A conserved serine-rich stretch in the glutamate transporter family forms a substrate-sensitive reentrant loop

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Neuronal and glial glutamate transporters remove the excitatory neurotransmitter glutamate from the synaptic cleft. The proteins belong to a large family of secondary transporters, which includes bacterial glutamate transporters. The C-terminal half of the glutamate transporters is well conserved and thought to contain the translocation path and the binding sites for substrate and coupling ions. A serine-rich sequence motif in this part of the proteins is located in a putative intracellular loop. Cysteine-scanning mutagenesis was applied to this loop in the glutamate transporter GltT of *Bacillus stearothermophilus*. The loop was found to be largely intracellular, but three consecutive positions in the conserved serine-rich motif (S269, S270, and E271) are accessible from both sides of the membrane. Single-cysteine mutants in the serine-rich motif were still capable of glutamate transport, but modification with *N*-ethylmaleimide blocked the transport activity in six mutants (T267C, A268C, S269C, S270C, E271C, and T272C). Two milimolars L-glutamate effectively protected against the modification of the cysteines at position 269–271 from the periplasmic side of the membrane but was unable to protect cysteine modification from the cytoplasmic side of the membrane. The results indicate that the conserved serine-rich motif in the glutamate transporter forms a reentrant loop, a structure that is found in several ion channels but is unusual for transporter proteins. The reentrant loop is of crucial importance for the function of the glutamate transporter.

Neuronal and glial glutamate transporters in the mammalian central nervous system remove the neurotransmitter glutamate from the synaptic cleft, thereby helping to end the excitatory signal and preventing neurotoxicity of high concentrations of glutamate (1–4). The glutamate transporters belong to a large family of transport proteins in which bacterial glutamate transporters also are found, including GltT of *Bacillus stearothermophilus* (5). Evidence is accumulating that the C-terminal half of the transporters, which is particularly well conserved, constitutes a major part of the translocation pathway and contains the binding sites for the substrate and cotransported ions (6–13). This part of the transporters contains a serine-rich sequence motif, which is conserved in all members of the enormously divergent family. The motif has been implied in substrate binding, but there is no experimental evidence for such a function (5, 14).

The membrane topology of the glutamate transporters has been studied with a number of experimental techniques, leading to a variety of models for part of the proteins (reviewed in ref. 5). The models agree on the presence of six membrane-spanning helices in the N-terminal part of the proteins, but the membrane topology of the C-terminal half remains somewhat controversial. A number of independent experiments, including cysteine-scanning mutagenesis, proteolytic digestion, and *phoA* gene fusion studies (13, 15), indicate that membrane-spanning segment 6 is followed by a large intracellular loop, in the middle of which the conserved serine-rich motif is located (Fig. 1). The subsequent outgoing membrane-spanning helix 7 contains two additional conserved stretches, one of which is involved in cation binding (6, 7) and the other possibly in substrate binding (5). Helix 7 is followed by an extracellular loop that reenters the membrane (15) and, finally, by an inward-going amphipathic membrane-spanning segment (helix 8) (13).

Structural and functional information about membrane proteins can be effectively obtained by cysteine-scanning mutagenesis (16, 17). In this report, the region containing the conserved serine-rich motif in the glutamate transporter GltT of *B. stearothermophilus* was subjected to cysteine-scanning mutagenesis. The results indicate that the loop between membrane-spanning helices 6 and 7 is largely intracellular, but that it reenters the membrane so that part of the conserved serine-rich motif is accessible from both sides of the membrane. The activity of mutants with single cysteines in the conserved motif is extremely sensitive to thiol modification with maleimides. The substrate L-glutamate protects against maleimide modification of the single cysteines in the conserved motif that are accessible from the outside. The data provide evidence for the functional importance of the serine-rich motif and suggest the presence of a water-filled pore in the membrane-embedded part of the glutamate transporters.

Materials and Methods

Cell Growth and Expression. *Escherichia coli* strains ECOMUT2, which lack the glutamate transporter GltP (18), and Top10 (Invitrogen) were grown in Luria broth medium at 37°C. Ampicilline was added to a final concentration of 100 μg/ml when required. For protein expression from pBAD24-derived plasmids (19), 0.1% L-arabinose was added at an optical density of 0.7 at 660 nm, and cells were harvested 1.5 hr after induction.

Recombinant DNA Techniques. General molecular biological techniques are described by Sambrook *et al.* (20). Enzymes for recombinant DNA work were obtained from Boehringer Mannheim. An L-arabinose inducible expression vector for GltT was made by cloning the *gltT* gene encoding the glutamate transporter GltT of *B. stearothermophilus* with an N-terminal His-tag from plasmid pGltThis (21) in a *Nco*I/*Xba*I opened pBAD24 vector (19). Mutations were introduced in the *gltT* gene with PCR by using the overlap extension method (22). Oligonucleotides were obtained from GIBCO/BRL. All PCR-amplified DNA fragments were sequenced by BMTC (Groningen, The Netherlands).

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Abbreviations: AMdiS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; NEM, *N*-ethylmaleimide.

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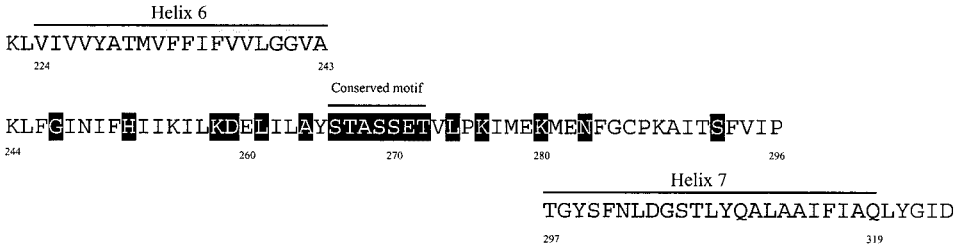

Fig. 1. Amino acid sequence of the region between membrane-spanning helices 6 and 7 in the glutamate transporter GltT of *B. stearothermophilus*. The positions of helices 6 and 7 and the conserved serine-rich motif are indicated. Residues that were changed to cysteine in single-cysteine mutants are shown in black boxes. Numbers refer to the GltT protein sequence.

Labeling of Whole Cells with Maleimides. *E. coli* Top10 (30-ml cultures) expressing GltT variants were washed with 50 mM potassium phosphate, pH 7, and resuspended in 300 μl of the same buffer. 3-(*N*-maleimidopropinyl)biocytin (biotin maleimide, Molecular Probes) was added to a final concentration of 250 μM. The cell suspension was incubated for 10 min at 30°C, and the reaction was stopped with DTT (final concentration 5 mM). When indicated, the reaction with biotin maleimide was preceded by incubation for 5 min at 30°C with 100 μM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMdiS, Molecular Probes). Subsequently, cells were broken with a Soniprep 150 sonicator (MSE Scientific Instruments, Crawley, UK) operated at an amplitude of 8 μm. Debris was removed by centrifugation at 9,000 $\times$ *g* for 10 min and membranes were collected from the supernatant by centrifugation at 250,000 $\times$ *g* and 4°C for 20 min and resuspended in 50 mM potassium phosphate, pH 7.

Preparation and Labeling of Membrane Vesicles with an Inside-Out Orientation. *E. coli* Top10 cells expressing GltT variants were washed with 50 mM potassium phosphate, pH 7, and resuspended in the same buffer containing 2 mM $MgSO_4$ and 10 μg/ml DNase. The suspension was passed once through a French Press cell operated at 10,000 psi. Removal of debris and collection of the membranes was done as described above. Membrane vesicles were resuspended in 50 mM potassium phosphate, pH 7, at a concentration of 5 mg/ml of protein. Membrane vesicles (2 mg protein) were labeled with the maleimides, as described for whole cells. For testing the effect of substrate on the labeling, membrane vesicles were prepared in the presence of 2 mM L-glutamate.

Preparation and Labeling of Membrane Vesicles with a Right-Side-Out Orientation. Membrane vesicles with a right-side-out orientation were prepared from *E. coli* ECOMUT2 expressing GltT variants by the osmotic lysis procedure, as described by Kaback (23). Membrane vesicles with a right-side-out orientation (5 mg/ml of protein in 50 mM potassium phosphate, pH 7) containing mutant S269C were incubated with 10 μM of *N*-ethylmaleimide (NEM) or AMdiS for 0, 10, 30, 60, and 120 s at room temperature. The reactions were stopped with DTT (final concentration 2 mM), and the vesicles were washed twice with 50 mM potassium phosphate, pH 7. Unreacted cysteines were subsequently labeled with 0.5 mM biotin maleimide for 30 min at room temperature.

Purification and Visualization of GltT Mutants. Purification of His-tagged GltT from membrane vesicles was done essentially as described (21) by using Ni^{2+}-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany). Purified proteins were run on SDS/polyacrylamide gels followed by transfer to poly(vinylidene difluoride) membranes (Boehringer Mannheim) and detected with monoclonal antibodies directed against a 6 His-tag (Dianova, Hamburg, Germany). Biotinylated proteins were detected with alkaline-phosphatase-labeled streptavidin. Antibodies and streptavidin were visualized by using the Western-light chemiluminescence detection kit (Tropix, Bedford, MA).

Glutamate Transport Assays. Glutamate uptake in right-side-out membrane vesicles was measured as described by rapid filtration (21). To test the effect of NEM and AMdiS on transport, membrane vesicles (5 mg/ml of protein in 50 mM potassium phosphate, pH 7) were incubated with 0.25 mM of the maleimides for 10 min at room temperature, immediately followed by the transport assay. When the incubations with the maleimides were done in the presence of L-glutamate, 5 mM DTT was used to stop the reactions. Subsequently, the vesicles were spun down at 25,000 $\times$ *g* for 15 min at 4°C and washed twice with excess buffer (50 mM potassium phosphate, pH 7), followed by the transport assay.

Results

Construction and Activity of Single-Cysteine Mutant Transporters. A cysteine-less version of His-tagged GltT of *B. stearothermophilus* was constructed to provide a background for cysteine-scanning mutagenesis. Wild-type GltT contains one cysteine at position 286 (Fig. 1), which was replaced with a serine residue. The mutant protein was expressed in *E. coli* and was found in the membrane at similar levels as the wild-type protein (Fig. 2 *Inset*). Glutamate transport activity of the cysteine-less mutant was measured in right-side-out membrane vesicles prepared from *E. coli* cells expressing the protein. The activity of the cysteine-less

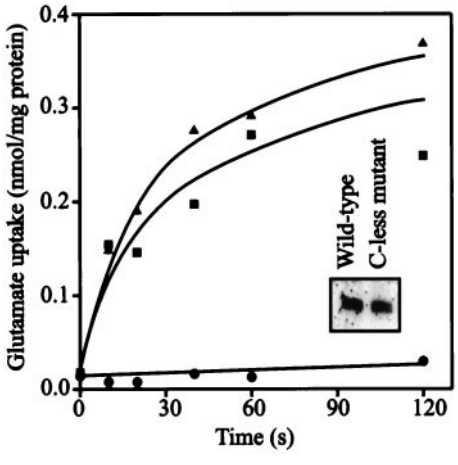

Fig. 2. Glutamate uptake activity of wild-type and cysteine-less GltT. Glutamate uptake in right-side-out membrane vesicles prepared from *E. coli* cells expressing His-tagged wild-type GltT (■), cysteine-less GltT (▲), or no GltT (●). (*Inset*) Expression levels of the wild-type and cysteine-less mutant on a Western blot stained with antibodies against the His-tag.

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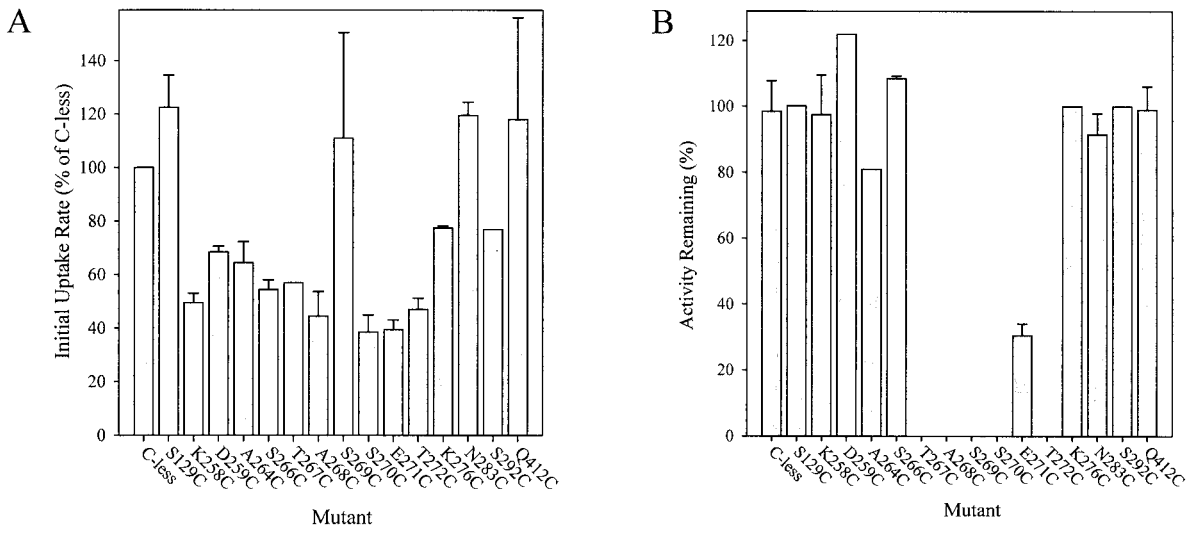

Fig. 3. Glutamate uptake activity of single-cysteine GltT mutants. (*A*) Initial rates of glutamate uptake in right-side-out membrane vesicles containing the single-cysteine mutants were expressed as percentage of the rate in membrane vesicles containing the cysteine-less mutant. (*B*) Initial rates of glutamate uptake in right-side-out membrane vesicles after treatment with NEM. Rates were expressed as percentage of the initial rates measured in untreated membrane vesicles.

mutant was similar to the wild-type glutamate transport activity (Fig. 2).

A set of 18 single-cysteine mutants of GltT was constructed in the region containing the conserved serine-rich stretch in the putative intracellular loop between membrane-spanning segments 6 and 7 (Fig. 1). As controls, mutants were created with a single cysteine in the loop between the third and fourth membrane-spanning segment (S129C) and one with a single cysteine at the hydrophilic C terminus (Q412C). The two positions have well-established extra- and intracellular locations, respectively (13, 15, 24). On expression in *E. coli,* 15 of the 20 single-cysteine mutants were found in the membrane at similar levels as the wild-type protein, whereas the remaining five mutants (G247C, H252C, L261C, L274C, and K280C) were not found in the membrane (not shown). The glutamate transport activity of the mutants that were expressed was measured in right-side-out membrane vesicles and varied from 40% to 130% of wild-type activity (Fig. 3*A*).

Effect of NEM on Transport Activity. The effect of cysteine modification on glutamate transport activity was assessed in right-side-out membrane vesicles prepared from *E. coli* cells expressing the single-cysteine mutants. NEM was chosen as cysteine-modifying maleimide, because it readily permeates the membrane and consequently will label both internal and external cysteines. Treatment with NEM did not affect the glutamate uptake activity of the cysteine-less mutant in membrane vesicles with a right-side-out orientation (Fig. 3*B*). In contrast, the glutamate transport activity of five single-cysteine mutants in the conserved serine-rich stretch, T267C, A268C, S269C, S270C, and T272C, was completely blocked by NEM modification, whereas the uptake activity of mutant E271C was partially inhibited by NEM. The activity of the remaining single-cysteine mutants was not affected by NEM (Fig. 3*B*), even though the cysteines in four of these mutants (S129C, N283C, S292C, and Q412C) were accessible for maleimide modification (see below).

Labeling Characteristics of Intra- and Extracellular Cysteines. The accessibility of the single-cysteine residues in the mutants to thiol-modifying reagents was tested in whole cells and in membrane vesicles with an inside-out orientation. Two different maleimide derivatives were used: biotin maleimide, which is uncharged, and AMdiS, which is charged and membrane impermeable (25). His-tagged GltT variants were solubilized from membrane vesicles and purified, followed by SDS/PAGE and Western blotting. GltT mutants labeled with biotin maleimide were visualized by using streptavidin coupled to alkaline phosphatase.

The cysteine-less transporter was used as a negative control and was not modified by biotin maleimide in whole cells or inside-out membrane vesicles (not shown). The single cysteine in mutant S129C is localized in the external loop between transmembrane helices 3 and 4. In whole cells, the cysteine at position 129 could be labeled by biotin maleimide, and this labeling was prevented by prior incubation with the charged AMdiS (Fig. 4, lanes 1 and 2). This behavior is consistent with the extracellular location of the cysteine and indicates that position 129 is accessible from an aqueous environment. The single cysteine in mutant Q412C is localized at the internal C terminus of the protein. In whole cells expressing mutant Q412C, no modification by biotin maleimide was observed (Fig. 4, lanes 3 and 4).

The labeling experiments were repeated in membrane vesicles

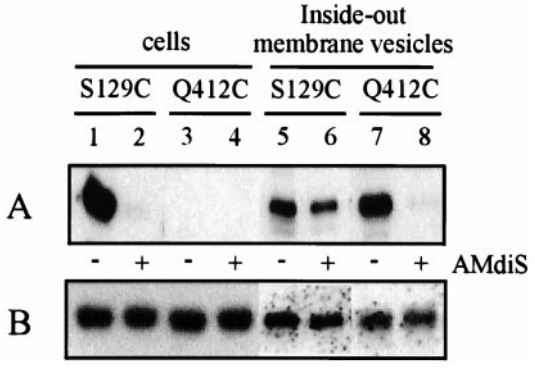

Fig. 4. Western blot analysis of maleimide-labeled and purified GltT mutants S129C and Q412C. Western blots were decorated with streptavidin coupled to alkaline phosphatase to detect biotinylated proteins (*A*) and antibodies against the His-tag to compare the amounts of protein (*B*). Single-cysteine mutants S129C and Q412C were labeled with biotin maleimide with (+) or without (−) prior incubation with AMdiS, as indicated, in whole cells (lanes 1–4) and inside-out membrane vesicles (lanes 5–8).

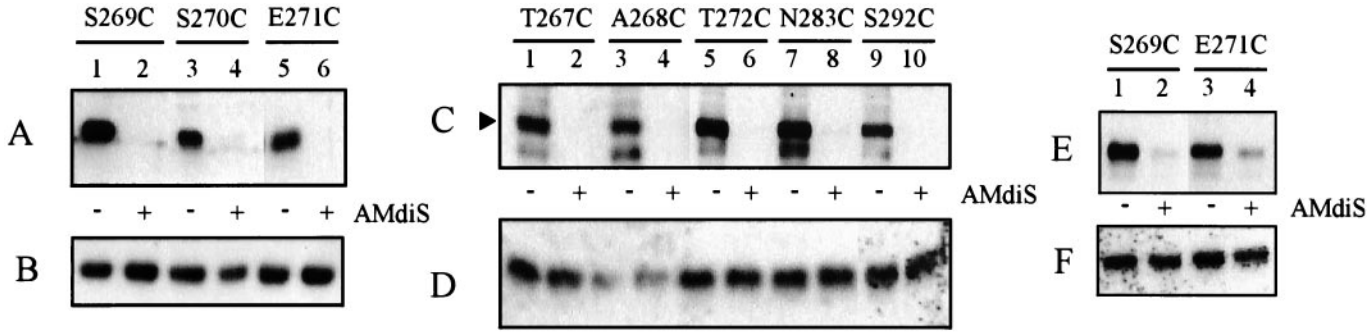

Fig. 5. Western blot analysis of maleimide-labeled and purified single-cysteine mutants. Western blots were decorated with streptavidin coupled to alkaline phosphatase to detect biotinylated proteins (*A, C,* and *E*) or antibodies against the His-tag to compare the amounts of protein (*B, D,* and *F*). Single-cysteine mutants were labeled with biotin maleimide with (+) or without (−) prior incubation with AMdiS, as indicated, in whole cells (*A* and *B*) and inside-out membrane vesicles (*C–F*).

with an inside-out orientation prepared from *E. coli* cells expressing the mutants. The cytoplasmic cysteine at position 412 in mutant Q412C is located at the outside of inside-out membrane vesicles. In the membrane vesicles, this cysteine could be labeled by biotin maleimide, and prior incubation with AMdiS effectively prevented labeling (Fig. 4, lanes 7 and 8). The labeling behavior of mutant Q412C in whole cells and membrane vesicles is consistent with the intracellular location of position 412 and indicates that biotin maleimide does not react with intracellular cysteines in whole cells under the conditions of the experiment. The periplasmic cysteine at position 129 in mutant S129C is located in the lumen of inside-out membrane vesicles. In contrast to what was observed with mutant Q412C in whole cells (Fig. 4, lanes 3 and 4), the internal cysteine at position 129 in membrane vesicles was readily labeled by biotin maleimide, indicating that the maleimide crosses the membrane (Fig. 4, lane 5). A possible explanation for the apparent contradiction between whole cells and membrane vesicles is that in whole cells the entering maleimide is efficiently scavenged in the reducing conditions of the cytoplasm. Prior incubation of inside-out membrane vesicles containing mutant S129C with the membrane-impermeable AMdiS did not completely prevent the labeling with biotin maleimide of mutant S129C (Fig. 4, lane 6). The fraction of cysteines that is inaccessible for AMdiS is likely to represent the fraction of closed membrane vesicles with an inside-out orientation in the preparation, whereas the fraction of cysteines that is accessible for AMdiS may represent the fraction of membrane vesicles with a right-side-out orientation or unsealed membranes. The experiments show that the maleimide reagents can be used to discriminate between cytoplasmic and periplasmic cysteines.

Membrane Topology of the Region Encompassing the Conserved Serine-Rich Stretch. In intact cells, 3 of the 13 active single-cysteine mutants in the region containing the conserved serine rich stretch, S269C, S270C, and E271C, reacted with the maleimide compounds in a similar way as mutant S129C (Fig. 5*A*). The cysteine residues at the three consecutive positions 269–271 were labeled with biotin maleimide, and the labeling was prevented by prior incubation with the membrane-impermeable AMdiS, indicating that these residues are accessible from the periplasmic aqueous environment.

The other mutants did not react with biotin maleimide in intact cells (not shown). In membrane vesicles with an inside-out orientation, the cysteine residues in five of these mutants (T267C, A268C, T272C, N283C, and S292C) were labeled with biotin maleimide, and the labeling was blocked by prior incubation with AMdiS (Fig. 5*C*). The labeling characteristics of these five mutants resemble those of mutant Q412C, indicating that the cysteines at positions 267, 268, 272, 283, and 292 are accessible from the cytoplasmic side of the membrane. The remaining active single-cysteine mutants, K258C, D259C, A264C, S266C, and K276C, did not react with the maleimides in the membrane vesicles or in the whole cells (data not shown). Most likely the cysteine residues in these mutants are protected from labeling by the folding of the protein.

Surprisingly, cysteines at positions 267, 268, and 272 that are accessible from the cytoplasm and not from the periplasm are immediately adjacent to the three cysteines at positions 269, 270, and 271, which are accessible from the periplasm (Fig. 1). This raised the question whether, in addition to their periplasmic accessibility, positions 269–271 might be accessible from the cytoplasm as well. Indeed, in inside-out membrane vesicles, mutants S269C, S270C, and E271C were labeled with biotin maleimide, and labeling was effectively blocked by prior incubation with AMdiS (shown for S269C and E271C in Fig. 5*E*), in contrast to what was observed for mutant S129C with a periplasmic cysteine (Fig. 4, lanes 5 and 6). Mutants S269C, S270C, and E271C had labeling characteristics similar to mutant Q412C, with a fixed cytoplasmic cysteine, in inside-out membrane vesicles (Fig. 4, lanes 7 and 8), and, similar to mutant S129C, with a fixed periplasmic cysteine in whole cells. This indicates that the cysteines in the three mutants are accessible from both sides of the membrane.

Protection of Modification of the Single-Cysteine Mutants by Glutamate. The serine-rich stretch is well conserved in the glutamate transporter family, suggesting functional importance. This is supported by the inhibition of glutamate transport by NEM in the six mutants with single cysteines at the consecutive positions 267–272 (Fig. 3*B*) and by the unusual topological organization of the region. To further examine the functional importance, the effect of the presence of the substrate L-glutamate on the labeling of the reactive cysteines was tested. Labeling of the cysteine at position 129 with biotin maleimide in whole cells was not affected by the presence of 2 mM L-glutamate (Fig. 6*A*, lanes 6 and 7). In contrast, L-glutamate prevented the reaction of biotin maleimide with the cysteines at positions 269 (lanes 1 and 2), 270 (not shown), and 271 (lanes 4 and 5). D-Glutamate, which is not a substrate of the transporter, did not affect the reaction with biotin maleimide (shown for mutant S269C in lane 3).

The effect of L-glutamate on cysteine labeling was also tested in inside-out membrane vesicles, which were prepared in the presence of 2 mM L-glutamate. None of the mutants with cytoplasmic cysteines (T267C, A268C, T272C, N283C, and S292C) were protected from the labeling with biotin maleimide (not shown). Interestingly, although 2 mM L-glutamate was able to prevent the labeling of mutants S269C, S270C, and E271C in

Fig. 6. Protection by L-glutamate against maleimide labeling. Western blot analysis of purified mutants S129C, S269C, and E271C labeled with biotin maleimide in whole cells (*A* and *B*) and inside-out membrane vesicles (*C* and *D*) in the absence (−) or presence (+) of 2 mM L-glutamate (L) or D-glutamate (D). Western blots were decorated with streptavidin coupled to alkaline phosphatase to detect biotinylated proteins (*A* and *C*) and antibodies against the His-tag to compare the amounts of protein (*B* and *D*).

intact cells, it was not able to do so in inside-out membrane vesicles (shown for S269C and E271C in Fig. 6*C*). These results suggest that L-glutamate, at the concentration used, blocks the accessibility of the cysteines at positions 269–271 from the periplasmic side, but not from the cytoplasmic side of the membrane.

The latter observation was confirmed by the effect of L-glutamate on the inhibition of transport activity by maleimide modification in right-side-out membrane vesicles prepared from cells expressing mutant S269C. Glutamate transport by this mutant was blocked by modification with NEM as well as AMdiS (Fig. 7), consistent with the accessibility of the cysteine at position 269 from the outside. The presence of 2 mM L-glutamate during the labeling reaction with the membrane-impermeable AMdiS fully protected against inactivation of the transporter. In contrast, L-glutamate was not able to protect against the inactivation with the membrane-permeable NEM (Fig. 7), showing that position 269 is accessible from the cytoplasmic side of the membrane in the presence of 2 mM L-glutamate. To exclude the possibility that NEM reacts much faster than AMdiS with the cysteine at position 269, which could obscure protection of L-glutamate against NEM, the reactivities of NEM and AMdiS with the cysteine at position 269 were measured. Incubation of membrane vesicles with a right-side-out orientation with 10 μM of the maleimides for 0, 10, 30, 60, and 120 s revealed a time course of labeling that was, within experimental error, identical for NEM and AMdiS (not shown).

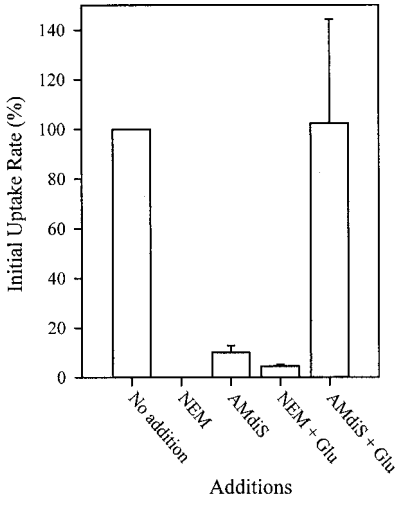

Fig. 7. Protection by L-glutamate against inactivation of glutamate transport by maleimides. The initial rates of glutamate in right-side-out membrane vesicles prepared from *E. coli* cells expressing mutant S269C were measured after treatment with AMdiS or NEM in the presence or absence of 2 mM L-glutamate. Initial rates are expressed as percentage of the rate in membrane vesicles that had not been treated with either of the maleimides.

Discussion

A multiple sequence alignment of more than 60 members of the glutamate transporter family revealed one sequence motif that is conserved in all members (5). This serine-rich motif is located in the putative cytoplasmic loop between membrane-spanning helices 6 and 7 in the C-terminal half of the transporters, which is thought to contain the translocation path and the binding sites for glutamate and the coupling ions (6–13). The loop comprising the serine-rich motif was studied by cysteine-scanning mutagenesis in the glutamate transporter GltT of the bacterium *B. stearothermophilus*. From the 18 single-cysteine mutants that were made in this region, 8 reacted with cysteine-modifying maleimides, and the cysteines in these 8 mutants were accessible from the cytoplasmic side of the membrane as shown in membrane vesicles with an inside-out orientation. The single cysteines in these mutants could be labeled with both charged and noncharged maleimides, indicating that they are facing the aqueous environment. Moreover, because the maleimides used (AMdiS and biotin maleimide) are bulky molecules, the route via which the modification takes place must be spacious. The cytoplasmic accessibility of the reactive single-cysteine mutants agrees with the cytoplasmic location of the loop between transmembrane helices 6 and 7 in most models for the membrane topology of the transporters.

Surprisingly, in addition to their cytoplasmic accessibility, cysteine residues at three consecutive positions in the middle of the loop (S269, S270, and E271) were also accessible from the exterior side of the membrane. The unusual pattern of accessibility could indicate that the region between membrane-spanning segments 6 and 7 enters the membrane from the cytoplasm and is partially accessible from the periplasm (Fig. 8). Immediately adjacent to the three residues with a double-sided accessibility are residues that are exclusively accessible from the cytoplasm. The absence of hydrophobic residues that separate residues facing the outside from those facing the cytoplasm suggests the presence of a water-filled pore in the protein that spans almost the entire membrane. Alternatively, this may indicate a conformational change by which the loop moves from one surface of the membrane to the other. The latter could be a manifestation of the reorientation of the empty binding site(s) that is pertinent to the catalytic cycle of a secondary transporter.

Reentrant loops have been postulated in a number of ion channels, and the presence of such a structure in the potassium channel of *Streptococcus lividans* has been confirmed by x-ray crystallography (26, 27). These loops line aqueous pores in the membrane-embedded part of the proteins. The first reentrant

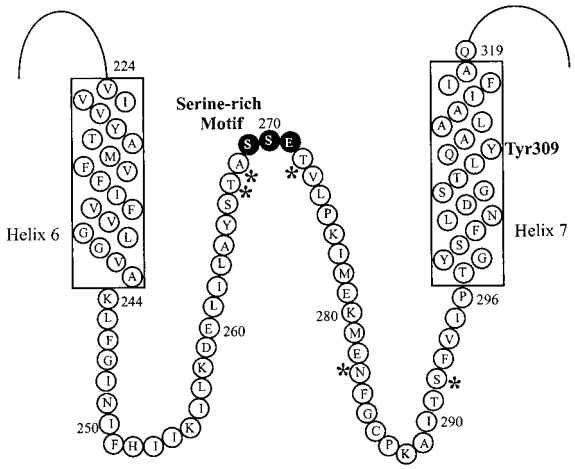

Fig. 8. Model for the membrane topology of the region between membrane-spanning helices 6 and 7 in GltT. The three residues that are accessible from both sides of the membrane, in the absence of L-glutamate, are indicated in black circles; those accessible exclusively from the cytoplasm are indicated with an asterisk.

loop in a secondary transporter was found by cysteine-scanning mutagenesis in the glutamate transporter EAAT2 of rat (15), which belongs to the same family as GltT of *B. stearothermophilus*. The reentrant loop in EAAT2 differs in three ways from the reentrant loop described in this study. First, the loop is extracellular (between membrane-spanning helices 7 and 8) and is partly accessible from the cytoplasm. Second, there are hydrophobic stretches of 8–10 residues that separate residues facing the outside from those facing the cytoplasm. Third, the loop between membrane-spanning helices 7 and 8 does not contain residues with a double-sided accessibility. Together with the present data, the C-terminal half of the glutamate transporters would be characterized by two reentrant loops, one entering the membrane from the cytoplasmic side and one from the periplasmic side. Recently, the reentrant loop between membrane-spanning helices 7 and 8 in EAAT2 was found to play a crucial role in the coupling of the fluxes of glutamate and cotransported ions (9).

The functional importance of the reentrant loop described here is suggested by the presence of the serine-rich motif, which is conserved in all members of the family of glutamate transporters (5). Two lines of experimental evidence support an important functional role of the serine-rich motif. First, modification with NEM of cysteines at six consecutive positions in and around the motif (position 267–272) leads to inactivation of the transporter. Second, the cysteines at positions S269, S270, and E271 are protected from modification with maleimides by the substrate L-glutamate at a concentration of 2 mM. This protection can occur by a direct blockade of the access to the cysteines by L-glutamate or by a L-glutamate-induced conformational change in the protein that closes the access to the cysteines. Interestingly, protection of the cysteines for maleimide modification by 2 mM L-glutamate is observed only from the exterior side of the membrane. L-Glutamate does not protect the cysteines when maleimides are added from the cytoplasmic side of the membrane. This may reflect a higher K_d for L-glutamate at the internal side of the membrane or may indicate that L-glutamate simply cannot protect the cysteines from modification with the maleimides from the cytoplasmic side of the membrane. The behavior of mutants S269C, S270C, and E271C is very similar to the behavior of mutant Y403C in the rat glutamate transporter EAAT2 (6). Y403 is located in membrane-spanning segment 7 at the corresponding position of Y309 in GltT (Fig. 1) and is involved in potassium ion coupling (7). The cysteine at position Y403C in EAAT2 is accessible from both sides of the membrane, but substrate protection occurs from the extracellular side of the membrane only.

The similar behavior of mutant Y403C in helix 7 of rat EAAT2 and mutants S269C, S270C, and E271C in the loop between helices 6 and 7 in GltT of *B. stearothermophilus* suggests that the two regions may be close to each other in space. Membrane-spanning segment 7 contains several hydrophilic residues and could line a water-filled pore, just like the reentrant loop. Other candidates to line a water-filled channel in the transporters are membrane-spanning segment 8, which is extremely amphipathic with a well-conserved face of hydrophilic and charged residues (13), and the reentrant loop between helices 7 and 8. Studies to determine the structural and functional relations between these domains are likely to yield information on the transport mechanism of the glutamate carriers.

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