Excitatory amino acid transporters (EAATs) regulate glutamate concentrations in the brain to maintain normal excitatory synaptic transmission. A widely accepted view of transporters is that they consist of a pore with alternating access to the intracellular and extracellular solutions, which serves to couple ion movement to the movement of substrate. However, recent observations that EAATs, and also a number of other neurotransmitter transporters, can also function as ligand-gated chloride channels have blurred the distinctions between transporters and ion channels. Here we show that mutations in the second transmembrane domain (TM2) of EAAT1 alter anion permeation properties without affecting glutamate transport and that a number of TM2 residues are accessible to the external aqueous solution. Furthermore, we demonstrate that the extracellular edge of TM2 is in close proximity to a membrane-associated domain that influences glutamate transport. This study will provide the foundation for beginning to understand how transporters can function as both transporters and ion channels.

In the mammalian central nervous system, glutamate is the predominant excitatory neurotransmitter, and EAATs selectively regulate synaptic glutamate concentrations to maintain a dynamic signaling process between neurons. EAATs are secondary active transporters in which glutamate is co-transported with a cation. This enables EAATs to maintain a 106-fold glutamate concentration gradient across the cell membrane. In addition to this coupled transport conductance, glutamate also activates a thermodynamically uncoupled chloride conductance through the transporter (2–4), which may serve a number of functions such as modulation of cell excitability, regulation of the rate of voltage-dependent glutamate uptake, and influencing ion homeostasis (4–7). A number of other neurotransmitter transporters, including transporters for dopamine, serotonin, norepinephrine, and γ-aminobutyric acid, also allow uncoupled fluxes of ions (8–11), and in the case of the dopamine transporter the substrate-activated chloride conductance regulates presynaptic excitability (8). At present there is little understanding of the molecular basis for how transporters can support these separate functions.

Five EAAT subtypes have been characterized (3, 12–16), and the highly conserved carboxyl-terminal half of EAATs has been identified, by the use of chimeric transporters and site-directed mutagenesis, as forming the glutamate binding and/or translocation domain (17–23) (see Fig. 1A). However, a number of mutations that disrupt transport do not affect chloride permeation (for example see Refs. 18 and 24), and several recent studies have reported selective modulation of the transport component without affecting the chloride component of transport (20–22), implying there are separate molecular determinants for glutamate transport and chloride permeation. In order to investigate this idea further, it is necessary to identify the molecular determinants of chloride permeation and to examine interactions between these residues and other residues involved in glutamate binding and translocation.

The ion permeation pathways of various ion channels are lined with amino acid residues with polar side chains such as serine, threonine, and tyrosine (25, 26), and it has been suggested that the chloride selectivity of the cystic fibrosis transmembrane regulator is the result of an energetically favorable interaction between chloride ions and a polarizable, as opposed to charged, pore (27). The second transmembrane domain is in the amino-terminal half of the EAATs and is separate, at least in primary structure, from the glutamate translocation region. It has positive charges at either end and contains a number of polar residues that are highly conserved between the transporter subtypes (Fig. 1). Based on the position and nature of the residues within TM2, we hypothesized that this domain forms part of the chloride permeation pathway of EAAT1. We have tested this idea by creating a number of conservative mutations in TM2, and we show that several residues within this domain selectively alter chloride channel function without affecting glutamate transport which suggests that this domain forms part of the chloride channel. Many of these residues are accessible to the aqueous environment that is consistent with this domain lining a water-filled pore, and finally we demonstrate that the extracellular edge of TM2 is in close proximity to a membrane-associated domain that influences glutamate transport.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—EAAT1 was subcloned into the plasmid oocyte transcription vector (pOTV) (15). Site-directed mutagenesis was performed using the QuikChange Site-directed Mutagenesis Kit (Stratagene), and all mutations were sequenced on both strands by Dye Terminator Cycle Sequencing (ABI PRISM, PerkinElmer Life Sciences). The wild type and mutant transporter cDNAs were linearized with SpeI and cRNA transcribed with T7 RNA polymerase using the Message mMachine kit (Ambion Inc.).
**Electrophysiology**—All chemicals were obtained from Sigma unless otherwise stated. Stage V oocytes were harvested from *Xenopus laevis* as described previously (28), and all surgical procedures were approved by the University of Sydney Animal Ethics committee. 50 nl of cRNA was injected into oocytes and incubated in standard frog Ringer’s solution (ND96: 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.55).

Current recordings were made using the two-electrode voltage clamp technique with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) interfaced with a MacLab 2e chart recorder (ADI Instruments, Sydney, Australia) using the chart software and a Digidata 1200 (Axon Instruments) controlled by an IBM-compatible computer using the pClamp software (version 7.0, Axon Instruments). The current-voltage relationships for substrate-elicited conductances were determined by subtraction of steady state current measurements in the absence of substrate, obtained during 75-ms voltage pulses from −30 mV to potentials between −100 and +80 in 10-mV steps, from corresponding current measurements in the presence of substrate.

Uptake of L-[3H]glutamate or D-[3H]aspartate (Amersham Biosciences) was measured under voltage clamp in oocytes expressing wild type or EAAT1 mutant transporters. Oocytes were voltage-clamped at 100 mV, and 100 μM L-[3H]glutamate or D-[3H]aspartate was applied for 1 min, followed by a 3-min washout. Oocytes were removed from the bath and lysed in 50 mM NaOH, and scintillation counting was performed.

Recording solutions for the chloride titration were normal frog Ringer’s solution with glucuronate substitution to the desired extra- and intracellular chloride concentration [Cl⁻]₀ and [Cl⁻]ᵢ, respectively. 100 μM Tris-Cl was added to frog Ringer’s solution to obtain 200 μM [Cl⁻]ᵢ. The anion permeability ratios were determined using recording solutions with 10 mM sodium salts of the various test anions chloride (Cl⁻), bromide (Br⁻), iodide (I⁻), or nitrate (NO₃⁻) plus 86 mM sodium gluconate to obtain 96 mM Na⁺, and 2 mM potassium gluconate, 1 mM magnesium gluconate, 1.8 mM calcium gluconate, 5 mM HEPES, pH 7.55. Recordings were made with the bath grounded via a 3 M KClagar bridge connected to a 3 nΩ DC resistor to minimize offset potentials. After testing the effects of the various anions, the measurements in Cl⁻ were repeated to ensure that anion loading of the cell was minimal. The reversal potential measurements in the different anions were used to calculate F/D[I] (X denotes Br⁻, I⁻, or NO₃⁻) using the Goldman-Hodgkin-Katz equation (29). The equilibrium potentials for chloride were calculated assuming [Cl⁻]ᵢ = 41 mM (20). The transporter-specific leak conductance was isolated by using L-threo-benzyloxyaspartate (TBOA) (Tocris, Bristol, UK).

Substrate dose responses were fitted by least squares as a function of current (I) to I/I₀ = [S]/[Kₛₐₜ] + [S], where I₀ is the maximal current, Kₛₐₜ the concentration of substrate that generates half-maximal current, and [S] the substrate concentration. Current as a function of cadmium concentration [Cd²⁺] was also fitted by least squares analysis to I/I₀ = 1 − [Cd²⁺]/IC₅₀ + [Cd²⁺] + Kₛₐₜ/[Cd²⁺] at half-maximal reduction in transport current, and C is the residual transport current at maximal Cd²⁺ inhibition of transport. All values presented are means of at least three cells ± S.E.

**Cysteine Modifications**—2-Sulfonatoethyl-methanesulfonate (MTS) and 2-(trithymethyleneammonium)-ethyl-methanesulfonate (MTSET) were obtained from Toronto Research Chemicals (Toronto, Canada). Stock solutions (0.1 nM) were prepared in MilliQ water, stored at −20°C, and diluted using frog Ringer's buffer immediately before use. The current-voltage relationships were determined, as described above, before and after a 5-min application of either 10 mM MTSES, 1 mM MTSET, or 30 μM HgCl₂. Current-voltage measurements were normalized to the current generated by 100 μM L-glutamate at −100 mV before application of the sulphhydryl reagent. Experiments were also carried out in the presence of HgCl₂ in the absence of L-glutamate. Time course of MTS modification of cysteine mutants was performed at −60 mV. A 100 μM L-glutamate control dose (Icontrol) was applied to oocytes expressing R90C, Q93C, or V96C, and after washout, the same L-glutamate dose was applied with the lowest concentration of MTS reagent that gave stable inhibition within 2 min. The time-dependent decrease in current was fitted with a single exponential giving the time constant of modification. The pseudo first-order rate constant was calculated as the inverse of the modification time constant, and dividing by the concentration of the MTS reagents provided the second-order rate constants.

**Cross-linking Experiments**—After a control dose of 100 μM L-glutamate, oocytes were unclamped, and the grounding electrode was removed followed by a 2-min treatment with 1 mM DTT; the oocyte was washed for 5 min, and the grounding electrode was replaced, followed by the application of 100 μM L-glutamate. The current-voltage measurements presented in Fig. 6 were normalized to the current generated by 100 μM L-glutamate at −100 mV after reduction by DTT. Cross-linking experiments were performed with L-[3H]glutamate or D-[3H]aspartate uptake after voltage-clamped (~100 mV) oocytes expressing wild type or EAAT1 mutant transporters. (UN)labeled oocytes values were subtracted from the data presented. Reversal potential (Eₘᵡ) measurements are from v-aspartate-elicited currents in the presence of 96 mM Cl⁻ or 96 mM NO₃⁻.

### Table I

| Substrate affinity (Kₛₐₜ) values were obtained from dose-response measurements at −60 mV; L-[3H]glutamate uptake was performed on voltage-clamped (~100 mV) oocytes expressing wild type or EAAT1 mutant transporters. (UN)labeled oocytes values were subtracted from the data presented. Reversal potential (Eₘᵡ) measurements are from v-aspartate-elicited currents in the presence of 96 mM Cl⁻ or 96 mM NO₃⁻. |
|---|
| Kₛₐₜ (L-glutamate) | Kₛₐₜ (D-glutamate) | [³H]Glutamate uptake (fmol/oocyte/min) | Eₘᵡ (mV) |
|---|
| EAAT1 | 20.0 ± 3.0 | 23.0 ± 2.0 | 371 ± 31 | 61.2 ± 1.2 |
| R90L | 19.5 ± 0.9 | 11.4 ± 1.8 | 388 ± 79 | 60.3 ± 0.7 |
| Q93A | 28.7 ± 2.2 | 10.3 ± 2.5 | 385 ± 43 | 67.0 ± 1.2 |
| P98Q | 21.4 ± 3.4 | 10.9 ± 0.9 | 385 ± 53 | 67.2 ± 3.9 |
| S102A | 16.5 ± 0.4 | 32.0 ± 0.9 | 385 ± 66 | 72.2 ± 2.0 |
| S103A | 42.0 ± 0.8 | 38.2 ± 0.6 | 385 ± 74 | 72.5 ± 2.0 |
| S103V | 13.0 ± 2.3 | 24.0 ± 2.3 | 385 ± 74 | 72.5 ± 2.0 |
| T106A | 23.2 ± 2.3 | 24.5 ± 2.3 | 385 ± 74 | 72.5 ± 2.0 |
| D112A | 9.2 ± 1.2 | 11.1 ± 1.4 | 213 ± 41 | 60.0 ± 1.1 |
| K114L | 11.7 ± 0.8 | 8.4 ± 1.3 | 255 ± 19 | 60.0 ± 1.1 |

* p < 0.001 when compared with wild type (EAAT1). Data shown are mean ± S.E. (n = 3).
* p < 0.01.
* p < 0.05.
* No current reversal could be measured at membrane potentials up to +80 mV.
The reversal potential of the L-glutamate- (data not shown) and D-aspartate activated currents measured at 60 mV was similar to that of the wild type, except for D112A and K114L (Table I) in which the rates of transport were reduced by ~30%. Relatively minor changes in the $K_{0.5}$ values for L-glutamate- and D-aspartate-elicited currents of the mutant transporters were also observed (Table I). These results demonstrate that residues in this region are unlikely to be directly involved in coupled glutamate transport.

Application of either L-glutamate or D-aspartate to oocytes expressing EAAT1 generates a current that is the sum of the inward coupled transport conductance and the un-coupled chloride conductance (2). The net current activated by L-glutamate reverses direction at $+61.4 \pm 2.5$ mV ($n = 10$), whereas the current activated by the alternate substrate D-aspartate reverses direction at $+11.2 \pm 1.2$ mV ($n = 10$, Table I and Fig. 2A). The more negative reversal potential of the D-aspartate-activated current is due to a larger chloride component than the current activated by L-glutamate. The substrate-activated anion conductance of wild type EAAT1 has a hydrotropic permeability sequence of $\text{Cl}^- < \text{Br}^- < \Gamma < \text{NO}_3^-$ (Table II and Fig. 3C), where $\text{NO}_3^-$ is more permeant than $\text{Cl}^-$ through the transporter (2). When the extracellular chloride is substituted with $\text{NO}_3^-$, the substrate-activated outward current is larger, and the reversal potential shifts to more negative membrane potentials because the proportion of current due to the anion conductance compared with the transport conductance is significantly greater (2) (Fig. 2A). At $+60$ mV most of the observed current is due to the influx of external anions with little contribution from coupled transport. The D-aspartate-activated currents measured at $+60$ mV in the presence of extracellular $\text{NO}_3^-$ of S103A, S103V, T106A, D112A and K114L were significantly reduced, whereas P98G and S102A showed larger currents compared with wild type (Fig. 2). Significant changes in the reversal potential of the L-glutamate- (data not shown) and D-aspartate-activated currents measured in the presence of either 96 mM $\text{Cl}^-$ or $\text{NO}_3^-$ are also observed (Table I). Thus, although small changes in $K_{0.5}$ values for D-aspartate and L-glutamate were observed, the large changes in the current-voltage relationships of the mutants are likely to be due to changes in gating and/or permeation properties of the chloride channel of the transporters.

To investigate the role of TM2 in anion permeation through the transporter, the permeability of various anions relative to chloride was determined for each of the mutant transporters. To estimate the relative anion permeabilities of the wild type and mutant transporters, it is necessary to subtract the conductance due to coupled transport from the total substrate-activated conductance. This was carried out using a two-step procedure. First, the voltage dependence of the coupled transport component of EAAT1 was estimated by varying the extracellular chloride concentration from 10 to 200 mM and measuring the residual current at the theoretical chloride reversal potentials for each concentration of chloride (Fig. 3A). The reversal potential for chloride at 10, 30, 60, 100, and 200 mM is 36, 8, −10, −22, and −40 mV, respectively, assuming $[\text{Cl}^-]_\text{in} = 41$ mM (2). At these potentials, the current due to the uncoupled chloride component, in the above buffers, is zero, and the result...
remaining current is due to coupled substrate transport. The current measurements at the various chloride reversal potentials were plotted and fit to an exponential function for $\delta$-aspartate- (e-fold per 41 ± 2 mV; n = 5) (Fig. 3A) and $\lambda$-glutamate (e-fold per 54 ± 5 mV; n = 5) (data not shown)-activated currents. The second step was to estimate the proportion of the current due to coupled transport and the anion conductance for the wild type and each of the mutant transporters. Substrate transport is coupled to the co-transport of 3 Na$^+$, 1 H$^+$, and the counter-transport of 1 K$^+$ ion (1), resulting in a net inward movement of two positive charges for every transport cycle. The proportion of the current at −100 mV that is due to coupled transport was estimated by performing $\delta$-[3H]aspartate and $\lambda$-[3H]glutamate uptake, whereas clamping the oocytes at −100 mV and calculating the quantity of charge translocated per transport cycle of the current at −100 mV that is due to coupled substrate transport for wild type EAAT1 and each of the mutants can be predicted. With this value and the calculated voltage dependence of the coupled substrate component of EAAT1 estimated in step 1 and assuming a similar voltage dependence of coupled transport for all transporters, we estimated the proportion of the current at each membrane potential due to coupled substrate transport for the mutant transporters. This conductance was then subtracted from the total substrate elicited conductance to leave the uncoupled anion conductance.

Examples of the current-voltage relationships of the $\delta$-aspartate-activated anion conductances of wild type EAAT1, P98G, S102A, and T106A are presented in Fig. 3C. These recordings were made by using 10 mM of the permeant anions in the extracellular solution to avoid anion loading of the oocyte. The Q93A, P98G, S102A, S103A, S103V, and T106A mutations showed significant changes in relative anion permeability compared with wild type for $\delta$-aspartate- and $\lambda$-glutamate-activated anion conductances.

### Table II

Relative anion permeability ratios for $\delta$-aspartate- and $\lambda$-glutamate-elicited conductances of wild type and mutant EAAT1 transporters

|     | $P_{\delta\text{-Asp}}$/$P_{Cl^{-}}$ | $P_{\lambda\text{-Glu}}$/$P_{Cl^{-}}$ | $P_{Na^{+}}$/$P_{Cl^{-}}$ | $P_{\delta\text{-Asp}}$/$P_{Cl^{-}}$ | $P_{\lambda\text{-Glu}}$/$P_{Cl^{-}}$ | $P_{Na^{+}}$/$P_{Cl^{-}}$ | n  |
|-----|-----------------------------------|-----------------------------------|-----------------------------|-----------------------------------|-----------------------------------|-----------------------------|----|
| EAAT1| 2.1 ± 0.1                        | 8.4 ± 0.3                         | 10.7 ± 0.4                   | 1.7 ± 0.1                        | 8.1 ± 0.2                         | 9.7 ± 0.2                    | 10 |
| R90L | 2.1 ± 0.1                        | 7.3 ± 0.6                         | 9.1 ± 0.8                    | 1.7 ± 0.1                        | 5.9 ± 0.5                         | 7.8 ± 0.8                    | 6  |
| Q93A | 1.5 ± 0.1                        | 4.7 ± 0.2                         | 5.7 ± 0.3$^a$                | 1.4 ± 0.1                        | 5.0 ± 0.3                         | 6.1 ± 0.4$^a$                | 6  |
| S102A| 2.9 ± 0.3$^a$                     | 12.5 ± 0.7$^a$                    | 15.1 ± 0.9$^a$               | 2.1 ± 0.1                        | 11.0 ± 0.9                        | 15.1 ± 1.2$^a$               | 5  |
| S103A| 3.6 ± 0.1$^a$                     | 23.6 ± 1.4$^a$                    | 34.0 ± 1.3$^a$               | ND$^d$                           | ND$^d$                           | ND$^d$                       | 6  |
| S103V| 0.9 ± 0.2$^a$                     | 2.8 ± 0.8$^a$                     | 3.3 ± 0.7$^a$                | 1.6 ± 0.2                        | 5.6 ± 0.6                         | 5.7 ± 0.6$^a$                | 6  |
| T106A| 1.1 ± 0.2$^a$                     | 2.7 ± 0.8$^a$                     | 3.1 ± 0.2$^a$                | 1.4 ± 0.5                        | 2.3 ± 0.9$^a$                     | 1.3 ± 0.7$^a$                | 5  |
|     | 1.6 ± 0.1                         | 5.9 ± 0.5$^a$                     | 7.1 ± 0.5$^a$                | 1.6 ± 0.1                        | 4.6 ± 0.7$^a$                     | 4.8 ± 0.7$^a$                |     |

$^a$p < 0.05 when compared with wild type (EAAT1). Data shown are mean ± S.E.  
$p < 0.01$.  
$^d$Not determined, no reversal potential was measured in the Cl−-based buffer.
vated conductances (Table II), and in the case of S103V the permeability sequence of the L-glutamate-activated conductance is changed to Cl<sup>-</sup> < NO<sub>3</sub> < Br<sup>-</sup> < I<sup>-</sup>. The change in relative anion permeability of several mutant transporters and in particular the change in permeability sequence for S103V suggest that there are changes in the interactions between the permeating anions and residues in TM2 and imply that these residues face the lumen of the chloride channel. It should be noted that there are small differences in the permeability ratios depending on which substrate is used to activate the uncoupled anion conductance. This may be due to subtle differences in the conformation of the anion pore when it is activated by D-aspartate compared with L-glutamate.

The D112A Mutant Transporter Has a Large Leak Conductance—In addition to the coupled substrate transport conductance and the substrate-activated uncoupled chloride conductance of glutamate transporters, a substrate-independent chloride conductance has also been described (31–34). The L-glutamate- and D-aspartate-activated currents of the D112A mutant do not reverse direction at membrane potentials up to +80 mV in the chloride based buffer. However, in the absence of substrate, the whole cell conductance of oocytes expressing D112A is significantly greater than oocytes expressing wild type EAAT1. This suggests that the D112A mutation may lock the chloride channel into a conformation that allows anions to leak through the transporter, and as a result, subsequent application of substrate does not activate an additional chloride conductance. We have tested this idea by employing the non-selective glutamate transport blocker TBOA. Application of 250 μM TBOA to oocytes expressing either wild type EAAT1 or D112A in ND96 (96 mM NaCl) blocks a small current that is similar for the two transporters. When the external buffer is changed to NO<sub>3</sub>-based buffer, there is a large difference in amplitude of the TBOA-blocked currents between the two transporters (207 ± 20 nA at +60 mV, n = 4 for EAAT1, and 968 ± 67 nA at +60 mV, n = 4 for D112A, Fig. 4). Although there are large differences in amplitudes of the TBOA-blocked leak conductances for D112A compared with wild type EAAT1, the reversal potentials of the leak currents were similar in the NO<sub>3</sub>-based buffer (−72.6 ± 0.9 mV, n = 4 for EAAT1, and −72.0 ± 1.2 mV, n = 4 for D112A) and also the Cl<sup>-</sup>-based buffer (−23.6 ± 1.8 mV, n = 7 for EAAT1, and −27.5 ± 1.3 mV, n = 11 for D112A), suggesting that relative anion permeability of the leak current is unchanged.

We investigated the nature of the leak conductance more closely by measuring reversal potentials in the 10 mM Cl<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup> and NO<sub>3</sub>-based buffers and calculating relative permeabilities of the TBOA-blocked leak currents. The relative anion permeability sequence and values for the leak conductance of EAAT1 are different from the substrate-activated anion conductance, but the anion permeability values for the leak conductance of D112A are not significantly different from the leak conductance of EAAT1 (Table III). Thus, the D112A mutation is unlikely to alter interactions between anions and the channel when operating in leak mode. However, the differences in amplitude of the leak suggest that this residue influences the gating of the channel. A number of additional mutations of Asp-12 were also generated to further characterize the role of this residue. Asp-12 was mutated to His, Asn, and Glu, and TBOA was applied to oocytes expressing the mutant transporters. The amplitude of the TBOA-blocked leak currents in these additional Asp-112 mutant transporters are similar to wild type EAAT1, and in each case, the reversal potential of the TBOA-blocked currents are also similar to wild type (data not shown), which suggests that the enhanced leak conductance is only present when Asp-112 is changed to the small non-polar residue alanine. These results are consistent with our suggestion that Asp-112 plays an important role in gating of the chloride channel but does not directly influence relative anion permeability. The K114L mutant showed similar reductions in the substrate-activated chloride conductance to the D112A mutant transporter, but it is interesting to note that the amplitude of the leak conductance is similar to wild type EAAT1 (data not shown).

Aqueous Accessibility of TM2—The results presented above suggest that TM2 plays an important role in determining the anion permeation properties of the channel. An additional feature of most ion channels is that pore-lining residues of ion channels are often accessible to the aqueous environment. Cysteine mutants of polar residues within this region and selected surrounding residues were generated in cysteine-less EAAT1 (CLE1) as described previously (21), and their reactivity with
water-soluble sulfur reagents was assessed by measurements of change in the L-glutamate-activated conductance. Application of 10 mM MTSES for 5 min to oocytes expressing CLE1 had no effect on the L-glutamate-activated conductance, but significantly changed the L-glutamate-activated conductance of R90C, Q93C, and V96C mutants (Fig. 5A). Similar results were found when MTSES was applied in the presence of L-glutamate (data not shown). However, none of the residues after Val-96 showed any change in activity after MTSES or MTSET application. The rate of modification by positively and negatively charged MTS reagents can also provide information about the charge selectivity of the environment surrounding an introduced cysteine residue (35, 36). Second-order reaction rates for MTSES and MTSET modification of the R90C, Q93C, and V96C mutants were calculated, and the relative reaction rates were estimated after normalizing the rates of MTSES and MTSET modification of cysteine in an aqueous solution ($k_{\text{MTSES}}/k_{\text{MTSET}} = 0.08$) (35). The relative reaction rates ($k_{\text{MTSES}}/k_{\text{MTSET}}$) for R90C, Q93C, and V96C were 251, 4.4, and 3.6, respectively, which is indicative of these residues being in an environment that selects negatively charged over positively charged molecules. This observation is consistent with the suggestion that these residues line the external vestibule of the chloride channel of EAAT1.

The minimum pore diameter of the chloride channel of EAAT1 has been estimated to be ~5 nm (32), whereas the width of the head group of MTSES is 5.8 nm (37), and therefore it is reasonable to assume that the geometry of the channel may limit accessibility of the bulky MTS reagent. To probe further the solvent accessibility of residues below Val-96, the cysteine mutants were treated with a smaller sulfur-reactive reagent Hg$^{2+}$, which has been used to modify the pore-lining residues of the CHIP28 water channel (38). Application of 30 μM HgCl$_2$ to oocytes expressing CLE1, L97C, and P98C in the presence of or absence of L-glutamate caused no significant change in the L-glutamate-activated conductance, but under similar conditions the L-glutamate-activated conductances of the L99C, S102C, and S103C mutants are greatly reduced (Fig. 5B). Thus, residues Arg-90, Gln-93, Val-96, Leu-99, Ser-102, and Ser-103 are accessible to the aqueous environment.

Although introduction of single cysteine residues in CLE1 cannot result in disulfide bonds within the same protein, it is possible for disulfide bonds to form between the introduced cysteine residues on separate transporter proteins and alter functional properties. Application of DTT to reduce any inter-protein disulfide bonds that may have formed spontaneously did not alter the functional properties of any of the cysteine mutants (for example see Fig. 6B). Furthermore, application of CuPh, which promotes the formation of disulfide bonds directly between cysteines (39), did not have any affect on the functional properties of the single cysteine mutants (see Fig. 6B). As no change in transporter function was observed in either case, it is unlikely that inter-protein disulfide bonds form between single cysteine mutants. A T106C mutant was also constructed, but the transporter was not functional. We ruled out the possibility of inter-protein disulfide bond formation as an explanation for the lack of transport activity by treating the oocytes with DTT. The lack of activity and the inability of DTT to induce any transport activity precluded any further investigation into the solvent accessibility of this residue.

The Extracellular Edge of TM2 Is in Close Proximity to a Membrane-associated Domain That Influences Glutamate Transport—Changing residues Arg-90, Gln-93, Val-96, L99, Ser-102, and Ser-103 to small, non-polar residues does not significantly alter substrate binding or transport, but the effects observed after the addition of MTSES or HgCl$_2$ to the cysteine mutants of these residues cannot be solely attributed to a reduction in the uncoupled chloride component. These results suggest that TM2, which contains molecular determinants for chloride permeation, may be in close proximity to areas in the carboxyl-terminal region of the transporter that have been implicated in substrate binding and translocation. Proximity relationships in membrane-bound proteins have been determined using the method of cross-linking pairs of introduced cysteine residues (39). We have used a function-based assay to determine whether pairs of cysteine residues in TM2 and a number of domains involved in glutamate transport can form a covalent disulfide bond by measuring glutamate transport currents before and after reduction by DTT or oxidation by CuPh.

V452C is accessible to extracellularly applied MTS reagents (21, 40) and is located in membrane-associated domain 10 (Fig. 1A), which has been suggested to exist as an aqueous pore or binding pocket that can be occluded when substrates or inhibitors bind to the transporter (41). Application of MTSET to the V452C transporter results in loss of glutamate translocation but does not interfere with glutamate binding and activation of...
the uncoupled chloride conductance (21). We created several cysteine mutant transporters of residues in TM2 that affect anion permeation and are accessible to the extracellular aqueous environment in a V452C background. All of the double mutant transporters, except the V452C/V96C double cysteine mutant, were functional when expressed in *X. laevis* oocytes with varying levels of activity (data not shown). The currents activated by 100 μM L-glutamate via the double cysteine transporter V452C/Q93C were very small compared with CLE1 and the other double mutant transporters, and it was postulated that this cysteine pair was forming a disulfide bond and inhibiting transporter activity. All of the double mutants were incubated with the reducing agent DTT to reduce any disulfide bonds that may have spontaneously formed between the introduced cysteines. The currents via V452C/Q93C were significantly increased (Fig. 6A), whereas CLE1 (Fig. 6B) and none of the other double mutant transporters were affected (data not shown). This suggests that this cysteine pair is forming a spontaneous disulfide bond, and these residues are very close in space.

Once any spontaneous disulfide bonds had been reduced by incubation with DTT, cross-linking between cysteine pairs was induced by incubating the cells with the oxidizing reagent CuPh. As expected the current via the double mutant transporter V452C/Q93C was significantly reduced after incubation with CuPh (Fig. 6A). The double mutant V452C/R90C does not form a spontaneous disulfide bond, but cross-linking can be induced by incubation with CuPh suggesting that these two cysteine residues are also close to one another in space (Fig. 6A). The currents via CLE1 (Fig. 6B) and the other double mutant transporters were not affected by CuPh (data not shown).

It has been suggested that the glutamate transporters form oligomeric structures (42, 43), and so it is possible that these cysteine residues are forming a disulfide bond with the same residue on another transporter subunit. To rule this out, the single cysteine transporters V452C, R90C, and Q93C were expressed in oocytes, and after a control dose of 100 μM L-glutamate oocytes were treated with DTT and CuPh as described above. No significant changes in current amplitude at any of the test membrane potentials were observed for the single cysteine mutants after application of either DTT or CuPh (Fig. 6B). The single cysteine transporter V452C was also co-expressed with the single cysteine mutants R90C and Q93C to test if cross-linking was occurring between these residues on separate transporter subunits. Again, incubation with DTT or CuPh did not result in any significant change in the L-glutamate-activated current when single cysteine transporters were co-expressed (Fig. 6C), suggesting that cross-linking is not occurring between transporter monomers, or if it is, it is not responsible for the change in function observed when the cysteine pairs are expressed within the same transporter protein. It is only when the two cysteine residues are in the same transporter that spontaneous cross-linking occurs between

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**Fig. 6.** Cross-linking of cysteine residues between TM2 and domain 10. Current-voltage relationships generated by the application of 100 μM L-glutamate in ND96 (open squares), after DTT treatment (closed squares), and after CuPh treatment (closed circles). The currents are normalized to the L-glutamate elicited current at −100 mV after DTT treatment. A, the double cysteine mutant transporters V452C/R90C and V452C/Q93C. B, CLE1 and the single cysteine mutant transporters V452C, R90C, and Q93C. C, V452C co-expressed with R90C and Q93C, respectively.
V452C/Q93C, and cross-linking can be induced between V452C/R90C.

We also investigated whether residues in TM2 were also in close proximity to other residues involved in glutamate transport. Tyr-405 has been implicated in co-transported ion binding and is alternately accessible from both sides of the membrane (24, 44, 45). Tyr-405 and Leu-404 are located in re-entrant loop 8 and are indicated in Fig. 1. We made the double mutants L404C/S103C and Y405C/Q93C and Y405C/D112C. The L404C/D112C and Y405C/D112C mutant transporters were non-functional, and the L-glutamate-activated currents of L404C/S103C and Y405C/Q93C were insensitive to DTT or CuPh (data not shown). Arg-477 (Fig. 1) is an aqueous accessible residue (40) that is close to Arg-479, which has been implicated in binding the γ-carboxyl group of glutamate (19). The double mutant transporters R477C/R90C and R477C/V96C were constructed but were insensitive to DTT and CuPh (data not shown), suggesting that these residues are not close enough to form a disulfide bond.

A complementary method used to determine proximity of cysteine residues is the formation of a high affinity cadmium-binding site in which a cadmium ion (Cd²⁺) interacts with the cysteinyl side chains of both introduced cysteine residues at the same time (46). After reduction of V452C/Q93C with DTT, subsequent application of Cd²⁺ inhibits transporter currents. The IC₅₀ value for Cd²⁺ inhibition of the L-glutamate-activated currents of V452C/Q93C is 0.9 ± 0.06 μM. Cd²⁺ had no effect on EAAT1, CLE1, V452C, or V452C/R90C at concentrations up to 100 μM (Fig. 7). The lack of high affinity Cd²⁺ inhibition of L-glutamate-activated currents of V452C/R90C suggests that these residues are not in sufficient proximity to coordinate Cd²⁺ binding, which is consistent with the previous observation that these residues do not form a spontaneous disulfide bond. However, disulfide bond formation can be induced by the application of CuPh, which suggests that these two cysteine residues may come into close contact during visits to rare conformational states. The observation that V452C and Q93C can form a disulfide bond places their α carbons within 3.8–6.8 Å of each other (47), and the fact that they also form spontaneous disulfide bonds suggests that they are within 5 Å of each other (48). In addition, β carbon atoms in cysteines bridged by Cd²⁺ are usually separated by 5–6 Å (49). By using two complementary approaches we have shown that a residue at the extracellular edge of TM2, a region involved in chloride permeation, is in close proximity to V452C, which is located in a membrane-associated domain involved in substrate binding and/or translocation.

An earlier cross-linking study on GLT-1 (the rat homologue of EAAT2) reported that the cysteine pairs Ala-364/Ser-440 and Ala-412/Val-427 (Ser-366/Gly-442 and Ala-414/Ile-429, EAAT1 numbering) were close enough to each other to form a disulfide bond and create a high affinity Cd²⁺-binding site in GLT-1 (39). V452C can form a disulfide bond with R90C and Q93C and a high affinity Cd²⁺-binding site with Q93C. We propose that chloride (Cl⁻) interacts with residues along TM2 and suggest that Ser-103 is a point of contact for permeating chloride ions. Asp-112 appears to play an important role in gating of the chloride channel. In the absence of glutamate, the channel has a low probability of opening resulting in a small chloride leak conductance, but upon glutamate (Glu) and Na⁺ (Na⁺) binding, the conformation of Asp-112 may be altered to increase the probability of channel opening leading to a greater chloride conductance. In this model, we suggest that glutamate and Na⁺ permeate the same pore as chloride but that there are separate molecular determinants for the two functions. Helical wheel representation of TM2, residues amino-terminal to Pro-98 (B), and residues carboxyl-terminal to Pro-98 (C). The dashed line separates residues in white lettering on black which are aqueous accessible. Note that T106C was not functional, and so the accessibility of this residue was not examined.

**FIG. 8. Structural predictions of TM2 and its relationship with the glutamate binding and translocation domain.** A, structural and mechanistic model for glutamate transport and chloride permeation of EAAT1. We have omitted the K⁺ ion and H⁺ for simplicity. The thick line is in the plane of the paper and the dashed line is behind the plane of the paper. Cysteine pairs A144C/I429C and S366C/G442C (EAAT1 numbering) are close enough to form a disulfide bond and a high affinity Cd²⁺-binding site in GLT-1 (39). V452C can form a disulfide bond with R90C and Q93C and a high affinity Cd²⁺-binding site with Q93C. We propose that chloride (Cl⁻) interacts with residues along TM2 and suggest that Ser-103 is a point of contact for permeating chloride ions. Asp-112 appears to play an important role in gating of the chloride channel. In the absence of glutamate, the channel has a low probability of opening resulting in a small chloride leak conductance, but upon glutamate (Glu) and Na⁺ (Na⁺) binding, the conformation of Asp-112 may be altered to increase the probability of channel opening leading to a greater chloride conductance. In this model, we suggest that glutamate and Na⁺ permeate the same pore as chloride but that there are separate molecular determinants for the two functions. Helical wheel representation of TM2, residues amino-terminal to Pro-98 (B), and residues carboxyl-terminal to Pro-98 (C). The dashed line separates residues in white lettering on black which are aqueous accessible. Note that T106C was not functional, and so the accessibility of this residue was not examined.
DISCUSSION

In this report, we have demonstrated that mutations of polar and charged residues within TM2 of EAAT1 to aliphatic residues of similar size and shape selectively alter anion permeability without changing the rate of L-[3H]glutamate transport. The selective manipulation of one function of the transporter without affecting the other suggests that the mutations have not caused large conformational effects and that the changes in anion permeability are likely to be due to small local structural changes. Furthermore, many of the polar and charged residues within TM2 are accessible to the external aqueous environment, and the extracellular edge of TM2 appears to be anion-selective as demonstrated by the greater reactivity of cysteine residues with the anionic MTSES compared with the cationic MTSET reagent. The combination of these observations is consistent with the suggestion that the altered side chains are directly responsible for altered anion interactions within the channel and that TM2 forms part of the chloride ion permeation pathway. However, an alternative interpretation of the results that cannot be definitively ruled out is that TM2 forms an aqueous accessible structure that indirectly influences other transmembrane domains that form the anion permeation pathway without affecting transport. At this stage, we favor the first interpretation for two reasons. First, TM2 is highly conserved between transporter subtypes, which implies a critical function for the domain. Second, it is difficult to conceive how a set of mutations of single residues along the entire length of the transmembrane domain all impact on the one function with no effect on the other, without those residues being responsible for the function. Nevertheless, further structural and functional studies are required to confirm our hypothesis.

An important feature of the results is that mutations at various sites along the full-length of TM2 alter the relative anion permeability, which suggests that this domain forms a structure that allows anions to pass from the extracellular to the intracellular side of the membrane. This allows us to make a number of predictions about the structure and roles of the various parts of TM2 in chloride channel function. From the pattern of accessibility of cysteine mutants and the changes in anion permeability ratios of mutants of Arg-90, Gln-93, Val-96, Leu-99, Ser-102, Ser-103, and Thr-106, it may be predicted that this transmembrane domain forms an α-helical structure with one face of the helix lining the channel. Proline residues within transmembrane α-helices have been predicted to introduce kinks in the helix and constrain conformational changes. Although Pro-98 is not accessible to the aqueous environment, the P98G mutant shows small yet significant changes in the relative anion permeability ratios and large increases in the outward currents measured at +60 mV. Therefore, this residue is unlikely to line the chloride channel but may influence the flexibility of the α-helix and thereby influence anion permeation and also regulate conformational changes required for opening the channel. If the residues of TM2 are plotted on an α-helical wheel diagram to identify residues that are likely to face into the pore of the chloride channel, the residues can be separated into two groups based on whether they are amino-terminal to or carboxyl-terminal to residue Pro-98. An α-helical wheel of residues amino-terminal to Pro-98 (Fig. 8B) shows residues Arg-90, Gln-93, and Val-96 facing a similar direction. A separate helical wheel plot of residues carboxyl-terminal to Pro-98 (Fig. 8C) suggests that residues Leu-99, Ser-102, Ser-103, and Thr-106 would all face a similar direction. Thus, we predict that Pro-98 introduces a kink into the helix that has the potential to bring the two sets of residues into alignment to form part of the chloride permeation pathway. Furthermore, residues below Pro-98 are not accessible to bulky MT8 reagents but are accessible to the smaller sulfhydryl-reactive reagent, Hg²⁺. This may be the result of a distortion in the helix that restricts accessibility of the MT8 reagents beyond this point. Mutations of Ser-103 show marked changes in anion permeability with the S103V mutant causing a change in permeability sequence. This residue is below Pro-98 and may be within a restricted segment of the pore and form a contact site with permeating anions.

The two charged residues, Asp-112 and Lys-114, at the intracellular edge of TM2 also play an important role in chloride channel function. Neutralizing either of these two charged residues markedly reduces the chloride component of both the D-aspartate- and L-glutamate-activated conductances. In the case of Lys-114, we envisage that it may form part of an intracellular electrostatic surface to facilitate chloride movement through the channel. The properties of the D112A mutant were investigated in more detail, from which we were able to gain some insights into gating of the channel. In the D112A mutant the substrate-independent leak current is significantly larger than in wild type EAAT1, and the amplitude of the L-glutamate- or D-aspartate-activated chloride current is greatly reduced. These observations have a number of implications. First, it is reasonable to suggest that the chloride leak current and the substrate-activated chloride current are both mediated by the same pore structure of the transporter. Second, Asp-112 may form part of the gate of the chloride channel. In the CLC channel (voltage-gated chloride channel), it has been postulated that a glutamate residue sits with the pore of the channel in the closed state preventing anion permeation, and that upon activation the glutamate residue moves in such a way as to allow anion passage (50). Asp-112 may play a similar role in EAAT1 to regulate chloride ion passage through the transporter. In the absence of substrate, Asp-112 may sit within the channel, and upon binding of substrate and Na⁺, conformational changes may cause Asp-112 to move out of the way and allow chloride ions to pass. The D112A mutation may lock the gate in an open state. If the leak and substrate-activated chloride currents are mediated by the same pore structure then it is noteworthy that the relative anion permeability ratios for the two functional states are significantly different. For the leak current the anion permeability ratios range from 1 to 2.6, where iodide is more permeant than nitrate. In contrast, the permeability ratios for the D-aspartate- and L-glutamate-activated anion current vary from 1 to 10.7 and 1 to 9.7 respectively, with nitrate more permeant than iodide. These differences suggest that substrate binding and/or transport alter the conformation of the channel and influence anion permeation. Furthermore, the slight differences in anion permeability for the D-aspartate- and L-glutamate-activated anion currents suggest that these two substrates generate subtly different conformations of the chloride channel. In a recent report (33), differences in anion permeabilities of the leak- and substrate-activated anion conductances were also observed for EAAT2 and EAAT4, which suggests that this property may be common to all glutamate transporter subtypes.

A question that arises from these observations is: what is the physical relationship between the glutamate translocation domain and the chloride channel of the transporter? Although it is possible to independently manipulate the two functions of glutamate transporters (20–22, 51, 52), the two functions can also influence the activity of one another. A number of models for the dual functions of glutamate transporters have been proposed. Wadiche et al. (2) have suggested that the chloride channel and glutamate translocation domain may be two distinct conformational states of the same pore region, whereas Sanders and Amara (33) suggest that there may be two perme-
Clathrin permeation. Also be of interest to identify other residues involved in chloride to these residues. Further cross-linking studies between these identified other residues or domains that are in close proximity for by a similar dual, but intersecting, pore model for glutamate transporters is that they contain two intersecting pores (54). In this scenario the separate pores may be independently regulated but also have the capacity to influence the activity of one another. The observations in this study could also be accounted for by a similar dual, but intersecting, pore model for glutamate transporters.

In this report we have demonstrated a relationship between the substrate-activated uncoupled chloride conductance, the substrate-independent leak conductance, and the ion-coupled substrate-activated uncoupled chloride conductance, the gated ion channels. The results presented in this study provide the first experimental evidence to allow distinction between these models. If TM2 forms part of the lining of the chloride channel pore, then from the model proposed by Eskandari et al. (43), it may be predicted that five copies of TM2 would come together to form the chloride channel. Our results do not support this model because several single cysteine mutants of parts of the pore primarily responsible for the different functional properties of glutamate transporters is that they contain two intersecting pores (54). In this scenario the separate pores may be independently regulated but also have the capacity to influence the activity of one another. The observations in this study could also be accounted for by a similar dual, but intersecting, pore model for glutamate transporters.

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The Chloride Permeation Pathway of a Glutamate Transporter and Its Proximity to the Glutamate Translocation Pathway
Renae M. Ryan, Ann D. Mitrovic and Robert J. Vandenberg

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