Glutamate transporters remove glutamate, the major excitatory neurotransmitter in the brain, from the synaptic cleft and thereby enable the post-synaptic receptors to sense glutamate released from the pre-synaptic nerve terminals. One of the major observations highlighting the importance of these transporters comes from the study of glutamate transporter knock-out mice. This study (1) indicates that glutamate transporters and in particular GLT-1 (2), play a central role in preventing both hyperexcitability and excitotoxicity. GLT-1 and the four glutamate transporter homologue GltPh (25) beautifully confirms and refines previously by several studies that each residue of this motif is extremely important for the function of the transporter (16, 29).

Moreover the equivalent amino acid residues from the eukaryotic glutamate transporters, which are asparagine 366 and aspartate 368. Substitution mutants N366Q and D368E, but not N366D and D368N, show glutamate-induced inwardly rectifying steady-state currents, but their apparent substrate affinity is dramatically decreased. Such currents, which reflect electrogenic net uptake of substrate are not observed with the reciprocal double mutant N366D/D368N. Remarkably, the double mutant exhibits slow substrate-induced voltage-dependent capacitative transient currents. These currents apparently reflect the reversible sodium-coupled glutamate translocation step, because the interaction of the double mutant with potassium is largely impaired. Moreover, when the analogous double mutant in the glutamate transporter GLT-1 is reconstituted into liposomes, a slow exchange of radioactive and unlabeled acidic amino acids is observed. Our results suggest that it is the interaction of asparagine 366 and aspartate 368 that is important during the glutamate translocation step. On the other hand, the side chains of these residues themselves are required for the subsequent potassium relocation step.

Glutamate transport is an electrogenic process (7–9), consisting of two distinct half-cycles (Fig. 1A): first, glutamate is co-transported with sodium and hydrogen ions and subsequently the binding sites reorient upon countertransport of potassium (10–12). The stoichiometry of the process has been determined to be 3Na⁺:1H⁺:1K⁺:glutamate (13, 14).

In addition to the ion-coupled glutamate translocation, glutamate transporters mediate a thermodynamically uncoupled chloride flux activated by two of the molecules they transport, sodium and glutamate (5, 15). In EAAC1 (also termed EAAT-3), lithium can replace sodium in coupled glutamate uptake but not in its capacity to gate the glutamate-dependent uncoupled anion conductance (16) and additional studies have reinforced the idea that the conformation gating of the anion conductance is different from that during substrate translocation (17–19). In addition, the uncoupled anion flux can be altered by substituting some of the amino acid residues of transmembrane (TM) domain 2, without significantly affecting the properties of coupled glutamate translocation (20). Despite these insights, little is known about the mechanism of glutamate-induced anion permeation, but it has been suggested that glutamate itself may gate the anion permeation (15).

Glutamate transporters have a non-conventional topology containing two re-entrant loops and two transmembrane domains (7 and 8) in their carboxyl-terminal half (21–23). Moreover the two re-entrant loops are in close proximity (24). The recently solved crystal structure of the glutamate transporter homologue Gltrp (25) beautifully confirms and refines both the topology and the proximity of the re-entrant loops. Moreover, the equivalent amino acid residues from the eukaryotic glutamate transporters, which are involved in potassium binding (12, 26), sodium specificity (16, 27), and the liganding of the γ-carboxy group of glutamate (28) are located at the binding pocket of Gltrp (25). Therefore, the Gltrp structure appears to be a good model for the study of its eukaryotic counterparts.

The two helical halves of TM7 are separated by an unwound part consisting of a highly conserved 5-amino acid stretch termed the NMDGT motif (Fig. 1B). It has been shown previously by several studies that each residue of this motif is extremely important for the function of the transporter (16, 29).

In the crystal structure of the archaeal homologue, the amino acid residues of this motif are found to form part of the substrate binding pocket (25). The side chains of the methionine...
and the threonine point toward it; but surprisingly, the side chains of the asparagine and aspartate of this motif are pointing away from the binding pocket. These two residues are seen to interact with each other and with additional residues of TM domains 3, 6, and 8. These interactions were suggested to be important for shaping and stabilizing the binding pocket structure (25).

In this study, we test the hypothesis that interaction of the corresponding asparagine and aspartate residues is important for the function of EAAC1. Our results suggest that besides shaping the substrate binding pocket, asparagine 366 and aspartate 368 of EAAC1 have additional functions in the transport process and are crucial for the execution of the potassium-translocation limb of the transport cycle.

EXPERIMENTAL PROCEDURES

Generation and Subcloning of Mutants—The rabbit glutamate transporter EAAC1 (4) with 10 histidines added immediately after the open reading frame followed by the stop codon (30) in the vector pBluescript SK− (Stratagene) was used as a parent for site-directed mutagenesis as described previously (31, 32). Restriction enzymes PinAI and PflMI were used to subclone the mutations into the construct containing the His-tagged WT EAAC1 residing in pOG1. The latter is an oocyte expression vector containing a 5′-untranslated Xenopus β-globin sequence and a 3′-poly(A) signal. This EAAC1 construct is termed WT in this study. The subcloned DNA fragments were sequenced on both strands between the two restriction sites noted above. In the case of the N396D/D398N-GLT-1 double mutant, the enzymes BsrGI and PshAI were used for subcloning. With the exception of Fig. 5, all figures document results with the mutant in the EAAC-1 background. In Fig. 5, the activity of the N396D/D398N-GLT-1 double mutant was compared with that of wild type GLT-1.

Expression in Oocytes—cRNA was transcribed using mMESSAGE-mMACHINE (Ambion), injected into Xenopus laevis oocytes, and maintained as described previously (16).

Surface Biotinylation—This was done as described previously (33), except that 4–5 oocytes, expressing wild type or mutant EAAC1, were treated with 1.5 mg/ml of sulfosuccinimidyl-2- (biotinamide)ethyl-1,3-dithiopropionate (Pierce) in 2 ml of ND96 and the streptavidin beads were eluted with a final volume of 70 μl of SDS-PAGE sample buffer. For samples of total cell transporter, 10% of the lysate was not treated with the streptavidin beads and these samples were run on the same gel alongside those eluted from the beads. The Western blots were probed with an affinity purified antibody directed against a peptide corresponding to amino acids 491–523 of rabbit EAAT3 (generously provided by N. C. Danbolt, University of Oslo; anti-C491 (Ab,371 (34)).

Oocyte Electrophysiology and Uptake—Oocytes were placed in the recording chamber, penetrated with two agarose-cushioned micropipettes (1%/2M KCl, resistance varied between 0.5 and 3 mΩ), voltage clamped using GeneClamp 500 (Axon Instruments), and digitized using Digidata 1322 (Axon Instruments) both controlled by the pClamp9.0 suite (Axon Instruments). Voltage jumping was preformed using a conventional two-electrode voltage clamp as described previously (30). The standard buffer, termed ND96, was composed of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Na-HEPES, pH 7.5). In sodium substitution experiments NaCl was replaced by an equimolar concentration of either LiCl or choline Cl. In sodium titration experiments the buffer was composed of 130 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Tris-HEPES, pH 7.5, and NaCl was replaced by equimolar concentrations of
Role of β-Bridge Residues in Glutamate Transporter

A. WT

B. N366Q

C. D368E

FIGURE 2. Substrate-induced currents by WT, N366Q, and D368E mutants. Oocytes expressing either WT EAAC1 (A, n = 3) or its mutants N366Q (B, n = 3) and D368E (C, n = 4) were voltage clamped and gravity perfused with ND96 as described under "Experimental Procedures." Currents in the absence of each of the amino acids: L-aspartate (L-Asp), L-glutamate (L-Glu), D-aspartate (D-Asp), or GABA at 2 (A) or 10 mM (B and C) were subtracted from those in their presence. GABA, which is not a substrate of this transporter, was used as a negative control. The membrane voltage was stepped from −25 mV to voltages between −100 and +40 mV in increments of +10 mV. Each potential was held clamped for 250 ms, which followed 250 ms of voltage clamped at a holding potential of −25 mV. The steady-state current from 210 to 240 ms at each potential was averaged. The currents induced by the indicated amino acids were normalized to the L-aspartate elicited current at −100 mV (I/I_{max}) and plotted against the holding potential (V_{holding}). Values are mean ± S.E. The L-aspartate-induced currents at −100 mV ranged from −250 to −850 nA in WT, and from −60 to −200 nA and −40 to −160 nA in N366Q and D368E mutants, respectively.

choline Cl. Offset voltages in chloride substitution experiments were avoided by the use of an agarose bridge (2%/2 M KCl) that connected the recording chamber to the Ag/AgCl ground electrode. For the determination of anion selectivity (Fig. 10), 10 mM NaCl of the perfusion solution was replaced isosmotically by the sodium salt of the tested anions. Voltage jumps in the presence of each of the tested anions (in the presence and absence of L-aspartate) was followed by a ~20-min washout with ND96 solution and the induced currents in chloride medium were measured again to ensure that the test anion was removed from the oocyte before testing the next anion. For uptake, four to five oocytes of each mutant were incubated for 20 min in ND96 containing D-[2,3-3H]aspartic acid as described previously (16).

Data Analysis—All current-voltage relations represent steady-state net currents (I_{current} – I_{background}) elicited by amino acids (AA) and were analyzed by Clampfit version 8.2 or 9.0 (Axon instruments). Even though there was a large variation in the absolute values of the substrate-induced currents between oocytes of different batches and sometimes even within a given batch, the voltage dependence of these currents as well as their kinetic parameters were always very similar when different oocytes were compared. Therefore the data are presented as normalized currents, usually those at −100 mV, as indicated in the figure legends.

Moreover the range of the values of the absolute currents is also stated in the figure legends. Kinetic parameters were determined by non-linear fitting to the generalized Hill equation using the build-in functions of Origin 6.1 (Microcal). For the determination of the apparent affinity for sodium, I_{max}, K_{app}, and n_H were allowed to vary, and for the determination of the apparent affinity for acidic amino acids, the value of n_H was fixed to 1. The time constants were estimated using the decay of the charge movements induced by amino acids during the "on" phase using non-linear fitting to the first order decay exponential function of Clampfit 8.2 (Axon instruments).

Cell Growth and Expression—HeLa cells were cultured (35), infected with recombinant vaccinia/T7 virus vTF7-3 (36), and transfected with plasmid DNA encoding WT-GLT-1, N396D/D398N-GLT-1, or the vector pBluescript SK− alone, as described (35). Solubilization of transporters expressed in the HeLa cells, their reconstitution in proteoliposomes (2, 12) and transport experiments (2, 12) were done as described. Exchange data are presented as net exchange, after subtracting the values obtained on proteoliposomes not containing 10 mM L-aspartate but only 0.12 M sodium phosphate, pH 7.4. Protein was determined by Lowry’s method (37).

RESULTS

Substrate Interactions in N366Q and D368E Mutants—In contrast to oocytes expressing wild type (WT) EAAC1, where a saturating concentration of each of the three substrates, L-aspartate, D-aspartate, and L-glutamate (2 mM), induced currents of similar size (Fig. 2A), this was not the case in substitution mutants N366Q and D368E, where currents induced by 10 mM D-aspartate or L-glutamate were much smaller than those induced by the same concentration of L-aspartate (Fig. 2B and C). In the N366Q mutant the I_{max} values for D-aspartate was only 39 ± 1% of that for L-aspartate. In the case of L-glutamate, even at 20 mM saturation was not yet reached (data not shown). In WT EAAC1, the K_m values for the three substrates (at −100 mV) were around 10 μM (Table 1). In N366Q, the K_m values for L-aspartate, D-aspartate, and L-glutamate were higher than WT EAAC1 by around 100-, 350-, and 1000-fold, respectively (Table 1). In the case of the D368E mutant the increase of the K_m for L-aspartate was not as dramatic as in N366Q, around 20-fold. On the other hand, L-glutamate-induced currents were too small for quantitative analysis and even at 10 mM the induced inward currents at −100 mV were smaller than 30 nA. In the case of D-aspartate, the K_m values of the two mutants were similar, around 2.5 mM (Table 1).

No uptake of D-[2,3-3H]aspartate by the N366Q and D368E mutants could be detected (Fig. 3) and the same was true for the uptake of L-[2,3-3H]aspartate (data not shown), apparently because of the dramatic increase of the K_m values of the mutants for the acidic amino acids (Table 1). Surface biotinyla-
Role of β-Bridge Residues in Glutamate Transporter

TABLE 1

|                | L-Asp | L-Glu | D-Asp |
|----------------|-------|-------|-------|
| WT             | 0.015 ± 0.002 | 0.013 ± 0.001 | 0.008 ± 0.007 |
| N366Q          | 1.66 ± 0.34 | 12.6 ± 0.76 | 2.90 ± 0.17 |
| D368E          | 0.32 ± 0.05 | 4.00 ± 0.08 | 2.40 ± 0.28 |
| ND/DN          | 0.073 ± 0.02 | 0.25 ± 0.08 | 0.067 ± 0.032 |
| WT, low Na±    | 0.053 ± 0.014 | 0.065 ± 0.013 | 0.039 ± 0.007 |

a Values of L-glutamate-induced currents in D368E were too low for apparent affinity determination.

FIGURE 3. Radioactive D-aspartate transport by EAAC1 mutants. D-[2,3-3H]Aspartate uptake of oocytes expressing either WT or the indicated mutants was performed as described under “Experimental Procedures.” The results are expressed as percent of the uptake by WT. The uptake in oocytes expressing WT ranged from 1.4 to 2.2 pmol/oocyte. Data are mean ± S.E. of at least three experiments each done using 4–5 oocytes for each mutant, and were corrected for uptake in non-injected oocytes. The double mutants are least three experiments each done using 4–5 oocytes for each mutant, and the first voltage step (from −40 mV back to −25 mV) and the last voltage step (from +40 mV back to −25 mV). The “low Na+” medium had the same composition as ND96, except that the external sodium concentration was 20 mM (choline substitution), n = 3. Data are given as mean ± S.E.

FIGURE 4. Cell surface biotinylation of the wild type and mutants. Oocytes expressing EAAC1-WT and the indicated mutants, were labeled and processed as described under “Experimental Procedures.” The six left lanes show the total samples, and the six right lanes show the biotinylated samples. The first lane of each group shows a sample from uninjected oocytes (Uninj.). The empty lane between the two groups contained the marker proteins and their positions (n in kDa) are indicated on the left. All samples were separated on the same SDS gel, transferred to nitrocellulose, and detected as described under “Experimental Procedures.” Shown is a representative of four separate experiments.

tion using an anti-EAAC1 antibody showed that the lower functional expression of N366Q and D368E was not due to lower expression of these transporters on the plasma membrane than the WT (Fig. 4). In the samples of the total protein of wild type and mutants, bands of around 55–60 and 65–70 kDa were observed, which apparently represent non-mature and mature monomeric forms of the transporter, respectively. Moreover bands of lower mobility, apparently representing aggregated transporters, were observed (Fig. 4), although in other experiments the monomeric form was more abundant (data not shown). In the biotinylated samples, representing those transporters located at the plasma membrane, the mature form was predominant among the monomeric transporters, except for the case of N366Q (Fig. 4). In other experiments the pattern of N366Q was not different from that of the other mutants (data not shown). The specificity of the bands is illustrated by the fact that the transporter bands were not observed in total and biotinylated samples of non-injected oocytes (uninj., Fig. 4). In the depicted experiment, the mutant transporters were present on the plasma membrane at similar or higher levels than those of WT (Fig. 4). Even though there was variation between experiments, the expression of the mutants was usually at least that of WT (data not shown). The proportion of monomeric to aggregated transporter also changed between experiments, such that sometimes the low-mobility bands were predominant (data not shown). It was difficult to control this variability, which is apparently related to the well known property of glutamate transporters to aggregate (38). Nevertheless, whatever the aggregation state of the transporter, these bands were never seen with uninjected oocytes, just as seen in Fig. 4.

In the N366D, D368N, N366A, and D368A mutants, none of the three substrates (tested at concentrations up to 10 mM) induced any measurable currents (data not shown) and no uptake of D-[2,3-3H]aspartate was observed (Fig. 3). Again, as found by surface biotinylation, the lack of functional expression of these four mutants is not due to lower expression of these transporters on the plasma membrane (Fig. 4 and data not shown).

As will be shown below, the N366Q had a lowered apparent affinity for sodium. However, lowering the external sodium concentration from 100 to 20 mM had only a relatively modest effect on the $K_m$ values for the three substrates in WT EAAC1 as compared with the impact of the mutations (Table 1). Thus it appears that the dramatic increase in $K_m$ for the three substrates in N366Q is not just a consequence of its reduced apparent affinity for sodium.
Radioactive Uptake by N366/D368 Double Mutants—In contrast to the single mutants at positions 366 and 368, oocytes expressing the N366D/D368N double mutant exhibited significant, albeit slow, uptake of D-[3H]aspartate (Fig. 3). As also shown in Fig. 3, such uptake was neither observed when the neutral alanine was introduced at both positions (N366A/D368A) nor in the N366Q/D366E and N366E/D366Q double mutants. Also in the case of the double mutants, we found that their expression level at the plasma membrane was similar to that of WT (data not shown). The observation of radioactive substrate uptake in the N366D/D368N mutant, which does not exhibit steady-state transport currents (see Figs. 6 and 7) is reminiscent of the E404D mutant of the glutamate transporter GLT-1, which has an impaired interaction with potassium and is therefore locked in the exchange mode (12). Because oocytes have high (around 12 mM) intracellular levels of acidic amino acids (13), oocytes expressing the E404D mutant are able to take up radioactive acidic amino acids (in exchange for their endogenous unlabeled counterparts) to almost the same levels as those expressing WT (12). One way to test if the N366D/D368N mutant is locked in the exchange mode is to use reconstituted systems using the transporters expressed in cell lines, such as HeLa cells. Because we anticipated low exchange levels in the double mutant, we used the equivalent N396D/D398N double mutant in the background of the glutamate transporter GLT-1, which is much more robustly expressed in the heterologous HeLa cell expression system than EAAC1. Under net flux conditions, created by dilution of potassium containing liposomes, inlaid with wild type GLT-1, into a sodium-containing transport medium in the presence of the potassium-specific ionophore valinomycin (2.5 μM), D-[2,3-3H]aspartate uptake was observed (Fig. 5, net flux). Such uptake was not seen when the liposomes were inlaid with similarly solubilized proteins from HeLa cells transfected with the vector alone (SK), whereas with N396D/D398N double mutant liposomes (ND/DN) net flux amounted for no more than 2% of that of WT (Fig. 5). On the other hand, much higher D-[3H]aspartate uptake was seen in sodium and L-aspartate (10 mM) containing N396D/D398N liposomes (increment over similar liposomes containing sodium without L-aspartate), even though exchange by wild type GLT-1 liposomes was more robust (Fig. 5, exchange). In the control liposomes (from cells transfected with the vector alone), very little exchange was observed (Fig. 5). As observed previously in GLT-1 liposomes (12), uptake was also seen in N396D/D398N liposomes containing L-glutamate, but not in those containing γ-aminobutyric acid (GABA), which is not a substrate of the glutamate transporters (data not shown).

Similar results were also obtained when uptake of L-[2,3,4-3H]aspartate was tested (Fig. 5, exchange).

Transport Currents in the N366D/D368N Double Mutant—In contrast to WT EAAC1 and single mutants N366Q and D368E, the substrate-induced currents by oocytes expressing the double mutant N366D/D368N were strikingly different. L-Glutamate induced extremely slow transient currents (τ around 100 ms), but very small steady-state currents (Fig. 6A), if at all because even after 1000 ms the steady state was not yet reached (data not shown). These transient currents, induced by glutamate, are in marked contrast to the response by WT EAAC1 where L-glutamate induces robust steady-state currents (Fig. 6D). Similar slow transient currents were also induced by D-aspartate (Fig. 6B) and L-aspartate (Fig. 7A) but not by γ-GABA, which is not a substrate of the glutamate transporters (Fig. 6C).

The amount of charge movement induced by each of the three substrates was dependent on their concentration. Half-maximal charge movements were observed at concentrations that were higher than the Kₘ values determined from steady-state transport currents in WT, but much lower than the corresponding values in the single mutants (Table 1). The voltage dependence of the transients was almost independent on the substrate concentration (data not shown).

The transients induced by L-aspartate were strictly dependent on the simultaneous presence of sodium (Fig. 7A); they were not observed when the sodium was replaced by lithium (Fig. 7B) or choline (data not shown). At reduced sodium concentrations, the L-aspartate-induced transients were still observed but their voltage dependence was shifted (data not shown).

The substrate-induced transients observed in the double mutant are reminiscent of those observed in the E404D mutant of the glutamate transporter GLT-1, which is locked in the...
exchange mode (12). The only difference is that in the N366D/D368N mutant these transients were around 5-fold slower, in harmony with the fact that exchange by E404D is similar to that by wild type and much faster than by N366D/D368N (Fig. 5).

Besides the biochemical approach used with the double mutant (Fig. 5), we have also used an electrophysiological approach to probe the interaction of potassium with WT and N366D/D368N transporters. In oocytes expressing glutamate transporters, elevation of external potassium induces reverse transport of endogeneous acidic amino acids (12). As a consequence the transporter-mediated anion conductance is activated and is readily observable when highly permeant anions, such as nitrate or thiocyanate, are present. In the E404D-GLT-1 mutant, which has lost the ability to interact with potassium, this cation cannot activate the anion conductance (12). In the presence of 48 mM sodium thiocyanate, 48 mM potassium induced an outward current at positive potentials (choline subtraction) in oocytes expressing WT EAAC1 (Fig. 8A, K⁺-Na⁺). These outward currents were not observed in oocytes expressing N366D/D368N (Fig. 8B), indicating a defective interaction of the mutant transporters with potassium. In WT, external amino acid substrates also induced the transporter-mediated anion conductance: the outward currents induced by L-aspartate, recorded at low potassium concentrations, were depend-
In the N366Q mutant no saturation was observed in the entire range where these experiments are feasible, 0–130 mM sodium and 0–100 mM potassium. Remarkably, in WT potassium attenuated the L-aspartate-induced currents, as long as it is induced by substrate rather than by potassium, on the presence of thiocyanate (Fig. 8A, L-Asp + Na⁺ versus L-Asp + SCN⁻). In contrast with the potassium-induced outward currents, the outward currents induced by L-aspartate were present in the double mutant (Fig. 8B, L-Asp + Na⁺). Thus the double mutant is capable of mediating the uncoupled anion conductance, as long as it is induced by substrate rather than by potassium. Remarkably, in WT potassium attenuated the L-aspartate-induced outward currents (Fig. 8A, L-Asp + K⁺ versus L-Asp + Na⁺), but potassium hardly had an effect in the double mutant (Fig. 8B), again pointing to a defective interaction of the mutant transporters with potassium. Neither the L-aspartate-nor potassium-dependent outward currents were observed in uninjected oocytes (data not shown). The inward currents induced by potassium, observed in wild type as well as in the mutant at negative potentials (Fig. 8, K⁺–Na⁺), were also seen in non-injected oocytes (data not shown) and presumably represent cation-leak currents.

Cation Interactions in the N366Q and D368E Mutants—When the L-aspartate (2 mM)-induced transport currents were monitored at varying sodium concentrations (choline substitution), saturation was observed in WT at around 100 mM sodium (16) with a half-maximal activation at 19.8 ± 1.4 mV at −100 mV and 24.8 ± 0.4 mV at −60 mV (n = 4). The Hill number was 1.43 ± 0.26 and 1.32 ± 0.04 at −100 and −60 mV, respectively. In the N366Q mutant no saturation was observed in the entire range where these experiments are feasible, 0–130 mM sodium (Fig. 9A). At around 80–90 mM sodium, approximately half the current of that measured at 130 mM sodium was observed (Fig. 9A). On the other hand, the Kₘ for sodium of the D368E mutant was similar to that of WT. The D368E L-aspartate-induced currents were saturated at around 100 mM sodium with half-maximal activation at 19.8 ± 1.4 mV at −100 mV and 32.1 ± 2.7 mV at −60 mV (Fig. 9B). In the case of D368E, the Hill numbers were similar to those of WT and did not differ significantly at the two potentials: 1.75 ± 0.21 at −100 mV and 1.56 ± 0.14 at −60 mV.

In contrast to WT (16), in N366Q (Fig. 9C) and D368E (Fig. 9D), L-aspartate (10 mM) was only able to induce currents in sodium-containing but not in lithium-containing media. GABA, which is not a substrate of the glutamate transporters, was not able to induce any currents (Figs. 9, C and D) and no L-aspartate-induced currents were observed in choline-containing media (data not shown).

Selectivity of the Anion Conductance in the Mutants—The N366Q and D368E mutants exhibit a severely disrupted interaction with the acidic amino acid substrates. It has been proposed that the substrate, when occupying its binding site on the transporter, may directly gate the anion conductance (15). If this is the case, one might expect that the altered substrate interaction observed in the single mutants (Fig. 2 and Table 1) may lead to a change in the selectivity sequence of the permeating anions. Using the size of the L-aspartate (2 mM)-induced outward currents at positive potentials in the presence of various external test anions as a qualitative measure of the anion selectivity, we found that the behavior of WT was in good agreement with that reported in the literature (39): Cl⁻ < I⁻ < NO₃⁻ < ClO₄⁻ < SCN⁻ (Fig. 10A). In the case of the N366Q mutant no detectable anion permeability was observed with Cl⁻, I⁻, or NO₃⁻, but in the presence of the other two anions outward currents at positive potentials were still detectable (Fig. 10B). The behavior of the D368E mutant was totally different. No outward currents were induced by L-aspartate in the presence of 4 of the 5 anions tested (Fig. 10D). Only in the presence of SCN⁻ did L-aspartate induced a permeability change in D368E but instead of increasing the outward current, it actually inhibited it (Fig. 10D). In the case of the N366D/D368N double mutant, where the Kₘ values for the three substrates were only moderately affected (Table 1), the selectivity sequence of the five anions was similar to that observed in the wild type (Fig. 10, A and C). Similar results were also observed at

![Figure 8](https://example.com/figure8.png)

**FIGURE 8. Anion conductance in WT and N366Q/D368N mutant.** The currents induced by L-aspartate in the presence of thiocyanate were obtained by subtracting steady-state currents recorded in ND96 from those obtained in ND96 plus 2 mM L-aspartate (squares, L-Asp − SCN⁻); currents induced by either L-aspartate or by potassium in the presence of thiocyanate were obtained by subtracting the currents recorded in 48 mM NaSCN, 48 mM choline Cl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4, from those either in the same medium with 2 mM L-aspartate (circles, L-Asp + Na⁺) or from those in a similar solution with 48 mM KCl instead of choline Cl with no L-aspartate present (triangles, K⁺–Na⁺); L-aspartate-induced currents in the presence of both potassium and thiocyanate are defined as the currents recorded in the KCl containing medium subtracted from those recorded in the same medium supplemented with 2 mM L-aspartate (inverted triangles, L-Asp + K⁺). Values were normalized to currents induced by L-aspartate in the NaSCN and choline-containing medium at +40 mV and are mean ± S.E. of five and three repeats for WT (A) and N366Q/D368N (ND/ND, B) respectively. Absolute values for L-aspartate-induced currents at +40 mV in NaSCN and choline-containing medium ranged from 370 to 900 nA in the N366Q/D368N mutant, and from 1500 to 1700 nA in WT. Currents were recorded using the same voltage protocol described in the legend to Fig. 2.
DISCUSSION

Individual mutation of two conserved residues of the neuronal glutamate transporter EAAC1, asparagine 366 and aspartate 368, leads to loss of substrate-induced steady-state transport currents, unless they are substituted by residues with a chemically similar side chain, namely N366Q and D366E. In WT these currents represent the sum of the contributions of electrogenic glutamate transport and the substrate-induced anion conductance (15). However, in the two mutants these currents predominantly reflect the electrogenic glutamate transport, because their substrate-induced anion conductance is impaired, at least in chloride containing media (Fig. 10). Thus the two single mutants are capable of carrying out both half-cycles of the transport cycle: 1) sodium- and proton-coupled acidic amino acid translocation and 2) the potassium translocating reorientation step of the transporter (Fig. 1A). In the crystal structure of the archaeal glutamate transporter homologue Gltph, the asparagine and aspartate residues, corresponding to asparagine 366 and aspartate 368 of EAAC1, are within hydrogen bond distance from each other (25). This structure appears to represent the substrate occluded state; an intermediate conformation between outward- and inward-facing forms of the substrate-loaded transporter complex $T_{\text{Na},\text{H},\text{Glu}}$ (Fig. 1A). Our results are fully consistent with the structure and indicate that in EAAC1 these two residues functionally interact with each other (Fig. 3). It appears that this interaction is important for the relatively low $K_m$ of the transporter for its acidic amino acid substrates (Table 1). On the other hand, the single mutants N366Q and D368E are profoundly impaired in their interaction with acidic amino acid substrates (Figs. 2 and 3, Table 1). Due to the modest resolution of the GLTph structure, there is not enough information on the position of the bound substrate and none on the positions of the occluded...
sodium ions. Therefore it is impossible to infer from energy minimization how the mutations might impact the binding pocket. However, from the experiments done here, it appears that functional interaction of the asparagine and the aspartate residues is fairly specific with tight constraints on the total length of the two side chains involved. For example, in the case of N366Q, there still is potential for the amide group to interact with the carboxyl group of the aspartate at position 368. Yet the apparent affinity for the substrate is profoundly affected (Table 1). Furthermore, no radioactive transport is observed in oocytes expressing the N366Q/D368E and N366E/D368Q mutants (Fig. 3).

Even though the N366D/D368N mutant (or N396D/D398N for GLT-1) has a relatively efficient interaction with acidic amino acid substrates (Table 1 and Fig. 3), its interaction with potassium is largely impaired (Figs. 5 and 8). As a consequence net transport currents cannot be observed (Figs. 6 and 7), but instead the double mutant mediates substrate-dependent transient currents, apparently reflecting the movement of the substrate-loaded transporter complex $T_{\text{NaCl,Glut}}$ through the membrane in response to the membrane potential (half-cycle I of Fig. 1A). This indicates that exchange of labeled with unla- beled substrate is still possible and this has in fact been demonstrated experimentally, at least in the background of the highly related GLT-1 transporter (Fig. 5). The expression of EAAC1 in HeLa cells is around 3-fold lower than that of GLT-1 and conversely, in the oocyte expression system, the substrate-induced currents obtained with EAAC1 are 5–8-fold larger than with GLT-1. This is the reason that electrophysiologial analysis of mutants with impaired transport activity is feasible in the background of EAAC1 but not in that of GLT-1. Nevertheless, the defective interaction of N366D/D368N (in the EAAC1 back-
Role of β-Bridge Residues in Glutamate Transporter

ground) with potassium could also be shown in oocytes by examining the effect of external potassium on anion conductance (Fig. 8). It is of interest to note that potassium attenuated the aspartate-induced anion conductance in WT (but not in N366D/D368N, Fig. 8). This appears to be due to the subtraction procedure used. In the presence of external potassium, reverse transport takes place and as we have shown earlier (28) this results in a partial activation of the anion conductance (corresponding to K\(^+\)-Na\(^+\) in Fig. 8). This current is subtracted from the L-aspartate-induced current resulting in a smaller net L-aspartate-induced current in the presence of potassium than in sodium (where a negligible current, observed without potassium, is subtracted).

The substrate-induced transients by N366D/D368N are much slower than those previously observed by the GLT-1 mutant E404D, which is locked in an obligatory exchange mode (12). Exchange by this mutant is as fast as that of WT GLT-1 (12) as opposed to the exchange mediated by the double mutant, which is much slower than that by WT (Fig. 5). This slow exchange is apparently due to a less efficient interaction of the asparagine and aspartate in their swapped locations, with surrounding residues. The slow transients apparently reflect the voltage-dependent transition of the positively charged T\(_{\text{Na}^\text{+},\text{H}^\text{+},\text{Glu}^\text{–}}\) (Fig. 1A) between their outward- and inward-facing conformations (12).

The inability of the N366D/D368N mutant to interact with potassium suggests that the asparagine at position 366 and/or the aspartate at position 368 themselves, are important for this interaction. In fact, as stated above, only in mutants where at these positions a chemically similar side chain was introduced, could an interaction with potassium be inferred from the transport currents, which reflect stoichiometric sodium and glutamate transport (Fig. 2). It is impossible to measure radioactive uptake in the N366Q and D368E mutants (Fig. 3), because such measurements have to be performed at low concentrations of substrate with a high specific radioactivity. Because of the high \(K_m\) for acidic amino acids of these mutants, the rate of transport of the mutants at these concentrations would be a much smaller fraction of \(V_{\text{max}}\) than that by WT.

Besides the different impact of the N366Q and D368E mutations on the apparent affinity of the acidic amino acid substrates, the N366Q mutation also has a pronounced effect on the \(K_m\) of the transporter for sodium (Fig. 9A). In the case of D368E, there was not much of an effect of the mutation on the \(K_m\) for sodium (Fig. 9B). It is of interest to note, that while this paper was in preparation a study was published where this aspartate residue was mutated to an asparagine (40). In agreement with our observations, this mutant did not exhibit any stoichiometric transport currents, but anion leak currents were observed. However, the sodium and glutamate concentrations needed to activate these currents were much higher than in WT (40). These findings were interpreted to indicate that the primary effect of the mutation is to impact the binding of the first sodium ion to the glutamate-free transporter (40). Our own observations indicate that the D368E and N366Q mutants impact the cation selectivity (Fig. 9, C and D) and the N366Q mutant strongly affects the \(K_m\) for sodium (Fig. 9A). The \(K_m\) for sodium of D368E is not affected very much, in agreement with

the conclusion that aspartate 368 is primarily important for sodium binding to the glutamate-free but not to the glutamate-bound form of the transporter (40).

The multiple effects of the mutations of asparagine 366 and aspartate 368 on the interaction both with sodium and potassium are reminiscent of results obtained by homology modeling of the Na\(^+\)-K\(^+\)-ATPase on the structure of the Ca\(^{2+}\)-ATPase (41). In the latter case it was found that the binding sites for sodium and potassium are partly overlapping, and this could also be the case for the neuronal glutamate transporter. However, in the absence of structures of this transporter with a resolution high enough to observe sodium and potassium ions directly, we cannot exclude the possibility that the effects of the mutations are indirect. In the case of a direct effect and assuming that the GltPh structure is physiologically relevant, a picture would emerge where the side chains of the two residues point toward the binding pocket in the glutamate-free form of the transporter. Binding of glutamate would then bring about a conformational change, so that the two side chains point to each other and away from the binding pocket that would also result in a redistribution of the bound sodium ions. Even though this idea is attractive, in the absence of independent evidence, it has to be considered speculative at the present time.

In the structure of the archaeal glutamate transporter homologue, no direct clue can be found regarding the structural basis for the mechanism of the uncoupled anion conductance. However, based on functional studies, it has been proposed that the acidic amino acid-mediated increase in anion conductance is due to a direct gating when the substrate is occupying the binding pocket (15). Our results on L-aspartate-induced anion conductance in the mutants with perturbed substrate binding (Fig. 10) are fully compatible with this idea. In both N366Q and D368E mutants, each of which is heavily impaired in their interactions with acidic amino acids (Fig. 2 and Table 1), the properties of the anion conductance are grossly changed. In contrast, the N366D/D368N double mutant closely resembles the wild type in the selectivity sequence of the anion permeation (Fig. 10) and at the same time there is not much impact on its apparent substrate affinity (Table 1). As observed in Fig. 10, the phenotypes of the N366Q and D368E mutants are not the same. The N366Q mutation affects the anion selectivity of the leak pathway. In the D368E mutant, the ability of L-aspartate to gate the anion conductance is lost, except when it is measured in a thiocyanate containing medium. But in this case L-aspartate actually inhibits anion conductance. Even though indirect effects cannot be excluded, the simplest explanation is that when the orientation of L-aspartate in the binding pocket is altered, this directly affects the gating (D368E) or selectivity (N366Q) of the anion conduction.

Acknowledgments—We thank Elia Zomot for advice on energy minimization in modeling studies and Dr. Niels Danbolt (University of Oslo) for the generous gift of the affinity purified antibody against EAAC-1.

REFERENCES

1. Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takashashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T., Okuyama, S.,
Role of β-Bridge Residues in Glutamate Transporter

Kawashima, N., Hori, S., Takimoto, M., and Wada, K. (1997) Science 276, 1699–1702
2. Pines, G., Danbolt, N. C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., and Kanner, B. I. (1992) Nature 360, 464–467
3. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10955–10959
4. Kanai, Y., and Hediger, M. A. (1992) Nature 360, 467–471
5. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10955–10959
6. Fairman, W. A., Vandenberg, R. J., Arriza, J. L., Kavanaugh, M. P., and Amara, S. G. (1995) Nature 375, 599–603
7. Dann, S., and Dantzig, M. P. (1992) J. Biol. Chem. 271, 10951–10959
8. Kanner, B. I., and Bendahan, A. (1982) Biochemistry 21, 6327–6330
9. Kanner, B. I., and Bendahan, A. (1982) Biochemistry 21, 6327–6330
10. Pines, G., and Kanner, B. I. (1990) Biochemistry 29, 11209–11214
11. Kavanaugh, M. P., Bendahan, A., Zerangue, N., Zhang, Y., and Kanner, B. I. (1997) J. Biol. Chem. 272, 1703–1708
12. Zerangue, N., and Kavanaugh, M. P. (1996) Nature 383, 634–637
13. Levy, I. M., Warr, O., and Attwell, D. (1998) J. Neurosci. 18, 9620–9628
14. Wadiche, J. I., Amara, S. G., and Kavanaugh, M. P. (1995) Neuron 15, 721–728
15. Borre, L., and Kanner, B. I. (2001) J. Biol. Chem. 276, 40396–40401
16. Seal, R. P., Shigeri, Y., Eliasof, S., Leighton, B. H., and Amara, S. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 15324–15329
17. Ryan, R. M., and Vandenberg, R. J. (2002) J. Biol. Chem. 277, 13494–13500
18. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8122–8126
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
20. Danbolt, N. C., Storm-Mathisen, J., and Kanner, B. I. (1992) Neuroscience 51, 295–310
21. Grunewald, M., Bendahan, A., and Kanner, B. I. (1998) Neuron 21, 623–632
22. Slotboom, D. J., Sobiczak, I., Konings, W. N., and Lolkema, J. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14282–14287
23. Grunewald, M., and Kanner, B. I. (2000) J. Biol. Chem. 275, 9684–9689
24. Brocke, L., Bendahan, A., Grunewald, M., and Kanner, B. I. (2002) J. Biol. Chem. 277, 3985–3992
25. Yernool, D., Boudker, O., Jin, Y., and Gouaux, E. (2004) Nature 431, 811–818
26. Zhang, Y., Bendahan, A., Zarbiv, R., Kavanaugh, M. P., and Kanner, B. I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 351–355
27. Zhang, Y., and Kanner, B. I. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1710–1715
28. Bendahan, A., Arnon, A., Madani, N., Kavanaugh, M. P., and Kanner, B. I. (2000) J. Biol. Chem. 275, 37436–37442
29. Zarbiv, R., Grunewald, M., Kavanaugh, M. P., and Kanner, B. I. (1998) J. Biol. Chem. 275, 14231–14237
30. Borre, L., and Kanner, B. I. (2004) J. Biol. Chem. 279, 2513–2519
31. Pines, G., Zhang, Y., and Kanner, B. I. (1995) J. Biol. Chem. 270, 17093–17097
32. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
33. Bennett, E. R., Su, H., and Kanner, B. I. (2000) J. Biol. Chem. 275, 34106–34113
34. Holmseth, S., Dehnes, Y., Bjornsen, L. P., Boulland, J. L., Furness, D. N., Bergles, D., and Danbolt, N. C. (2005) Neuroscience 136, 649–660
35. Keynan, S., Suh, Y. J., Kanner, B. I., and Rudnick, G. (1992) Biochemistry 31, 1974–1979
36. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8122–8126
37. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
38. Danbolt, N. C., Storm-Mathisen, J., and Kanner, B. I. (1992) Neuroscience 51, 295–310
39. Wadiche, J. I., and Kavanaugh, M. P. (1998) J. Neurosci. 18, 7650–7661
40. Tao, Z., Zhang, Z., and Grewer, C. (2006) J. Biol. Chem. 281, 10263–10272
41. Ogawa, H., and Toyoshima, C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15977–15982