Cysteine-scanning Mutagenesis Reveals a Conformationally Sensitive Reentrant Pore-Loop in the Glutamate Transporter GLT-1*

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Glutamate transporters in the brain ensure that the synaptic levels of the transmitter are kept below the toxic levels (1–6). These transporters, located in the plasma membrane of nerve and glial cells, play an important role in limiting the duration of synaptic excitation (7–10). The uptake process is electrogenic (11–13), involving co-transport of three sodium and glutamate fluxes. Here we used cysteine-scanning mutagenesis of the GLT-1 transporter to test the idea that this loop undergoes conformational changes following sodium and substrate binding. 15 of 22 consecutive single cysteine mutants in the stretch between Gly-422 and Ser-443 exhibited 30–100% of the transport activity of the cysteine-less transporter when expressed in HeLa cells. The transport activity of 11 of the 15 active mutants including five consecutive residues in the ascending limb was inhibited by small hydrophilic methanethiosulfonate reagents. The sensitivity of seven cysteine mutants, including A438C and S440C, to the reagents was significantly reduced by sodium ions, but the opposite was true for A439C. The non-transportable analogue dihydrokainate protected at almost all positions throughout the loop, and at two of the positions, the analogue protected even in the absence of sodium. Our results indicate that reentrant loop II forms part of an aqueous pore, the access of which is blocked by the glutamate analogue dihydrokainate, and that sodium influences the conformation of this pore-loop.

Removal of glutamate from the synaptic cleft by (Na+ + K+) coupled transporters prevents neurotoxicity due to elevated concentrations of the transmitter. These transporters exhibit an unusual topology, including two reentrant loops. Reentrant loop II plays a pivotal role in coupling ion and glutamate fluxes. Here we used cysteine-scanning mutagenesis of the GLT-1 transporter to test the idea that this loop undergoes conformational changes following sodium and substrate binding. 15 of 22 consecutive single cysteine mutants in the stretch between Gly-422 and Ser-443 exhibited 30–100% of the transport activity of the cysteine-less transporter when expressed in HeLa cells. The transport activity of 11 of the 15 active mutants including five consecutive residues in the ascending limb was inhibited by small hydrophilic methanethiosulfonate reagents. The sensitivity of seven cysteine mutants, including A438C and S440C, to the reagents was significantly reduced by sodium ions, but the opposite was true for A439C. The non-transportable analogue dihydrokainate protected at almost all positions throughout the loop, and at two of the positions, the analogue protected even in the absence of sodium. Our results indicate that reentrant loop II forms part of an aqueous pore, the access of which is blocked by the glutamate analogue dihydrokainate, and that sodium influences the conformation of this pore-loop.

The five known eukaryotic glutamate transporters, GLT-1 (19), GLAST-1 (20), EAAC-1 (21), EAAT-4 (18), and EAAT-5 (22), have an overall amino acid identity of ~50%. The homology is significantly higher in the carboxyl-terminal half of the transporters. Topology studies suggest that this region of the protein has an intriguing arrangement containing two oppositely oriented reentrant loops, the two transmembrane domains (TMs)1 7 and 8, and an outward-facing hydrophilic region (Refs. 23–25, and see Fig. 1). Some of the features of the membrane topology remain under debate (26).

Several amino acid residues critical for the function of glutamate transporters are located in the carboxyl-terminal half. Two adjacent amino acid residues of GLT-1, Tyr-403 and Glu-404, are located in TM7 (Fig. 1) and are conserved in all other glutamate transporters. Both were implicated in the binding of potassium ions (16, 27) and appear to be close to one of the sodium binding sites (27). A conserved arginine residue, Arg-477 in GLT-1, is located in TM8 (Fig. 1) and has been shown to play a pivotal role in the sequential interaction of the transporters with amino acid substrates and with the potassium ion (28).

Cysteine residues engineered into some positions of reentrant loop I are accessible to sulfydryl reagents from both sides of the membrane (24, 25). Modification of these cysteines results in inactivation of the transporter, but this can be prevented by L-glutamate applied from the extracellular side (24, 25). Two serine residues at the carboxyl-terminal extremity of reentrant loop II (Fig. 1) of GLT-1 play a role in the sensitivity of the transporter to the non-transportable glutamate analogue dihydrokainate (29). Mutation of serine 440 to glycine results in an alteration of the sodium specificity of glutamate transport (29). Moreover, when this residue is replaced by cysteine, the S440C transporters become sensitive to membrane-impermeant sulfhydryl reagents in a substrate- and substrate analogue-protectable fashion (29). These observations suggest that the reentrant loops play an important role in glutamate transport. To address the question of whether reentrant loop II undergoes conformational changes during the transport cycle and participates in the formation of the pore of the transporter, we replaced all residues of this loop, one at a time, by cysteines. We used the functional impact of exposure of these single cysteines to small hydrophilic sulfhydryl reagents as an indicator for their accessibility to the aqueous space. It appears that the majority of the engineered cysteines are accessible, indicating that the loop forms part of an aqueous pathway through the transporter. We conclude from the dependence of the accessibility pattern on sodium and dihydrokainate that this loop forms a dynamic structure.

The abbreviations used are: TM, transmembrane domain; CL-H, cysteine-less GLT-1 with a decahistidine tail at the carboxyl terminus; MTS, methanethiosulfonate; MTSET, (2-trimethylammoniomethyl)-methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate; DHR, dihydrokainic acid; BM, 3-N-maleimidylpropionylbiocytin.

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Position 431 is accessible to the large sulfhydryl reagent BM but only from the inside of the cells (23). Consistent with this, the activity of the A431C mutant was not affected by preincubation with 1 mM membrane-impermeant MTSET (Fig. 3). MTSEA is a sulfhydryl reagent smaller than MTSET, and it is also membrane-permeant (34, 35). In contrast to MTSET, preincubation with 2.5 mM MTSEA potently inhibited the A431C transporters (Fig. 3). Neither of the two reagents had any effect on the cysteine-less transporter (Fig. 3). In the ascending limb of the loop (the stretch from positions 431 to 443) six of the seven single cysteine mutants with significant activity were sensitive to one or both of the MTS reagents (Fig. 3). Only in the case of T432C was no inhibition of D-[3H]aspartate transport by either of these reagents observed. The activity of S443C and the five single cysteine mutants in the consecutive residues 436–440 was inhibited by MTSEA (Fig. 3). The activity of S443C and the mutants of positions 438–440 was also inhibited by the impermeant MTSET (Fig. 3). As will be shown later (Fig. 5), I436C and G437 also became sensitive to MTSET in sodium-free medium.

We have previously shown that S443C is accessible to the large sulfhydryl reagent BM from the extracellular space (23). This was also the case with the three consecutive positions 438–440 (Fig. 4). Thus a biotinylated band of the size of the mature plasma membrane form of the transporter (23, 25) was detected in the case of A438C, A439C, and S440C (Fig. 4). This biotinylation could be prevented by reaction of the cells expressing these mutant transporters prior to solubilization and nickel chromatography with MTSET (Fig. 4). Thus, cysteines introduced at position 436 or 437, which are located deeper down in the loop, cannot react with the larger BM (Fig. 4) but only with the smaller MTS reagents.

In the descending limb, position 422 was accessible to BM from the extracellular side. However, its modification by MTSEA or MTSET did not have a functional impact (Fig. 3). V424C, T426C, and V427C were partly inhibited by MTSEA, and the former two were also partly inhibited by MTSET. T426C, V427C, and S428C did not react with the large BM (data not shown). No effects of the MTS reagents could be detected with S428C and L429C, whereas in T430C only MTSEA inhibited, just as in A431C (Fig. 3).

Effects of Sodium Ions on the Accessibility of Engineered Cysteines—Serine 440 is a determinant of the sodium specificity of glutamate and aspartate transport, implying that the loop plays a role in the interaction of the transporter with sodium ions (29). Therefore we examined the effect of sodium ions on the accessibility of the cysteine residues introduced in the pore-loop. The experiments summarized in Fig. 5 illustrate the ability of the MTS reagents to inhibit the transport of the single cysteine mutants upon preincubation with or without sodium ions (choline replacement). To be able to evaluate a possible differential effect at those positions that are very sensitive, namely 436–440 and 443, we probed them with lower concentrations of the reagents than those used in Fig. 3. In the ascending limb, sodium conferred a significant protection against inhibition of I436C, G437C, A438C, and S440C by both reagents (Fig. 5, A and B). On the other hand, sodium potentiated the inhibition of A439C by the two reagents (Fig. 5, A and B). A similar potentiation by sodium was observed on S443C in the case of MTSET (Fig. 5B). The sensitivity of this mutant toward MTSEA was so extreme that total inhibition was obtained using concentrations as low as 5 μM. Therefore we did not continue to try to see whether the effect of sodium is also observed with MTSEA (Fig. 5A). In the descending limb only the inhibition of V427C was protected by sodium, and this
was true for both reagents (Fig. 5, A and B). At the apex of the reentrant loop, T430C and A431C were sensitive only to MT-SEA (Fig. 3), and in both mutants sodium markedly protected

![Graph](image1)

**FIG. 2.** Transport activity of single cysteine mutants located in the reentrant loop II. Each residue of the second reentrant loop was replaced by a cysteine. Sodium-dependent D-[^3]H]aspartate uptake was measured in HeLa cells expressing these single cysteine mutants or the CL-H transporter as described under “Experimental Procedures.” Values are expressed as percentage of the CL-H transport measured. Each bar is the mean ± S.E. of 3–10 independent determinations, each done in triplicate.

![Graph](image2)

**FIG. 3.** Effect of MTS reagents on single cysteine mutants at reentrant loop II. Sodium-dependent D-[^3]H]aspartate uptake was measured in HeLa cells expressing CL-H or the indicated mutants. Before the transport assay, the cells were incubated for 5 min at room temperature in the standard NaCl-containing transport medium with or without 1 mM MTSET (black bars) or 2.5 mM MTSEA (gray bars). The cells were then washed twice and assayed for D-[^3]H]aspartate uptake for 10 min. The data shown are percentage of activity of that of cells preincubated in NaCl medium without MTS reagents. Each bar is the mean ± S.E. of two to seven independent determinations, each done in triplicate.

was true for both reagents (Fig. 5, A and B). At the apex of the reentrant loop, T430C and A431C were sensitive only to MT-SEA (Fig. 3), and in both mutants sodium markedly protected

![Graph](image3)

**FIG. 4.** Biotinylation of single cysteine mutants. HeLa cells expressing the CL-H transporter or the indicated single cysteine mutants were washed twice with phosphate-buffered saline and incubated with or without 1 mM MTSET as indicated. The cells were washed and incubated for 30 min at room temperature in phosphate-buffered saline containing 500 μM BM. Excess BM was removed by two washes with phosphate-buffered saline containing 2% 2-mercaptoethanol. The solubilized cellular proteins were purified on nickel-nitrilotriacetic beads, and the eluted proteins were subjected to SDS-PAGE and then transferred onto nitrocellulose. Biotinylated proteins were detected with streptavidin conjugated to peroxidase and ECL (Amersham Biosciences). The numbers on the left indicate the apparent molecular mass (in kDa) of markers (New England Biolabs).
The activity of these mutants remained insensitive to MTSET also in the absence of sodium (Fig. 5B).

Effects of Transportable and Non-transportable Glutamate Analogues—The mutants, whose activity was sensitive to MTSET, were significantly protected against it by the non-transportable glutamate analogue DHK. The only exception was S443C where the analogue potentiated the inhibition by MTSET (Fig. 6B). Similar effects of DHK were observed against inhibition by MTSEA, except for T426C where no effect was observed. In the case of S443C we did not determine the effect due to the extreme sensitivity of this mutant to MTSEA (Fig. 6A). The activity of these mutants remained insensitive to MTSET also in the absence of sodium (Fig. 5A).

Further analysis of the effect of transportable substrates on the protection of A438C and S440C against inhibition by MTSET revealed an intriguing result. Although D- and L-aspartate both protected, a saturating concentration of L-glutamate (1 mM) did not protect A438C and only had a slight protective effect on S440C (Fig. 8A). The specificity of the protection by D- and L-aspartate was shown using /H9253-aminobutyric acid. This amino acid neurotransmitter, which does not interact with GLT-1, also does not protect (Fig. 8A). The lack of a protective effect by L-glutamate does not reflect the binding to a different site than that of aspartate because L-glutamate prevented the protection afforded by aspartate when both were present (Fig. 8B).

**DISCUSSION**

The great majority of the cysteines engineered in reentrant loop II are accessible to the aqueous phase as evidenced by the inhibitory effects of small hydrophilic sulfhydryl reagents on the transport of these mutants (summarized in Fig. 9). 15 of 22 mutants retained greater than 30% of the activity of the parent construct (Fig. 2), and 11 of these 15 were inhibited by MTSEA (Fig. 3). It is possible that in the four mutants where no sig-
significant effect of MTSEA was observed, the introduced cysteines are still accessible to the hydrophilic reagents, but their modification has no functional impact. This is the case for at least one of these four, namely G422C. The activity of this single cysteine mutant was neither affected by MTSEA nor by MTSET (Fig. 3). However, this cysteine is labeled by the large BM, and this labeling is prevented by preincubation with MTSET (23). Although BM has a definite membrane permeability, it reacts only with cysteines exposed to the aqueous phase. Our results showed that the reentrant loop is accessible to the aqueous phase at many positions and therefore behaves like a pore-loop rather than two membrane-embedded segments. Our accessibility data are in good agreement with those reported in the study by Seal et al. (26) do not draw this conclusion, the latter observation is consistent with the assignment of position 430 together with 431 at the apex of the reentrant loop, facing the intracellular side (Ref. 23, and Figs. 3 and 5).

In the presence of sodium, five of the 11 single cysteine mutants were sensitive to MTSEA but relatively resistant to MTSET: V427C, T430C, A431C, I436C, and G437C (Fig. 3). Since MTSET is membrane-impermeant (34, 35) and also larger than MTSEA, this difference in sensitivity could be due to internal versus external exposure of the engineered cysteine. Alternatively the smaller MTSEA may reach positions with a restricted access, and the larger MTSET cannot access those.

The results of our accessibility studies are in good agreement with the membrane topology we determined for GLT-1 (Refs. 23 and 25, and see Fig. 1). Thus, going down the ascending limb, which connects the external S443C with the internal A431C (Fig. 1), we found that cysteines introduced at positions 440, 439, and 438 are accessible to the small reagents MTSEA and MTSET (Fig. 3) and also the large BM (Fig. 4). The cysteines introduced at positions 437 and 436 of the pore-loop did not react with BM (Fig. 4). They were not very accessible to MTSET in the presence of sodium but became markedly more accessible to MTSET in its absence (Figs. 3 and 5). Both were accessible to the smaller MTSEA even in the presence of sodium (Fig. 3). Accessibility of five consecutive positions rules out a membrane-embedded α-helix facing an aqueous pore but is compatible with pore helices such as those observed in the potassium channel KcsA (36) and the water and glycerol channels (37, 38). In the descending limb, which connects the external G422 with the internal A431, the cysteines introduced at positions 425, 426, and 427 were accessible to MTSEA, and the two at 425 and 426 were also somewhat accessible to MTSET (Fig. 3). The residues at the apex of the loop, which face the inside of the cell (Thr-430 and Ala-431), were only accessible to the permeant MTSEA but not to MTSET (Fig. 3).

Reentrant loop I in the related bacterial glutamate trans-
the access of the MTS reagents to all these residues. Moreover, in the case of positions 439 and 443, sodium potentiates the accessibility, and this must be due to a conformational change induced by sodium binding. It is likely that this difference between the pore-loop of the glutamate transporter and those of the channels is related to differences in the permeation through channels and transporters.

The observations on the effects of aspartate and DHK on accessibility of residues of the pore-loop (Figs. 6–8) are consistent with the proximity of serine 440 to the substrate binding site (29). The accessibility of many of the cysteines introduced in the loop was limited by DHK (Fig. 6). Although in most cases the protection by DHK required the presence of sodium, in the two positions 427 and 437 this was not the case (Figs. 7 and 9). Therefore, binding of DHK in such a manner to protect at these positions does not require any sodium-induced conformational changes. The sodium dependence of protection by DHK of the other residues is probably due to the sodium-dependent conformational changes of the loop described above. Following these changes, DHK may sterically protect a larger part of the loop. D-Aspartate protected only at the two positions 438 and 440 but interestingly not at position 439, although DHK did protect at the latter position (Fig. 6). DHK is a bulkier molecule than D-aspartate, and it appears that this extra mass physically restricts access to the residues of the pore-loop from the outside. This is perhaps also the reason why DHK could protect T426C against MTSET but not against MTSEA (Fig. 5). An easy explanation is that permeant MTSEA can approach the cysteine introduced at position 426 from the inside, whereas in the case of the impermeant MTSET the approach has to be from the outside. The observation that D- and L-aspartate protect much better than L-glutamate at position 440 and even more dramatically at position 438 (Fig. 8) seems counterintuitive because of the larger mass of L-glutamate. However, it has been demonstrated that the distance between the oxygens of the two carboxyl groups of aspartate is in fact larger than that of glutamate (41). Although it appears that DHK can protect at some positions without the involvement of conformational changes, its binding is likely to induce the movement of other residues. This is exemplified by the potentiation by DHK of the inhibition of S443C by MTSET (Fig. 6B).

A conserved arginine located in the middle of TM8 appears to be an important binding determinant for one of the carboxyl groups of the substrate (28). Moreover, the access to TM8 appears to be much more restricted than that of the pore-loop (42). A particularly intriguing question is how, after binding in the vicinity of the pore-loop, the accessibility of glutamate to TM8 is achieved. One way to shed light on this question may be to examine proximity relationships (39) of pairs of cysteines introduced in the pore-loop and TM8 in the presence and absence of glutamate.

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