55C/A364Q or D) the aspartate-bound outward-facing conformation (V216C/A391C). The amino acids mutated to cysteines are in magenta or green, and the protein backbone is in wheat. (B and E) Nonreducing SDS/PAGE of (B) K55C L130W C321S A364Q GltPh or (E) L130W V216C C321S A391C GltPh. (F) As a reference for the relative L-aspartate induced fluorescence changes, the data from untreated L130W GltPh (±SEM; n = 5) are shown. In the double cysteine mutants, the error bars represent the SEM of (C) three or (F) four experiments. (G) The factor Xa cleavage site in a ribbon presentation of the aspartate-bound L130W GltPh protomer with the N- and C-terminal cleavage products in green and wheat, respectively. (H) An SDS/PAGE of the factor Xa cleavage products of AGH111-114IDGR L130W GltPh. (I) The mean relative fluorescence response to the addition of 500 μM aspartate to the cleaved and uncleaved mutant in 500 mM NaCl (bars). The values of individual experiments are shown as filled circles.
we studied the effect of cross-linking the transporter in either the inward- or outward-facing conformation on the observed fluorescence changes. The oxidative cross-linking of two inserted cysteines, C55 and C364, traps GltPh in the inward-facing conformation (5) (Fig. 4A). After cross-linking L130W K55C C321S A364C GltPh with Cu(II)(1,10-phenanthroline)_3 (CuPh), W130 fluorescence is independent of L-aspartate (Fig. 4C). When cross-linking is prevented by the addition of N-ethylmaleimide before CuPh, the mutant transporters show a significant fluorescence increase on aspartate addition. These data show that the observed fluorescence changes rely on conformations other than an inward-facing GltPh. We noticed a lower relative fluorescence change in the mutant compared with L130W GltPh (Fig. 4F), which may have resulted from N-ethylmaleimide treatment or other incubation conditions by an unknown mechanism.

To test whether the fluorescence signals report on conformational changes of GltPh, in the outward-facing conformation, we sought to trap the transporter in the outward-facing conformation with the help of suitable cysteine insertions. V216 and A391 show sufficient proximity for cysteine cross-linking in the outward-facing conformation (Fig. 4D) (Cm distance: 6.6 Å) but not the inward-facing conformation (Cm distance: 16.4 Å). SDS/PAGE analysis shows two electrophoretic species of L130W V216C A391C GltPh (Fig. 4E). Whereas CuPh treatment does not change the proportions of these two electrophoretic species, the apparent lower molecular weight species disappears after treatment with DTT and therefore, corresponds to an intramolecularly linked (outward-facing) conformation. This species accounts for 75–80% of the transporters after CuPh treatment (Fig. S5B), and a fluorescence change of ≈15% is observed on L-aspartate addition, which is roughly identical to 75% of the fluorescence change that is observed after amino acid binding to L130W GltPh (Fig. 4F). The identity of the remaining 25% remains unclear. GltPh trapped in the outward-facing state displays similar fluorescence signals as compared to when it is free to move.

A recent study showed substrate induced changes of the TM3–TM4 loop in susceptibility to protease cleavage and that cutting either end of this loop abolishes radioactive aspartate uptake by GltPh (21). We inserted a factor Xa site at the end of the TM3–TM4 loop that is remote from W130 into L130W GltPh (Fig. 4G). Fig. 4F shows that cleavage at this site does not interfere with the W130 fluorescence changes on aspartate binding.

Another possible explanation for the observed W130 fluorescence variation is large collective motions that bring the trans-side currents and the rising phase of the anion currents to report on the substrate occluding movements of HP2. The corresponding rates for EAAT1 (1,000 s⁻¹) (26), EAA1/EAAT3 (1,200 s⁻¹) (27), and EAAT4 (340 s⁻¹) (28) are slower than the rates of GltPh (k_{off} at high [aspartate] as extrapolated to 22 °C is ~2,700 s⁻¹), whereas EAAT2 (~4,000 s⁻¹) (29) exhibits faster rates. Thus, HP2 closure by GltPh resembles its mammalian counterparts. In experiments exploiting the temperature dependence of the above rates, an enthalpy of activation for EAAC1/EAAT3 was determined that is considerably larger (121 kJ/mol) (25) than our results for the Arrhenius energy of activation (68 kJ/mol) on GltPh. This difference might be because of isoform-specific variations. Alternatively, although the activation energy of EAAC1/EAAT3 was assigned to the closure of HP2, the electrophysiological approach might reflect more extensive conformational changes, including the transition from the outward- to inward-facing state. When comparing the association of the amino acid and transporter that precedes the conformational change, we find similar rates among GltPh (k_{on}: ~3 × 10⁷ M⁻¹ s⁻¹) and the mammalian glutamate transporters (EAAT3: 2 × 10⁷ M⁻¹ s⁻¹) (30). In conclusion, our data define GltPh as an accurate model for EAAT substrate recognition.

The small transport rates of GltPh must, therefore, originate from processes that either precede or follow substrate association. Slow transitions associated with Na⁺ binding show an
increase in the overall rate with increasing [Na\(^+\)] (Fig. S1), which indicates that conformational change(s) occur after the association of Na\(^+\). Whereas Na\(^+\) has, at most, only a minor impact on the distribution of inward- and outward-facing transporters (20), the opening of HP2 has been shown to rely on the binding of Na\(^+\) (31). Furthermore, large heat capacity changes on Na\(^+\) binding were reported that cannot be attributed to HP2 opening alone (24). Therefore, the slow conformational change(s) after Na\(^+\) binding might represent slow HP2 opening and/or more extensive rearrangements, which can explain the small transport rates of GltPh. However, L130W GltPh displays significantly slowed aspartate transport compared with WT (2), whereas the kinetics of Na\(^+\) binding are similar (Fig. 2 and Fig. S1). Therefore, the processes associated with Na\(^+\) binding cannot be rate-limiting in L130W GltPh.

Carrier-mediated transport requires tight interaction on one membrane side—to enable selection among various transport substrates—as well as fast release on the other side to support effective transport. GltPh exhibits similar binding affinities for outward- and inward-facing conformations (5), and substrates with low unbinding rates will, thus, stay bound in the binding pocket and reduce transport rates. Our data illustrate how an effective transport. GltPh exhibits similar binding affinities (5), and substrates that have low unbinding rates will, thus, stay bound in the binding pocket and reduce transport rates. Our data illustrate how an effective transport.

Materials and Methods

Mutagenesis, Expression, and Purification of GltPh. The expression constructs were generated using the QuikChange Kit and WT GltPh, with a C-terminal 8x histidine tag cloned into a pBAD24 vector (provided by Eric Gouaux, Oregon Health and Science University, Portland, OR). Heterologous expression was induced by the addition of 1% \(\alpha\)-arabinose to Escherichia coli Top10 or BL21-AL cells at OD\(\text{OD}_{600}\) = 0.5–0.7. The purification of GltPh was performed as previously described (32) using 1 mM n-dodecyl-\(\beta\)-D-maltopyranoside (DDM) and omitting sodium glutamate. If necessary, the protein was concentrated using ultrafiltration cartridges. Na\(^+\) and potentially bound amino acids were removed by replacing NaCl with choline chloride using disposable salt exchange columns followed by an additional salt exchange to an NaCl-containing buffer if necessary. All buffers contained 20 mM Tris (pH 7.4), 1 mM DDM, and choline chloride or NaCl as noted in the figures.

Fluorescence Measurements. The [GltPh] was between 0.05 and 4 \(\mu\)M. The steady state fluorescence intensities of the WT and mutant GltPh were determined in a Quantamaster 4 spectrofluorometer (Photon Technology Inc.). The solutions were stirred in a 1-cm cuvette thermostatted to 22 °C. The changes in GltPh fluorescence on the rapid addition of Na\(^+\) or amino acids were measured in a stopped-flow apparatus (HITECH SF-61Xk2; TgK Scientific). Excitation was performed at 297 nm, and emission was selected using long-pass filters with cut on wavelengths of 320 or 306 nm; the latter resulted in a higher signal-to-noise ratio. If not otherwise specified, the mixing system and observation cell were at 6 °C. The measured dead time of the system was 2.3 ± 0.1 ms (n = 4) at this temperature. To determine the rates at higher temperatures, a smaller observation cell with a reaction volume of 5 \(\mu\)L (dead time = 0.9 ± 0.1 ms; n = 5) instead of 22.5 \(\mu\)L was used. The [amino acid] was maintained in at least fivefold excess over [GltPh] to fulfill pseudo-first order conditions (14).

Cysteine Cross-Linking and Factor Xa-Mediated Proteolysis. CuPh-mediated cross-linking was performed as previously described (5). Cleavage of the L130W GltPh backbone at the N-terminal border of the TM3–TM4 loop was essentially performed as previously described (21).

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Exciting WT GltP shows that of C10 on L130W GltP binding steps to the observed transition to L130W GltP L3 L binding properties are only marginally resembles the of kL at 295 nm, of the tyrosine To test the accuracy of the derived model parameters of binding. The complexity of the kinetics, emission Lfl 3 μfl Lfl ≈ than Lfl > than Lfl in addition to the uncertainties inherent in the model predictions an initial lag phase at low concentrations of amino acids that is most pronounced with L-CS. To ensure comparability, the simulated traces were treated the same way as the experimental data by extrapolating to time 0 after fitting with an exponential function (SI Materials and Methods).

Fig. S4 shows the simulated traces after such treatment along with the normalized experimental traces after t-aspartate, d-aspartate, and L-CS addition, which resulted from a range of starting parameters. The curve shapes of the simulated and experimental traces for all substrates agree over the entire substrate concentration range. To evaluate the sensitivity of the kinetics to changes in the model parameters in relation to experimental scatter, we produced additional simulations with single parameters outside the 95% confidence intervals, which are presented in Table 1. We focused on the parameters that were determined to be significantly different between the substrates, such as the dissociation rate constants of the first step (koff) as well as the forward rate constants of the conformational change (ki). For such simulations, values equidistant to the respective confidence interval boundaries were selected. The resulting traces, after exponential fitting and normalization, in some cases show remarkable changes in curve shape (e.g., when increasing k1 of L-CS binding at high concentrations) (Fig. S4C). Other deviations are more subtle but are found over the entire range from lower to higher micromolar concentrations, such as the deviations arising from decreasing the k1 of d-aspartate binding (Fig. S4B). When decreasing the k1 of t-aspartate binding (Fig. S4A), the traces originating from a small range of concentrations (5–10 μM t-aspartate) clearly indicate inadequacy of the model that contains the altered parameter. Altogether, the simulations show the sufficient sensitivity of our experimental system to discriminate between the tested model parameters of the different substrates.

**SI Results**

**Tyrosinate Fluorescence in WT GltP**. Exciting WT GltP at 295 nm, which is commonly done to elicit tryptophan emission (1), results in fluorescence with a maximum at ~320 nm, which is similar to the values observed for L130W GltP (Fig. 1D). The tyrosine emission spectra normally peak at wavelengths close to 300 nm; however, ionization of tyrosine residues causes a red shift in excitation as well as emission maxima (2), which explains the observed WT GltP fluorescence properties in the absence of any tryptophan residues. The small increase that is only found at lower wavelengths after Na+ addition to WT GltP resembles the effect of Na+ on L130W GltP fluorescence and might have the same origin. After 289 nm excitation, the WT GltP emission peaks at 306 nm (Fig. 1E), which indicates that the emission of protonated tyrosine residues instead of tyrosinates is dominating at this excitation wavelength.

**Slow Transitions Associated with Na+ Binding.** The observed [Na+] dependence of the time course of the fluorescence signals on aspartate addition (Fig. 2) indicates slow processes that precede fast amino acid binding to GltP. To study these slow processes in greater detail, we performed the simultaneous rapid addition of varying [Na+] and a fixed [t-aspartate] of 500 μM, which resulted in a clear increase in the overall rate of fluorescence change with increasing [Na+] (Fig. S1). This observation shows the contribution of one or more Na+ binding steps to the observed transitions. The observed left shift of the curves on the time axis with increasing [Na+] indicates an increase in the rate of one or more processes as opposed to an increase in the amplitude of faster processes. Thus, at least one of the processes involved occurs after Na+ binding. The complexity of the kinetics, in addition to the uncertainties inherent in fitting the sums of many exponential functions to the kinetic data, precludes a detailed quantitative analysis here.

The equilibrium Na+ binding properties are only marginally affected by the L130W mutation (Fig. 2D). Nevertheless, L130W still could have changed the kinetics of Na+ binding. To test this hypothesis, we measured the stopped-flow kinetics of Na+ (250 mM) binding to WT GltP by monitoring tyrosine fluorescence after excitation at 289 nm. We observed a similar time course (Fig. S2) as compared to the time course after the simultaneous addition of t-aspartate and Na+ to L130W GltP. The comparison of t1/2 values as a measure of overall rate (0.42 s; after the addition of 250 mM Na+ and 500 μM t-aspartate to L130W GltP, 0.1 s) (Fig. S2) shows that the slow kinetics of Na+ binding to L130W GltP are not caused by the introduced tryptophan and that L130W is a reasonable reporter mutation to monitor the kinetics of substrate binding to GltP.

**L-Cysteine Sulfonic Acid Is Transported with a Lower Affinity than t-Aspartate.** L-cysteine sulfonic acid (L-CS) exhibits an ~10-fold lower binding affinity to GltP than t-aspartate, which was shown using the variation in fluorescence of W130 (2). To examine whether this difference is maintained during transport, we measured 3H-t-aspartate uptake by GltP into proteoliposomes. Fig. S3A shows that, in presence of 10 μM cold t-aspartate, the rate of 3H-t-aspartate transport was reduced to the level of uptake into liposomes without GltP. In contrast, 10 μM L-CS did not completely abolish 3H-t-aspartate transport but resulted in an ~90% inhibition (Fig. S3B). Therefore, L-CS displays a lower affinity to GltP than t-aspartate also under transport conditions.

To assess the relative transport activities of t-aspartate and L-CS, we compared the ability of saturating concentrations of the amino acids inside the proteoliposomes to stimulate 3H-t-aspartate uptake by counterflow. A similar assay was previously used to compare transport activities of potential substrates of mammalian glutamate transporters, with the difference that the ability of external amino acids to stimulate efflux of d-aspartate from synaptosomes was tested (3). Fig. S3C shows that t-aspartate and L-CS inside the proteoliposomes increase the amount of 3H-t-aspartate that is transported after 5 min to the same degree, whereas D,L-threeβ-hydroxyaspartate (TBOA), which is thought to be a non-transportable inhibitor, does not cause an increase. We conclude that L-CS is a transported substrate of GltP, and that t-aspartate and L-CS have comparable transport activities.

**Simulation of Time-Dependent Fluorescence Changes on Amino Acid Addition.** To test the accuracy of the derived model parameters of amino acid binding (Table 1), we simulated time courses on substrate addition through the integration of the corresponding differential equations. The model predicts an initial lag phase at low concentrations of amino acids that is most pronounced with L-CS. To ensure comparability, the simulated traces were treated the same way as the experimental data by extrapolating to time 0 after fitting with an exponential function (SI Materials and Methods).

Fig. S4 shows the simulated traces after such treatment along with the normalized experimental traces after t-aspartate, d-aspartate, and L-CS addition, which resulted from a range of starting parameters. The curve shapes of the simulated and experimental traces for all substrates agree over the entire substrate concentration range. To evaluate the sensitivity of the kinetics to changes in the model parameters in relation to experimental scatter, we produced additional simulations with single parameters outside the 95% confidence intervals, which are presented in Table 1. We focused on the parameters that were determined to be significantly different between the substrates, such as the dissociation rate constants of the first step (koff) as well as the forward rate constants of the conformational change (ki). For such simulations, values equidistant to the respective confidence interval boundaries were selected. The resulting traces, after exponential fitting and normalization, in some cases show remarkable changes in curve shape (e.g., when increasing k1 of L-CS binding at high concentrations) (Fig. S4C). Other deviations are more subtle but are found over the entire range from lower to higher micromolar concentrations, such as the deviations arising from decreasing the k1 of d-aspartate binding (Fig. S4B). When decreasing the k1 of t-aspartate binding (Fig. S4A), the traces originating from a small range of concentrations (5–10 μM t-aspartate) clearly indicate inadequacy of the model that contains the altered parameter. Altogether, the simulations show the sufficient sensitivity of our experimental system to discriminate between the tested model parameters of the different substrates.

**Derivation of the Kinetic Equations.** For the reaction scheme,

\[
P + L \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} PL \overset{k_1}{\underset{k_2}{\rightleftharpoons}} P^*L,
\]

assuming [L] >> [P], [PL], and [P*L], the time-dependent relaxation of [P], [PL], and [P*L] to the new equilibrium after ligand concentration changes can be described by the transition matrix (TM):
\[
TM = \begin{bmatrix}
-Lk_{\text{on}} & k_{\text{off}} & 0 \\
Lk_{\text{on}} & -k_1 - k_{\text{off}} & k_2 \\
0 & k_1 & -k_2
\end{bmatrix}.
\]

Solving the eigenvalue problem of TM yields a set of two nonzero eigenvalues and corresponding eigenvectors, with
\[
k_{\text{eig}1} = -\lambda_1 \quad \text{and} \quad \lambda_1 = \frac{[L]k_{\text{on}}}{k_{\text{off}}},
\]
\[
k_{\text{eig}2} = -\lambda_2.
\]

Finding the eigenvalues of TM is equivalent to transforming TM to a singular matrix with a determinant zero by subtracting a scalar matrix \( \lambda I \) (\( I \) being an identity matrix of the same size as TM):
\[
det(TM - \lambda I) = 0
\]
or
\[
det(TM + k_{\text{obs}}) = 0 : \begin{bmatrix} k_{\text{obs}} - [L]k_{\text{on}} & k_{\text{off}} & 0 \\ [L]k_{\text{on}} & k_{\text{obs}} - k_1 - k_{\text{off}} & k_2 \\ 0 & k_1 & k_{\text{obs}} - k_2 \end{bmatrix} = 0.
\]

Calculating the determinant leads to
\[
k_{\text{obs}}^3 - k_2 k_{\text{obs}}^2 - k_2^2 k_{\text{obs}} - k_2 k_{\text{obs}} k_{\text{off}} - [L]k_{\text{obs}} k_{\text{on}} + [L]k_{\text{obs}} k_{\text{on}} + [L]k_2 k_{\text{obs}} k_{\text{on}} = 0
\]
and
\[
k_{\text{obs}}^2 - k_{\text{obs}}(k_2 + k_{\text{off}} + k_1 + [L]k_{\text{on}}) + (k_2 k_{\text{off}} + [L]k_1 k_{\text{on}} + [L]k_2 k_{\text{on}}) = 0.
\]

Nonzero solutions for \( k_{\text{obs}} \) are obtained by setting the second factor to zero:
\[
k_{\text{obs}}^2 - k_{\text{obs}}(k_2 + k_{\text{off}} + k_1 + [L]k_{\text{on}}) + (k_2 k_{\text{off}} + [L]k_1 k_{\text{on}} + [L]k_2 k_{\text{on}}) = 0.
\]

The two solutions are
\[
k_{\text{obs}1} = \frac{1}{2} \left( k_{\text{on}}([L] + k_{\text{off}} + k_1 + k_2) + \sqrt{k_{\text{on}}([L] + k_{\text{off}} + k_1 + k_2)^2 - 4(k_{\text{on}}(k_1 + k_2)k_{\text{off}})} \right)
\]
\[
= \frac{1}{2} \left( k_{\text{on}}[L] + k_{\text{off}} + k_1 + k_2 \right) + \sqrt{k_{\text{on}}[L] + k_{\text{off}} - k_1 - k_2^2 + 4k_1 k_{\text{off}}}
\]

and
\[
k_{\text{obs}2} = \frac{1}{2} \left( k_{\text{on}}([L] + k_{\text{off}} + k_1 + k_2) - \sqrt{k_{\text{on}}([L] + k_{\text{off}} + k_1 + k_2)^2 - 4(k_{\text{on}}(k_1 + k_2)k_{\text{off}})} \right)
\]
\[
= \frac{1}{2} \left( k_{\text{on}}[L] + k_{\text{off}} + k_1 + k_2 \right) - \sqrt{k_{\text{on}}[L] + k_{\text{off}} - k_1 - k_2^2 + 4k_1 k_{\text{off}}} \quad (4).
\]

SI Materials and Methods
Mutagenesis, Expression, and Purification of Glt

L130W was inserted into pBAD24-Glt, which encodes the WT Glt, with a C-terminal 8x histidine tag (provided by Eric Gouaux, Oregon Health and Science University, Portland, OR) using the QuikChange Kit (Stratagene). Escherichia coli Top10 or BL21-AI cells (Invitrogen) harboring one of the resulting plasmids and in the case of Top10, pLysN to facilitate later bacterial lysis (5) were grown to OD500 = 0.5–0.7. After induction with 1% L-arabinose, the cells were incubated for 4 h. The following steps were performed at 4 °C. The cells were harvested by centrifugation. Lysis was performed by sonication followed by centrifugation at 15,000 × g to remove cellular debris. The supernatant was centrifuged at 100,000 × g. The resulting crude membranes were resuspended in 20 mM Tris (pH 7.4), 200 mM NaCl and 250 mM sucrose and solubilized by the dropwise addition of 40 mM n-dodecyl-β-D-maltopyranoside (DDM) (GLYCON Biochemicals) while stirring. After 1 h, DDM was diluted to 10 mM, and imidazole was supplemented to 30 mM. The insoluble material was pelleted by centrifugation at 65,000 × g for 30 min; 1 mL nickel-nitrilotriacetic acid agarose (Qiagen) was added per 1 mg expected Glt protein yield, and the mixture was incubated overnight at 4 °C. Subsequently, the affinity resin with bound proteins was washed with six volume equivalents of 20 mM Tris (pH 7.4), 200 mM NaCl, 41.25 mM imidazole, and 1 mM DDM. The elution was performed with 20 mM Tris (pH 7.4), 200 mM NaCl, 500 mM imidazole, and 1 mM DDM. No preparative size exclusion chromatography was performed. The purity and size distribution of Glt was assessed by SDS/PAGE and analytical size exclusion chromatography on a Superdex 200 10/300GL column (GE Healthcare) equilibrated in a buffer containing 100 mM Tris (pH 8), 200 mM NaCl, 5 mM EDTA, and 1 mM DDM. The [protein] was estimated from the absorbance of the solution at 280 nm using a molar extinction coefficient of 57,400 M⁻¹ cm⁻¹ for WT Glt (6). To obtain the molar extinction coefficient for L130W Glt (62,400 M⁻¹ cm⁻¹), we added to this value the contribution of a single additional tryptophan (7). The purified protein was flash frozen and stored at ~80 °C until use.

To remove Na⁺ and potentially bound amino acids, NaCl was replaced by choline chloride in four successive purification steps using disposable desalting columns (GE Healthcare) followed by two additional steps with background buffer if necessary. All buffers contained 20 mM Tris (pH 7.4), 1 mM DDM, and choline chloride or NaCl as reported in the figures. If necessary, the proteins were concentrated using ultrafiltration cartridges (100-kDa exclusion limit; Millipore) after thawing.
Cysteine Cross-Linking. Cu(II)(1,10-phenanthroline)₂-mediated cross-linking was performed as previously described (8). For the cross-linking of L130W K55C C321S A364C Glt₃ph, samples were incubated for 1 h with 10 mM DTP to reduce cysteines. Samples of both cysteine mutants were subjected to three successive rounds of buffer exchange to 20 mM Tris (pH 7.4), 1 mM DDM, and 200 mM choline chloride using disposable desalting columns. The oxidative cross-linking was performed for 30 min or 1 h with 0.1 mM Cu²⁺ and 1,10-phenanthroline freshly mixed at a 1:2 molar ratio. The reactions were quenched with 10 mM N-ethylmaleimide. Before fluorescence measurements, L130W K55C C321S A364C Glt₃ph was subjected to two successive rounds of buffer exchange into 20 mM Tris (pH 7.4), 1 mM DDM, and 200 mM NaCl were performed before nonreducing SDS/PAGE and fluorescence measurements. To compare the electrophoretic mobility of single and double cysteine mutants unbiased by the oxidative treatments, electrophoresis was performed directly after affinity chromatography. To quantify the relative amounts of protein in Coomassie Brilliant Blue-stained gels, the OD was compared with the Ods of a calibrated transmission step wedge (T2115; Stouffer).

Fluorescence Measurements and Data Analysis. The fluorescence spectra of the WT and mutant Glt₃ph were determined in a Quantamaster 4 spectrofluorimeter (Photon Technology Inc.), with the excitation and emission slits set to a band pass of 4 and 5 nm, respectively. The sample solution was stirred in a 1-cm cuvette thermostated to 22 °C. The measured photomultiplier sensitivity was calibrated through a monochromator set at 297 nm unless otherwise specified. Ewers et al. (11) described (11). Briefly, the protein sample buffer was exchanged to 20 mM Tris/Hepes (pH 8), 100 mM NaCl, 5 mM CaCl₂, and 7 mM DDM using disposable desalting columns. Cleavage was performed by the addition of Factor Xa (activated bovine Factor X; Sigma) at a ratio of 40 μg to 1 mg protein for 48 h at 37 °C. Thrombin was added at 30 U/mg protein after 8 h. Later, this step was omitted, because the His-tag does not modify the fluorescence properties. The reaction was stopped by the addition of 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride and 10 mM EDTA.

Reconstitution of Glt₃ph into Liposomes and Transport Assay. Glt₃ph was reconstituted into liposomes according to information in ref. 12; control liposomes without Glt₃ph were prepared in parallel under identical conditions. Proteoliposomes and control liposomes (20 mg lipid/mL) were flash frozen in liquid nitrogen and stored at −80 °C. Proteoliposomes and control liposomes were loaded with 100 mM KCl and 20 mM Hepes (pH 7.5) by at least three freeze/thaw cycles and extrusion (10 times through two 400-μm pore size polycarbonate filters; Avestin). The uptake reaction was initiated by the addition of 1 μL proteoliposomes/ control liposomes (100–125 mg lipid/mL) to 199 μL external buffer (100 mM NaCl, 20 mM Hepes, pH 7.5, 1 μM valinomycin, 100 nM 3H-l-aspartate, additives as indicated). External buffers were preequilibrated at 30 °C, and after the addition of liposomes, the mixture was briefly mixed. At each time point, the reactions were quenched by addition of 1.8 mL ice-cold 150 mM KCl followed by immediate suction filtration over nitrocellulose filters (0.22-μm pore size; GSWP, Millipore). The filters were washed three times with 1.8 mL ice-cold quench buffer. Radioactivity trapped on the filters was measured by addition of 10 mL Filter-Count (PerkinElmer), and subsequent counting in a TRI-CARB 2000CA scintillation counter (PerkinElmer). The signal-to-background ratio (dpm proteoliposomes/dpm control liposomes) was 400–500 at a reaction time of 6 min. Initial rates were calculated from the linear portion of the uptake time courses (first 60 s).

For countercurrent experiments, proteoliposomes were loaded with 100 mM NaCl, 20 mM Hepes (pH 7.5) alone or with buffer, and 50 μM l-aspartate, l-CS, or dl-TBOA, respectively. The proteoliposomes were concentrated and washed by centrifugation (270,000 × g, 25 min, 4 °C). The pellets were resuspended in 100 mM NaCl and 20 mM Hepes, pH 7.5 (60 mg lipid/mL). The reaction was initiated by the addition of 194–198 μL prewarmed external buffer (100 mM NaCl, 20 mM Hepes, pH 7.5, 100 mM 3H-l-aspartate) to 2–6 μL proteoliposomes. The mixture was briefly vortexed, and at each time point, the reactions were quenched by the addition of 1.8 mL ice-cold quench buffer (100 mM NaCl, 20 mM Hepes, pH 7.5) followed by immediate filtration over nitrocellulose filters. The filters were washed, and the radioactivity was measured as described above. Data analysis was done with GraphPad Prism (GraphPad Software).
Simulation of Time-Dependent Fluorescence Changes. Amino acid binding to GltPh was simulated using COPASI (13). Differential equations corresponding to Scheme 1 were solved by the implemented Livermore Solver for Ordinary Differential Equations (14) using the values presented in Table 1. To ensure the comparability of the simulated and experimental curves, the simulations were started from the end of the dead time of the stopped-flow apparatus (2.3 ms), and the resulting curves were fitted with an exponential function (one exponential term) extrapolating to time 0. The results were normalized to values between zero and one, P was assigned a relative fluorescence change of zero, and P* was assigned a change of one. Through this procedure, which is analogous to the treatment of the experimental data, deviations from a single exponential behavior in the simulated curves (i.e., the initial lag phase that was observed at low amino acid concentrations) that are not contained in the experimental curves are absent in the simulated data as well. Therefore, the progress of curves from time 0 to the end of the dead time is determined by the extrapolation of the fitted exponential only, which reproduces the information loss concerning the signal amplitude in the dead time of the stopped-flow apparatus.


Fig. S1. [Na+] dependence of the slow processes preceding the amino acid binding to L130W GltPh. Shown are the fluorescence traces after the simultaneous addition of 500 μM L-aspartate and various [Na+] to L130W GltPh.

Fig. S2. Slow Na+ binding to WT GltPh. Representative fluorescence trace after the addition of 250 mM Na+ to WT GltPh. Excitation was 289 nm.
Fig. S3. L-CS specificity of GltPh. (A) The time course of $^3$H-$\text{L}$-aspartate (100 nM) uptake into GltPh-containing liposomes driven by an inwardly directed Na$^+$ gradient in the absence or presence of L-CS and L-aspartate (10 μM each). Shown are the first 120 s of a representative experiment with GltPh-free liposomes as control. Duplicate measurements were performed for reaction times up to 30 s. (B) Initial rates of uptake in the absence or presence of competitor (mean ± SEM, three experiments). (C) Uptake of radiolabeled L-aspartate under counterflow conditions with buffer alone or buffer supplemented with L-aspartate, L-CS, or DL-TBOA (50 μM each) inside the proteoliposomes. Experiments were performed at equimolar Na$^+$ concentrations on both sides of the liposome. Data are given as mean ± SEM from six measurements after 5 min of reaction time in three experiments. The means of uptake with intraliposomal L-aspartate and L-CS are significantly different from the means of uptake with buffer or DL-TBOA inside the liposomes ($P < 0.05$ according to Tukey’s Multiple Comparison Test, ANOVA one-way).
Fig. S4. The fluorescence changes predicted by Scheme 1 agree with experimental results. The results of the fluorescence simulations after addition of the indicated amino acids to GltPh are shown together with the corresponding experimental traces (gray). The traces originating from the best fit parameters in Table 1 are in black, and the traces originating from varying $k_{\text{off}}$ or $k_1$ are in orange or blue, respectively. The value of $k_{\text{off}}$ was (A and C) 122 or (B) 417 s$^{-1}$. Values of $k_1$ were (A and C) 351 or (B) 623 s$^{-1}$. 

Ewers et al. www.pnas.org/cgi/content/short/1300772110
Fig. S5. An intramolecular disulfide bridge is formed by 75–80% of L130W V216C C321S A391C Glu$_{\text{mut}}.$ (A) Nonreducing SDS/PAGE showing double and single cysteine mutants (Left) after treatment aiming at cysteine oxidation or (Right) directly after elution from affinity resin. The SDS/PAGE sample buffer was with or without DTT as indicated. Note that the spontaneous formation of disulfide bridges in this mutant depends on the buffer conditions. (Right) In the presence of NaCl, the proportion of cross-linked species in the absence of DTT is lower than in the presence of ChCl (Fig. 4E). (B) Proportion of the cross-linked species as quantified by densitometry of Coomassie-stained gels like in A Left. Error bars represent SEM of two experiments.
Table S1. W130 fluorescence kinetics after the addition of 100 μM l-aspartate to protein preincubated with Na⁺: best fit parameters of exponential fits to the data

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<th>[Na⁺] (mM)</th>
<th>A∗</th>
<th>k†</th>
<th>A</th>
<th>k</th>
<th>A</th>
<th>k</th>
<th>A</th>
<th>k</th>
<th>A</th>
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<td>0.043</td>
<td>0.259</td>
<td>0.172</td>
<td>0.178</td>
<td>0.662</td>
<td>0.246</td>
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<td>0.227</td>
<td>1.579</td>
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*Amplitude.
†Rate constant.
‡Amplitude of largest rate was used in Fig. 2C.