Cysteine Scanning of the Surroundings of an Alkali-Ion Binding Site of the Glutamate Transporter GLT-1 Reveals a Conformationally Sensitive Residue*

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Glutamate transporters remove this transmitter from the extracellular space by cotransport with three sodium ions and a proton. The cycle is completed by translocation of a potassium ion in the opposite direction. Recently we have identified two adjacent amino acid residues of the glutamate transporter GLT-1 that influence potassium coupling. Using the scanning cysteine accessibility method we have now explored the highly conserved region surrounding them. Replacement of each of the five consecutive residues 396–400 by cysteine abolished transport activity but at several other positions the substitution is tolerated. One residue, tyrosine 403, was identified where cysteine substitution renders the transporter sensitive to modification by positively charged methanethiosulfonate derivates in a sodium-protectable fashion. In the presence of sodium, the nontransported glutamate analogue dihydrokainate potentiated the covalent modification, presumably by binding to the glutamate site and locking the protein in a conformation in which tyrosine 403 is accessible from the external bulk medium. In contrast, transported substrates significantly slowed the reaction, suggesting that during the transport cycle residue 403 becomes occluded. On the other hand, transportable substrates are not able to protect Y403C transporters against N-ethylmaleimide, which is highly permeant but unable to modify cysteine residues buried within membrane proteins. These results indicate that tyrosine 403 is alternately accessible from either side of the membrane, consistent with its role as structural determinant of the potassium binding site.

Glutamate transporters, located in plasma membranes of nerve and glial cells, are instrumental in keeping the synaptic concentration of the transmitter below neurotoxic levels (1–4). Moreover, together with diffusion, they may help to terminate its action in synaptic transmission (5, 6). They achieve this by an electrogenic process (7–9) where the transporter is cotransported with three sodium ions and a proton (10), followed by countertransport of a potassium ion (10–13).

A glutamate transporter has been purified to near homogeneity and reconstituted (14, 15). This transporter, termed GLT-1, has been cloned (16), and its physiological importance is illustrated in knock-out experiments (3, 4). Four other glutamate iso transporters have been cloned (17–20) and the homology between these family members is around 50%.

Recently we identified two residues of GLT-1 important for potassium coupling (13, 21). These residues, tyrosine 403 and glutamate 404, are located in the most conserved region of the transporter. Nearby in the primary sequence is aspartate 398, which is also critical for transporter function (22). These and other observations, suggest that this region may form part of the translocation pathway of the transporter. The scanning cysteine accessibility method (SCAM)† has permitted the systematic identification of residues lining ion channels (for instance, 23–25). We have therefore used this method to probe the accessibility of residues 395–407. Strikingly, this study has led to the identification of a conformationally sensitive residue. We suggest that it is alternatingly accessible from either side of the membrane in a substrate-dependent manner.

EXPERIMENTAL PROCEDURES

Cell Growth and Expression—HeLa cells were cultured (26), infected with recombinant vaccinia/T7 virus vTF7–3 (27), and transfected with plasmid DNA encoding wild type or mutant GLT-1 (26). Transport experiments with the GLT-1 substrate D-[3H]aspartate were done as described (22). Data presented are after subtracting the values obtained from cells transfected with the vector, pBluescript SK+ alone. In the case of some of the mutants and in particular if they were subcloned into the cDNA encoding the cysteine-less GLT-1 (see below), the sodium concentration of the transport medium was lowered to 15 mM (supplemented with 135 mM choline chloride) as the sodium affinity of aspartate transport endogenous to the HeLa cells is much lower than that of GLT-1 and its derivatives (data not shown). This enables us to obtain acceptable signal/background ratios, at least 4-fold, even in the case of relatively low activities exhibited by the cysteine-less transporter and its derivatives.

Inhibition Studies with Sulphhydryl Reagents—Prior to the transport measurements, the cells, adhered to 24-well plates, were washed with the 150 mM NaCl containing transport medium. Each well was then incubated at room temperature with 200 μM of this solution (in case of different composition this is indicated in the figure legends) and the indicated concentration of reagent under study. After 5 min the medium was aspirated, and the cells were washed twice with 1 ml of the transport solution. Subsequently they were assayed for D-[3H]aspartate transport using solution to which the labeled amino acid was added (22). The hydrophilic methanethiosulfonate (MTS) reagents used were purchased from Toronto Research Chemicals, Inc. Positively charged MTSEA and MTSET are approximately 1 nm in length, differing only at the charged head group, which measures 0.28 nm in MTSET and 0.36 nm in MTSEA (23). MTSES is negatively charged and has an intermediate size (23). Whereas MTSEA is membrane permeant to some extent, MTSET and MTSES are membrane impermeant.

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‡‡ The abbreviations used are: SCAM, scanning cysteine accessibility method; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate; MTSET, (2-trimethylammonium)ethylmethanethiosulfonate; MTSES, (2-sulfonatoethyl)methanethiosulfonate; NEM, N-ethylmaleimide.

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Site-directed Mutagenesis—Mutagenesis (22, 29) was done using uracil-containing single strand DNA derived from the shortened GLT-1 clone (30) or its cysteine-less homologue. Most results reported in this study concern individual replacements of selected residues of the wild type GLT-1 by cysteine, but cysteine mutants in a cysteine-less background were also tested to confirm the specificity of the effects observed in the wild type background. The cysteine-less GLT-1 was prepared and characterized as follows: Cysteines 536, 549 and 562 are clustered together at the carboxyl-terminal tail. Because we have evidence that the major part of this tail is not required for transport,2 the stretch from residue 533 to residue 563 was deleted by site-directed mutagenesis. At the same cycle of the mutagenesis another primer was annealed simultaneously to the uracil-containing single-stranded DNA. It was designed to convert cysteine 38 to serine. After verifying by DNA sequencing that these changes were indeed incorporated, the construct was expressed and shown to be unimpaired for D-[3H]aspartate transport. Subsequently uracil-containing single-stranded DNA was prepared from this construct, and new cycles of mutagenesis, sequencing, and expression were performed to mutate the remaining cysteines to serine. In the second cycle, cysteines 60, 293, and 296 were converted, in the third 184, and in the fourth 373. In the subsequent cycle a deca-histidine tail was introduced between the last residue of GLT-1, lysine 573, and the stop codon. A major reason to prepare the above construct was for the determination of the membrane topology of GLT-1 by one of several approaches,3 namely, biotinylation of single cysteine-containing transporters. In this approach artifactual biotinylated bands are observed running with a similar mobility as the above described construct. To detect transporter-related signals, it was necessary to “mass-tag” the construct so that the transporter band would be resolved from the artifactual ones. We have shown previously that most of the aminoterminal tail of the GABA transporter GAT-1 is not required for its functional expression (31). Therefore, we have inserted upstream of the original first methionine the coding sequence for the first 41 amino acids of GAT-1 preceded by the DNA sequence 5'-ATGCATTTCGTTGCTCCGAGAC-3', encoding for the amino acids MHFVLRD; the latter was added to facilitate its insertion using standard molecular biology approaches. Using GAT-1 as a template, the primers used for amplification were designed such that on both ends of the amplified fragment a StuI site was created. After digestion with StuI, the fragment was ligated into blunt ends created in the cysteine-less histidine-tagged GLT-1 described above, by cutting it with PflMI and removal of the 3'-overhangs with bacteriophage T4 DNA polymerase. Plasmid DNA of several of the transformants was isolated and one of those, which gave rise to aspartate transport after expression, was retained. The result is that the 48 amino acids are inserted immediately amino-terminal to the original start codon of GLT-1. All the expected features of the modified cysteine-less GLT-1 were verified by sequencing in both directions and upon expression its D-[3H]aspartate transport activity was found to be

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2 G. Pines and B. I. Kanner, unpublished observations.

3 M. Grunewald, A. Bendahan, and B. I. Kanner, submitted for publication.
44.5 ± 1.5% of that of the wild type GLT-1 (n = 7), and the level of expression as estimated by immunoprecipitation was comparable to that of the wild type (data not shown). The characteristics of transport of this cysteine-less histidine-tagged GLT-1 transporter were very similar to that of the wild type GLT-1, as judged by affinity to D-aspartate (Fig. 1A) and sodium (Fig. 1B). Single cysteine-containing transporters were made using uracil-containing single strand DNA derived from this construct. Mutants were verified by DNA sequencing. Mutations Y403C and E404C were subcloned into wild type or cysteine-less GLT-1 using the enzymes \textsc{Bsr}GI and \textsc{Bst}EII. Subcloned DNAs were sequenced in both directions between these restriction sites.

**RESULTS**

Cysteine-scanning Accessibility of Residues 395–407—Residues 395–407 of the glutamate transporter GLT-1 have been changed one at a time to cysteine. After transient expression of the wild type and the cysteine replacement mutants in HeLa cells, transport of D-[3H]aspartate was monitored. No transport can be detected when cysteine is introduced in each of the five consecutive residues from 396 to 400 (Fig. 2). The possible role of these residues in the transport process will be addressed under “Discussion.” Right now we focus on the remaining residues, where cysteine replacement leaves significant biological activity (Fig. 2).

Transport activity of wild type GLT-1 is not inhibited by 2.5 mM of the MTS derivative (MTSEA), and the same is true for all the active cysteine replacement mutants with the exception of Y403C (Fig. 3). When the preincubation with the sulfhydryl reagent is carried out in a sodium-free medium (choline substitution) E404C transporters become sensitive to the reagent as well (Fig. 4). It appears that sodium protects against covalent modification at this position, since similar data are obtained when the E404C mutation is introduced in a cysteine-less GLT-1 background (data not shown). Although not visible in the experiments depicted in Figs. 3 and 4, there is also a partial protection of Y403C transporters by sodium against inhibition by MTSEA. This can be readily observed at lower concentrations of this sulfhydryl reagent (Fig. 5). Similar results have been observed with the Y403C mutation introduced in a cysteine-less background (data not shown, but see Fig. 9). Other cations are not effective except for lithium, which affords a small but significant protection (data not shown). It is of interest to note that also in the case of E404C, lithium is the only cation besides sodium that can give a significant protection against inhibition by MTSEA (data not shown).

Characterization of the Inhibition of Y403C by MTS Reagents—As will be shown below, the most striking observation described in this paper is the opposite effect of transportable
and nontransportable substrates on the inhibition of Y403C by MTS reagents in a sodium-containing medium. In contrast, E404C transporters are not inhibited very much by MTSEA in the presence of sodium (Fig. 3), and the same is true when transporter substrates are added (data not shown). Therefore, we have focused on the characterization of functional effects of covalent modification of Y403C transporters. Wild type GLT-1 is not inhibited by the positively charged MTSEA and MTSET (Figs. 3, 4, and 6A) and also not by MTSES (data not shown). In contrast with wild type, not only MTSEA (Fig. 3) but also MTSET potently inhibit Y403C transporters (Fig. 6A). On the other hand, negatively charged MTSES does not (Fig. 6B). Because MTSES is intermediate in size between MTSEA and MTSET (23), it appears that the positive charge in the reagent determines its ability to inhibit. After preincubation of Y403C transporters with MTSES, they still can be inhibited by a subsequent exposure to MTSEA (Fig. 6B). This speaks against the possibility that the residue can be covalently modified MTSES but that this does not lead to inhibition of transport.

In the experiment depicted in Fig. 7, Y403C transporters are preincubated with 0.25 mM MTSEA in a sodium-containing medium. When transport of D-[3H]aspartate is subsequently measured, an inhibition of almost 80% is observed. When a saturating concentration (1 mM) of transportable acidic amino acids such as D- and L-aspartate or L-glutamate is added during the preincubation, this causes a marked protection of the transporters against the inhibition by MTSEA (Fig. 7). This protection is only observed in the presence of sodium ions (data not shown). Amino acids that are not substrates of GLT-1, such as GABA or glycine, do not protect (Fig. 7). The protective effect of D-aspartate is dose dependent; half-maximal effects are observed with 30–50 μM (data not shown), which is in good agreement with the apparent $K_m$ of D-aspartate transport in HeLa cells expressing GLT-1 (22). The protection by D-aspartate is observed at all MTSEA concentrations examined (Fig. 8A). The substrate similarly affords protection against the impermeant MTSET (Fig. 8B). It is also apparent that the larger MTSET inhibits less potently than MTSEA, presumably because of steric constraints. Again, inhibition by the MTS reagents and the protection by transportable substrates are the consequence of a direct modification of the cysteine at the 403 position, because similar results are obtained with transporters in which this is the only cysteine (Fig. 9). Strikingly, the competitive inhibitor dihydrokainate (12, 32), a nontransportable glutamate analogue (32–34), does not protect, and in fact even potentiatates the inhibition by the MTS reagents. Thus transportable substrates induce a conformational change making the residue less accessible to the impermeant reagent, whereas the blocker seems to lock the transporter in a conformation where it is more accessible to the extracellular bulk medium. Importantly, the wild type as well as the cysteine-less GLT-1 are not inhibited by MTSEA and MTSET even in the simultaneous presence of dihydrokainate (data not shown).

Effects of NEM—The conformational change induced by aspartate may cause burial of the 403 residue within the core of the transporter. Alternatively, it may reflect the transport step itself, and the residue may become accessible to the inside bulk medium. In the former case the residue is not expected to be reachable by NEM, which permeates lipid bilayers readily but is unable to modify cysteine residues within transmembrane-spanning α helices (35, 36). The maleimide does not inhibit wild type GLT-1 (data not shown) but inhibits the activity of the Y403C transporter (Fig. 10). In contrast with its protection against MTS reagents, D-aspartate does not protect the Y403C transporters against NEM (Fig. 10). This is consistent with the idea that its protection against relatively impermeant MTS reagents is because of the movement of the 403 residue from...
the outside of the cell to the inside. Similar results are obtained with the mutation introduced in a cysteine-less background (data not shown). The partial protection observed with dihydriokainate will be discussed below.

**DISCUSSION**

Analysis of amino acid residues 395–407 of GLT-1 by SCAM leads to two major conclusions: 1) Within the putative pore-forming domain of GLT-1 are five consecutive residues (396–400) where mutation to cysteine leads to complete loss of function, a result consistent with the structural conservation and functional importance of this region. 2) Residue 403, which has been previously shown to interact with cotransported cations (21), behaves as if it is alternately accessible from either side of the membrane in such a way that glutamate transport promotes its exposure to the inside, whereas binding of a nontransportable analog promotes its exposure to the outside. This residue is located in the middle of transmembrane α-helix seven (37).

One of the five residues that do not tolerate replacement by cysteine (Fig. 2) is aspartate 398. Its replacement by either asparagine, glycine, or glutamate also leads to loss of function, and this is not because of a defective biosynthesis or targeting (22). Injection of D398E cRNA into *Xenopus* oocytes results in neither glutamate transport currents nor transient currents reflecting sodium binding. It is possible therefore that this residue, together with asparagine 396, methionine 397, glycine

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Fig. 6. Effect of other MTS reagents on Y403C transporters. HeLa cells expressing wild type or Y403C transporters were preincubated in the standard NaCl medium containing 2.5 mM MTSEA (A) or 1 mM MTSET (A) or 10 mM MTSES (B) for 5 min. In B an additional 5 min of incubation with 2.5 mM MTSEA was carried out. Subsequently transport was measured using the 15 mM NaCl + 135 mM choline chloride-containing medium.

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Fig. 7. Effect of amino acids on inhibition of Y403C transporters by MTSEA. HeLa cells expressing Y403C were incubated in the standard medium in the presence and absence of 0.25 mM MTSEA and the indicated amino acids (at 1 mM final concentrations). After washing, transport in the low sodium medium was measured, and the data are expressed as percent activity remaining relative to the respective control without MTSEA.

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4 J. I. Wadiche, B. I. Kanner, and M. P. Kavanaugh, unpublished experiments.
399, and threonine 400, plays a crucial role in sodium binding. Recent experiments in our laboratory indicate that sodium and potassium binding sites are close to each other (21). Glutamate 404 and tyrosine 403 are involved in potassium binding (13, 21), and residues 396–400 are in fact nearby. Consistent with this is the protection of E404C and Y403C transporters by sodium against thiol modification (Figs. 3–5). This modification occurs at these very positions, as evidenced by the fact that the same results are also obtained in a cysteine-less background. This rules out a scenario in which the introduction of a new cysteine residue induces a change in the transporter’s structure such that modification now may occur at one of the nine endogenous cysteines of GLT-1. The cysteine-less GLT-1 transporter has around 40% of the wild type activity, and therefore most of the experiments have been done in the wild type background. However, they have been validated in the cysteine-less background as shown, for instance, in Fig. 9. It is of interest to note that the only ion that can partially substitute for sodium in the protection experiments is lithium. This is not unexpected as only this cation can replace some of the three sodium ions in the transport process (38). The fact that only positively charged MTS reagents inhibit at the 403 position (Fig. 6), also supports the idea that it is part of a negatively charged binding pocket for cations, which repels the negatively charged MTSES.

On the other hand, the protection by transportable acidic amino acids against inhibition by positively charged MTS reagents (Figs. 7–9) is because of a conformational change induced by their binding at a distinct site. This is inferred because the opposite effect is induced by binding to this site of the nontransportable dihydrokainate, which locks the transporter in a conformation where tyrosine 403 is accessible from the external bulk medium. Transportable substrates cause a conformational change where this residue becomes occluded. Further evidence for a close association of this phenomenon with the transport step itself is that it was only observed in the presence of the cosubstrate sodium. The sensitivity to NEM during transport conditions (Fig. 10), in contrast to the protection against MTS reagents (Figs. 8 and 9), suggests that now tyrosine 403 becomes accessible to the aqueous intracellular compartment.

Determinants of the binding site for the acidic amino acids are located on a stretch of 76 amino acid residues (39), which also contains the cation binding site (13, 21). One explanation for the partial protection by dihydrokainate against NEM may
be that the former is bound close enough to hamper the access of NEM, which is bulkier than the MTS reagents.

Tyrosine 403 is involved in the interaction of the transporter with internal as well as external potassium (21) and it appears, together with glutamate 404 (13), to be a structural determinant of the potassium site. Because of the role of potassium, which is to promote the return of the unloaded transporter to the side of the membrane from where substrate can be moved (11–13), it is to be anticipated that during the transport cycle the potassium binding site should be alternately accessible to either side of the membrane. The findings documented here are entirely consistent with this. We anticipate that application of SCAM to other interesting regions of GLT-1 and to other transporters, may lead to important structural and functional insights as well.

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