Structural Rearrangements at the Translocation Pore of the Human Glutamate Transporter, EAAT1*

Barbara H. Leighton 5, Rebecca P. Seal 5, Spencer D. Watts 1, Mary O. Skyba 5, and Susan G. Amara 1

From the 4Howard Hughes Medical Institute, 5Vollum Institute, Oregon Health and Science University, Portland, Oregon 97201, the 6Department of Neurology, University of California, San Francisco, California 94107, and the 7Department of Neurobiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Structure-function studies of mammalian and bacterial excitatory amino acid transporters (EAATs), as well as the crystal structure of a related archaeal glutamate transporter, support a model in which TM7, TM8, and the re-entrant loops HP1 and HP2 participate in forming a substrate translocation pathway within each subunit of a trimmer. However, the transport mechanism, including precise binding sites for substrates and co-transported ions and changes in the tertiary structure underlying transport, is still not known. In this study, we used chemical cross-linking of introduced cysteine pairs in a cysteine-less version of EAAT1 to examine the dynamics of key domains associated with the translocation pore. Here we show that cysteine substitution at Ala-395, Ala-367, and Ala-440 results in functional single and double cysteine transporters and that in the absence of glutamate or DL-threo-oxyaspartate (DL-TBOA), A395C in the highly conserved TM7 can be cross-linked to A367C in HP1 and to A440C in HP2. The formation of these disulfide bonds is reversible and occurs intramolecularly. Interestingly, cross-linking A395C to A367C appears to abolish transport, whereas cross-linking A395C to A440C lowers the affinities for glutamate and DL-TBOA but does not change the maximal transport rate. Additionally, glutamate and DL-TBOA binding prevent cross-linking in both double cysteine transporters, whereas sodium binding facilitates cross-linking in the A395C/A367C transporter. These data provide evidence that within each subunit of EAAT1, Ala-395 in TM7 resides close to a residue at the tip of each re-entrant loop (HP1 and HP2) and that these residues are repositioned relative to one another at different steps in the transport cycle. Such behavior likely reflects rearrangements in the tertiary structure of the translocation pore during transport and thus provides constraints for modeling the structural dynamics associated with transport.

Mammalian excitatory amino acid transporters (EAATs)2 reside on the plasma membrane of neurons and glia and are responsible for removing glutamate from the extracellular space, maintaining its concentration below neurotoxic levels (1, 2). Disruption of this process is associated with several pathological conditions, including ischemia, stroke, and amyotrophic lateral sclerosis (3, 4). This transporter family is comprised of five EAAT subtypes (EAATs 1–5) (5–10) and two-related neutral amino acid transporters (ASCT1 and -2) (11, 12), as well as a number of homologous prokaryotic amino acid and dicarboxylic acid transporters (13). In eukaryotes, the transport of glutamate against its concentration gradient is driven by the co-transport of three sodium ions and a proton, and the counter transport of a potassium ion (14), resulting in a stoichiometric transport current. Glutamate binding to the transporter in the presence of sodium also activates an anion conductance that is not stoichiometrically coupled to transport (7, 15). The prokaryotic carriers use sodium and/or protons to drive the concentrative uptake of glutamate across the cell membrane, and it is not yet known whether they also mediate an anion conductance (13).

The current model of the transporter topology is based on cysteine-scanning accessibility studies of the mammalian and bacterial carriers (1, 16, 17), as well as a recently reported 3.5 Å crystal structure of an archaeal transporter, GltPb1 (18). In this model, the first half of the protein appears to form six TM helices, and the second half is composed of two re-entrant loops in opposite orientations (HP1 and HP2), a seventh TM divided by a β-linker into two helices (TM7) and a final amphipathic transmembrane helix (TM8). Biochemical studies of the rat EAAT1–3 and a related bacterial transporter, GltPb-sc, as well as the crystal structure indicate that the transporter exists as a trimer, although substrate binding and translocation are thought to occur within each subunit (19–22). Indeed, within each monomer of the crystal the C-terminal domains form what appears to be a pore or binding pocket for a “trapped” glutamate molecule (18).

Our current understanding of the role of the C-terminal domains in transport is based on numerous structure-function and topology studies of the mammalian and bacterial transporters. In these studies, it was determined that consecutive, conserved serine residues in HP1 are in an aqueous environment and are accessible from both sides of the membrane (23–25). Moreover, the modification of cysteines substituted for each of these residues abolishes transport, supporting a model in which the residues are closely associated with the translocation pore (23–25). Similarly, most residues in the highly conserved TM7 are also in an aqueous environment and accessible from one or both sides of the membrane (26–28). Modification
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of a majority of these residues, after substitution with cysteine, also abolishes transport, and A395C is protected from modification by substrates and nontransported inhibitors (27). Two of these residues influence the binding of co-transported ions, sodium and/or potassium (Tyr-403 and Glu-404 in Glt1), and when mutated shift the carrier from a unidirectional transport mode into an exchange mode (26, 28, 29). In HP2, two residues (Ser-440 and Ser-443 in Glt1) influence whether lithium can substitute for sodium to support transport (30), and one residue appears to be accessible from the cytoplasmic side of the membrane (31). In addition, most all of the residues in the C-terminal portion of HP2 form a helix and reside in an aqueous environment, near what appears to be the extracellular mouth of the pore (32). Cross-linking of a cysteine introduced into HP1 to one introduced into HP2 suggested that these loops reside in close proximity to one another when the transporter is in the sodium-bound state (33). Based on this finding and on the crystal structure, the two re-entrant loops are hypothesized to act as internal (HP1) and external (HP2) gates in an alternating access model of transport (18, 33). Finally, previous work showed that TM8 forms an amphipathic helix with one face lining an aqueous pathway (34). An arginine residue at the N-terminal side of this helix (Arg-77 in Glt1) is thought to interact with the γ-carboxylate group of glutamate and to influence potassium binding (35). Mutation of a second arginine (Arg-475 in Glt1) in this helix changes the substrate selectivity and transforms the carrier into a substrate-gated cation channel (36). Thus, many of the previous studies, as well as the recent crystal structure, support a model in which these C-terminal domains are directly associated with the translocation pore.

To better understand how these domains are spatially oriented relative to one another during the transport cycle, we carried out a cysteine cross-linking study. We created 22 double cysteine transporters by introducing pairs of cysteine residues into C-terminal domains of a cysteine-less version of EAAT1 (27), and we then measured their transport activity before and after application of copper phenanthroline (CuPh), a catalyst of disulfide bond formation. We also determined the effect of cross-linking on the kinetics of transport, as well as the effects of substrates, inhibitors, and sodium ions on the extent of cross-linking. Results from this work suggest that Ala-395 in TM7 resides at a central point in the translocation pathway near a residue at the tip of each re-entrant loop, Ala-367 in HP1 and Ala-440 in HP2, and that the spatial relationship between these domains is altered during the transport cycle.

EXPERIMENTAL PROCEDURES

Constructs and Cell Transfections—To make the cysteine substitution mutants, cysteine residues were introduced into the Cys-less-EAAT1 transporter using PCR (QuikChange® site-directed mutagenesis kit; Stratagene). PCR products were subcloned into the pcMV5 vector and sequenced by dye terminator cycle sequencing (PerkinElmer Life Sciences). COS-7 cells were passaged and plated into 24-well plates and then transfected by the DEAE-dextran method. Experiments were performed 2 days post-transfection. Cys-less-EAAT1 mutant constructs were assessed for their ability to accumulate 10 μM L-[3H]glutamate (100 nM L-[3H]glutamate, 9.9 μM unlabeled L-glutamate (24 Ci/mmol)). Cells were lysed in 0.1% SDS and counted in a scintillation counter. Cells transfected with the pcMV5 vector served as a control for endogenous l-glutamate uptake under all conditions.

Kinetics—Cells expressing cysteine substitution mutants or Cys-less-EAAT1 were assayed for the ability to accumulate L-glutamate (200 μM; 100 nM L-[3H]glutamate, 199.9 μM nonlabeled L-glutamate) as a function of time (6). Uptake remained linear for 10 min. For V_{max} and K_{m} calculations, cells were incubated with 100 nM L-[3H]glutamate in final unlabeled L-glutamate concentrations of 1, 3, 10, 50, 100, 300, 500, and 1000 μM for 10 min at room temperature. Endogenous L-glutamate uptake measured in cells transfected with the pcMV5 vector was subtracted from each concentration. The Cys-less-EAAT1 transport activity was typically at least 5-fold over the endogenous background activity. The V_{max} is expressed as a percent of Cys-less-EAAT1. K_{m} and V_{max} values were derived by the Michaelis-Menten equation using KaleidaGraph (Synergy Software, Reading, PA).

CuPh-catalyzed Disulfide Cross-linking—COS-7 cells expressing Cys-less-EAAT1 or Cys-less-EAAT1 with single or double cysteine mutations were washed with phosphate-buffered saline (PBS) and then incubated for 5 min at room temperature with various concentrations of copper phenanthroline (CuPh) (0.1–1.5 mM) in PBS. Prior to experimental use, CuSO_4 and 1,10-phenanthroline were combined in a 1:2 ratio to produce the CuPh reagent. Cells were then washed two times with PBS plus 0.1 mM calcium chloride, 1 mM magnesium chloride (PBS/CM), and transporter-mediated uptake was assayed with 10 μM L-[3H]glutamate (100 nM L-[3H]glutamate, 9.9 μM unlabeled L-glutamate (24 Ci/mmol)) in PBS/CM for 10 min at room temperature. After washing twice with PBS/CM, cells were dissolved in 0.1% SDS, and activity was measured by a scintillation counter. Data were plotted as a percentage of the carrier activity without CuPh-catalyzed cross-linking under the same conditions. Percent activity = 100 × uptake after/uptake before.

DTT Application after CuPh Treatment—CuPh-catalyzed cross-linking was performed as described above. Cells were then washed twice with PBS/CM and incubated with 20 mM dithiothreitol (DTT) in PBS/CM for 5 min. Cells were washed twice with PBS/CM, and uptake was performed as described above. Data were plotted as a percent of transport activity measured in the absence of CuPh.

CuPh Treatment without Sodium—COS-7 cells expressing mutant or Cys-less-EAAT1 transporters were washed once in choline buffer containing 138 mM choline-Cl, 8.1 mM Tris-H_3PO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 (pH 7.4). CuPh was applied at 8 or 300 μM for A395C/A440C or 300 or 700 μM for A395C/A367C for 5 min in either choline buffer or PBS. Incubation at these concentrations reduces transport activity maximally or half-maximally. Cells were then washed twice in PBS/CM before radiolabeled glutamate uptake was performed in PBS/CM as described above. Data are plotted as the percent of the carrier activity without CuPh treatment. Percent activity = 100 × uptake after/uptake before.

CuPh Treatment in the Presence of Substrate—COS-7 cells expressing mutant or Cys-less-EAAT1 transporters were washed once with PBS and then incubated for 5 min in PBS or 10 mM L-glutamate with or without the addition of 300 μM...
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CuPh. Cells were then washed twice with PBSCM, and 10 μM L-[3H]glutamate uptake was measured as described above.

MTSET Modification after CuPh Treatment—Cells expressing mutant cysteine transporters were washed once with PBS, and then CuPh was applied at a concentration that results in maximal inhibition of transport. To remove the potential contribution of uncross-linked transporters, cells were then washed twice with PBS and incubated for 5 min with 1 mM MTSET, an impermeant sulfhydryl-modifying reagent established to completely inhibit A367C, A395C, and A440C (24). Cells were again washed twice, and 10 μM L-[3H]glutamate uptake was measured as described above.

DL-TBOA Inhibition of L-Glutamate Uptake—COS-7 cells were transfected with the cysteine substitution mutants and washed once with PBSCM and then preincubated with DL-TBOA, a nontransported substrate analog, at concentrations ranging from 64 nM to 5 mM in PBSCM for 5 min, and uptake of 10 μM L-glutamate (100 nM L-[3H]glutamate) was measured, as described above. Cells were then washed and processed as described above for scintillation counting. Results are expressed as the inhibition of L-glutamate uptake as a function of the concentration of DL-TBOA. IC50 values were calculated using GraphPad Prism (GraphPad Software, Inc., San Diego). The IC50 values are the mean ± S.E. of 3–4 independent experiments done in triplicate.

CuPh Treatment in the Presence of TBOA—COS-7 cells expressing cysteine substitution mutants or Cys-less-EAAT1 transporters were washed once with PBS or choline containing buffer and then incubated for 5 min in PBS or choline with and without 1 mM TBOA and in the presence or absence of CuPh (300 μM A395C/A440C or 700 μM A395C/A435C). Cells were then washed twice with PBSCM prior to 10 μM glutamate uptake as described above.

RESULTS

Effects of Cysteine Cross-linking on Transport—We constructed 22 double cysteine transporters for this cross-linking study using as the template a previously characterized and fully functional version of EAAT1 that lacks endogenous cysteines (Cys-less-EAAT1) (27). We focused on residues that we knew were similar in accessibility and in phenotype following modification (24) and that could be protected from modification by substrates and inhibitors (data not shown).

To determine whether the cysteine pair introduced into each transporter is capable of forming a disulfide bond, we expressed each transporter in COS-7 cells and then measured the accumulation of radiolabeled L-glutamate before and after exposure to the cross-linking reagent CuPh. From this assay, we identified two double cysteine transporters, A395C/A435C and A395C/A440C (Fig. 1), that exhibit a dramatic decrease in transport activity following exposure to CuPh. The other transporter mutants showed either impaired transport activity in the absence of 300 μM CuPh (A395C/T362C, A395C/S363C, A395C/S366C, A395C/I371C, A395C/A414C, A395C/T428C, A395C/I429C, A395C/A441C, A395C/G442C, A395C/T450C, A395C/M451C, and A395C/S457C) or no change in transport activity after exposure to CuPh (A367C/A435C, A367C/I438C, A395C/A441C, A395C/G442C, A395C/T450C, A395C/M451C, and A395C/S457C) or no change in transport activity after exposure to CuPh (A367C/A435C, A367C/I438C, A395C/A441C, A395C/G442C, A395C/T450C, A395C/M451C, and A395C/S457C).
A367C/G439C, A367C/A440C, A367C/T462C, and A395C/G439C; see Fig. 2).

To better characterize the effect of CuPh on the A395C/A367C and A395C/A440C transporters, we measured glutamate transport activity as a function of CuPh concentration. For both transporters, we observed that increasing concentrations of the cross-linking agent (1–1500 µm) lead to a greater reduction in glutamate transport (data not shown). Because the transport activity of our control Cys-less-EAAT1 transporter declines slightly at 1 mM CuPh, in subsequent experiments, we used CuPh concentrations that maximize cross-linking but do not affect the Cys-less-EAAT1 transporter (300 µm for the A395C/A440C transporter and 700 µm for the A395C/A367C transporter). However, even at the highest concentrations of CuPh, we still observed some residual transport activity in the A395C/A367C and A395C/A440C transporters (data not shown). To determine whether this residual activity results from incomplete cross-linking, we modified uncleaved sulfhydryl groups with MTSET, a compound shown to abolish covalent links between cysteine and other residues and thus the reversibility in the presence of DTT confirms the formation of a disulfide bond. To determine whether the cross-linking in these carriers occurs intra-molecularly or inter-molecularly, we examined the effect of CuPh on the transport activity of the single cysteine mutants, A367C, A395C, and A440C expressed either individually or in pairs (A395C with A367C and A395C with A440C) (Fig. 3, A and B). As shown in Table 1, the uptake activity of the individual transporters is similar to that observed for Cys-less-EAAT1 (A395C $K_m = 41 \pm 10 \mu M$, A440C $K_m = 30 \pm 7 \mu M$, and A367C $K_m = 42 \pm 6 \mu M$) and when expressed either alone or with the two single mutants together is unaffected by incubation with CuPh (Table 1). Thus, disulfide bonds formed in the double cysteine transporters are reversible and occur within single subunits rather than between subunits. These findings reinforce the idea that any functional effects of cross-linking observed in the A395C/A367C and A395C/A440C transporters likely arise from constraints within the translocation core of a single monomer.

Cross-linking Alters Transport Kinetics—To examine the effect of intra-molecular cross-linking on the transport kinetics of these carriers, we measured the apparent transport affinity ($K_m$) and maximum transport rate ($V_{max}$) of glutamate before and after exposure to CuPh. In the absence of CuPh, the double cysteine transporters exhibit $K_m$ values that are comparable with Cys-less-EAAT1 (Cys-less-EAAT1, $K_m = 33 \pm 6 \mu M$; A395C/A440C, $K_m = 24 \pm 3 \mu M$; and A395C/A367C, $K_m = 55 \pm 9 \mu M$) (Table 1). After cross-linking, however, A395C/A367C exhibits the same apparent transport affinity ($K_m = 37 \pm 7 \mu M$) but a reduced $V_{max}$ (Table 1 and Fig. 4B). In contrast, A395C/A440C has a significantly lower apparent transport affinity ($K_m = 167 \pm 25 \mu M$) but the same $V_{max}$ (Table 1 and Fig. 4B).
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Cross-linking alters transport kinetics of glutamate uptake. The apparent affinity and transport velocity of the A395C/A440C transporter is dramatically reduced in the A395C/A440C mutant (Fig. 5A). These data suggest that disulfide bond formation between A395C in TM7 and A367C in HP1 (the proposed intracellular gate) abolishes transport function, whereas a disulfide bond formed between the A395C and A440C in HP2 (the proposed extracellular gate) can surprisingly still permit maximal transport activity, albeit with a lower apparent transport affinity.

Cross-linking Alters DL-TBOA Affinity of the A395-A440C Transporter—To further assess how cross-linking A395C to A440C decreases the apparent affinity of this carrier for glutamate, we measured the IC50 for DL-TBOA after cross-linking. As glutamate, we measured the IC50 of DL-TBOA after cross-linking. As glutamate, we measured the IC50 for DL-TBOA after cross-linking. As glutamate gets lower (8 μM) but is less effective at retarding formation of the disulfide bond between A395C and A440C (Fig. 4A). These data suggest that disulfide bond formation between A395C in TM7 and A367C in HP1 (the proposed intracellular gate) abolishes transport function, whereas a disulfide bond formed between the A395C and A440C in HP2 (the proposed extracellular gate) can surprisingly still permit maximal transport activity, albeit with a lower apparent transport affinity.

Effect of Glutamate and DL-TBOA on Cross-linking in Double Cysteine Transporters—Our results on substrate transport and inhibitor binding after disulfide cross-linking of cysteine pairs support the idea that Ala-367, Ala-395, and Ala-440 are closely associated with substrate and inhibitor-binding sites at the translocation pore. To investigate this further, we examined whether co-incubating glutamate or DL-TBOA with CuPh prevents or retards disulfide bond formation. Indeed, addition of 10 μM glutamate significantly reduces disulfide bond formation in both double cysteine transporters (Fig. 5A). In the case of the A395C/A367C transporter, the addition of glutamate prevents disulfide cross-linking at the highest concentration of CuPh (700 μM) tested, whereas for the A395C/A440C transporter, glutamate inhibits cross-linking only at lower (8 μM) but not at higher (300 μM) concentrations of CuPh. Our results suggest that the binding and/or transport of glutamate is effective at blocking the disulfide bond formed between A395C and A367C but is less effective at retarding formation of the disul-

| TABLE 1 Cross-linking alters transport kinetics |  |
|-----------------|-----------------|-----------------|
| $K_m$ and $V_{max}$ values were calculated for each transporter mutant with and without CuPh preincubation. $V_{max}$ is expressed as the percent untreated Cys-less-EAAT1 transporter activity at saturating substrate concentration ($K_m$) and $V_{max}$ values for glutamate and suggests that disulfide bond formation. Indeed, as was observed with glutamate, the IC50 for DL-TBOA is assumed to bind at the same site on the transporter as glutamate but not to undergo translocation. Thus, by measuring the IC50 for DL-TBOA after cross-linking, we aimed to further establish whether substrate binding is impaired by disulfide bond formation. Indeed, as was observed with glutamate, the IC50 for DL-TBOA is dramatically reduced in the A395C/A440C mutant after CuPh treatment (IC50 = 341 ± 44 μM after comparing with IC50 = 20 ± 3 μM before), whereas no change is observed for the A395C/A367C transporter (IC50 = 9 ± 3 μM after comparing with IC50 = 10 ± 1 μM before) or the Cys-less-EAAT1 carrier (IC50 = 23 ± 4 μM before versus IC50 = 20 ± 2 μM after; see Table 2). The A395C/A367C transporter is nonfunctional after disulfide bond formation, and thus the IC50 value for DL-TBOA after CuPh treatment is derived from carriers that were not cross-linked and remains the same as that measured before cross-linking. On the other hand, after cross-linking of the IC50 value of the A395C/A440C transporter shifts in parallel to the $K_m$ value for glutamate and suggests that disulfide bond formation between these two residues alters the external binding site for both the substrate glutamate and for DL-TBOA.

Effect of Glutamate and DL-TBOA on Cross-linking in Double Cysteine Transporters—Our results on substrate transport and inhibitor binding after disulfide cross-linking of cysteine pairs support the idea that Ala-367, Ala-395, and Ala-440 are closely associated with substrate and inhibitor-binding sites at the translocation pore. To investigate this further, we examined whether co-incubating glutamate or DL-TBOA with CuPh prevents or retards disulfide bond formation. Indeed, addition of 10 μM glutamate significantly reduces disulfide bond formation in both double cysteine transporters (Fig. 5A). In the case of the A395C/A367C transporter, the addition of glutamate prevents disulfide cross-linking at the highest concentration of CuPh (700 μM) tested, whereas for the A395C/A440C transporter, glutamate inhibits cross-linking only at lower (8 μM) but not at higher (300 μM) concentrations of CuPh. Our results suggest that the binding and/or transport of glutamate is effective at blocking the disulfide bond formed between A395C and A367C but is less effective at retarding formation of the disul-

| TABLE 2 Cross-linking alters the DL-TBOA IC50 of the A395C/A440C transporter |  |
|-----------------|-----------------|-----------------|
| DL-TBOA IC50 values were measured after preincubation with 300 μM (A395C/A440C) or 700 μM (A395C/A367C) CuPh. Results are expressed as the average ± S.E. from 4 to 5 experiments done in triplicate. ** denotes a significant change in the IC50 after CuPh treatment. |

| Cyst-less-EAAT1 | 20 ± 2 | 23 ± 4** |
|-----------------|-----------------|-----------------|
| A395C/A440C     | 20 ± 3 | 341 ± 44** |
| A395C/A367C     | 10 ± 1 | 9 ± 3 |
fide between A395C and Ala-440. To determine whether glutamate prevents cross-linking as a consequence of its direct interaction with the substrate-binding site, we tested whether DL-TBOA, a nontransported substrate analog, could also prevent cross-linking. As shown in Fig. 5B, DL-TBOA prevents cross-linking in both double cysteine transporters to a similar extent as was observed with glutamate. Hence, it appears that the binding of glutamate or DL-TBOA to the carrier is sufficient to prevent disulfide bond formation between these residues.

**The Effect of Sodium on Disulfide Bond Formation**—Previous studies have shown that both substrate transport and activation of the anion channel require sodium binding and that sodium binding changes the conformation of the transporter (15,37–40). To determine whether sodium binding influences the extent of disulfide bond formation in the double cysteine transporters, we performed the cross-linking reactions in choline (a cation that does not support either transport or the anion conductance) rather than sodium, and we then measured glutamate uptake in normal sodium buffer. Under these conditions, disulfide bond formation was significantly reduced in the A395C/A367C carrier but was unchanged in the A395C/A440C transporter (Fig. 5C). These data suggest that the A395C and A367C residues are brought into close proximity when sodium is bound to the carrier, whereas A395C and

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**FIGURE 5. Effect of glutamate, DL-TBOA, and sodium substitution on disulfide bond formation.** Cells expressing the Cys-less-EAAT1, A395C/A367C, or A395C/A440C transporters were incubated with CuPh in the presence and absence of 10 mM glutamate (A), 1 mM DL-TBOA (B), or with choline substituted for sodium (C), and then glutamate uptake was measured. CuPh was applied at concentrations that maximally (300 μM for A395C/A440C and 700 μM for A395C/A367C) or half-maximally (8 μM for A395C/A440C and 300 μM for A395C/A367C) inhibit transport. Data are expressed as a percent of the uptake measured in the absence of CuPh treatment and are the average ± S.E. of 3–6 experiments done in triplicate.
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A395C and A440C are close to one another in both the sodium-bound and -unbound states.

DL-TBOA Binding without Sodium Prevents Cross-linking of A395C/A440C—A previous study on the accessibility of cysteines substituted for residues in HP2 of Glt-1 showed that DHK, a large nontransported, competitive inhibitor of Glt-1, could prevent the modification of two residues (A439C and S443C) when choline was substituted for sodium (31). In Fig. 5, A and B, we show that disulfide bond formation between A395C and A440C is inhibited by glutamate and DL-TBOA and that this occurs in the presence or absence of sodium. To test whether DL-TBOA could prevent cross-linking of A395C to A440C in the absence of sodium, we applied the inhibitor with CuPh in choline buffer. Under these conditions, we found that DL-TBOA could prevent the cross-linking of A395C and A440C (Fig. 6). These data suggest that DL-TBOA can bind to the carrier in the absence of external sodium and thus requires neither a direct interaction with sodium nor a sodium-dependent conformational change to bind to the carrier. Moreover, these results support the idea that DL-TBOA binding directly blocks A395C and/or A440C from forming a disulfide bond.

DISCUSSION

To assess the proximity and functional significance of residues in conserved regions of the human glutamate transporter, EAAT1, we engineered pairs of cysteine residues into HP1, HP2, and the β-bridge region in TM7 and examined the impact of disulfide cross-linking with copper phenanthroline on transport activity. The findings of these studies confirm that the conserved β-bridge of TM7 (Ala-395) is in close proximity to conserved regions of HP1 (Ala-367) and HP2 (Ala-440) in the mammalian carrier, and that these residues are repositioned with respect to each other at different steps in the transport cycle. Moreover, we show that conformational constraints introduced by cross-linking A395C to either HP1 (A367C) or to HP2 (A440C) have distinct effects on the translocation rate and on the apparent substrate affinity. Limiting the movement of HP1 appears to abolish transport, whereas constraints on HP2 alter the apparent affinities for glutamate and DL-TBOA with no effect on the maximal transport rate. These results, which link the movement of HP2 to initial substrate binding events and movement of HP1 to later steps in translocation, provide insight into the mechanism by which these two domains may function as outer and inner gates of the translocation pore.

Our initial screen of 22 cysteine pairs indicated that two pairs, A395C/A367C and A395C/A440C, could form disulfide cross-links, thus demonstrating the close interaction between the central β-bridge region of TM7 and both HP1 and HP2. Analyses of topology and structure-function relationships for the mammalian and bacterial glutamate carriers have suggested that TM7 and the loops of HP1 and HP2 are exposed to an aqueous environment and that these three regions contribute to substrate binding and translocation (23–25, 27, 31, 32, 34, 41). Consistent with the close proximity of the two hairpin loops as demonstrated here, cysteine substitution experiments performed on the glutamate transporter subtype, Glt-1, identified a distinct pair of residues, located at the tips of HP1 and HP2, which was capable of forming a disulfide bridge (33). The first report of a high resolution x-ray crystallographic structure for a glutamate transporter family member from P. horikoshii (GltPh) provided a model of a glutamate transporter structure consistent with many of these results (18). The model shows regions corresponding to the loops of HP1 and HP2 to be exposed to an aqueous basin extending halfway through the membrane bilayer, in close proximity to the conserved central region of TM7. An additional feature of the structural model is that these regions are in close proximity to the putative substrate-binding site. Within the model, analogous residues to Ala-367, Ala-395, and Ala-440 of EAAT1 are indeed located in close proximity to each other (Fig. 7A). The fidelity with which the structural model confirms the biochemical data summarized above, strengthens the argument that the archaeal structure provides a useful model for the mammalian carriers. However, the GltPh structure reflects the transporter in a single, substrate-bound state; approaches that assess dynamic aspects of domain movements combined with high resolution structures of carriers in other conformations will be required for further insight into the mechanism of substrate binding and translocation.

The dynamics of the interactions between these regions in different states of the transporter were assessed by whether co-transported ions, substrates, and inhibitors had an effect on disulfide formation. Indeed we found that residues in these regions are repositioned with respect to each other at different steps in the transport cycle. For the A395C/A367C transporter, disulfide formation was most efficient in the presence of...
sodium, whereas the A395C/A440C cross-link occurred both in the presence and absence of sodium (Fig. 5 and depicted in Fig. 7, B and C). This is an indication that sodium binding induces a conformational change in the transporter in the absence of substrate that leads to a change in the relative association of HP1 with TM7, while not affecting that of HP2 to TM7. This conclusion is further supported by results obtained with other carriers and by other approaches, which provided evidence of conformational changes induced by the binding of sodium (31, 33, 38, 42). A recent report examining residues thought to contribute to the sodium-binding site has implicated an aspartate residue in TM7 of EAAC1, Asp-367 (Asp-400 in EAAT1), as involved in the binding of sodium to the substrate-free state of the transporter (40). It is possible that sodium binding at or near this residue in EAAT1 induces a conformational change, which brings the loop of HP1 and the central β-bridge of TM7 into closer proximity. Our observation that there is no apparent change in the relative positioning of A440C with A395C induced by sodium could imply that A367C (i.e. HP1) is the domain that reorients most dramatically in the presence of sodium.

We also examined the kinetics of transport and substrate and inhibitor binding after cross-linking the double cysteine transporters. Interestingly, we found that conformational constraints, introduced by cross-linking A395C to either HP1 (A367C) or to HP2 (A440C), have distinct effects on the translocation rate and on the apparent substrate affinity. In the A395C/A367C pair, treatment with CuPh to induce disulfide bond formation abolishes the transport activity (Table 1 and Fig. 4B). Based on the current model, with Ala-395 and Ala-367 located near the substrate-binding site and translocation pathway, the reduction in transport activity observed upon cross-linking these residues may be caused by directly obstructing glutamate and/or sodium binding or by constraining the conformational changes required for their movement through the translocation pathway. This is also consistent with data showing that glutamate and DL-TBOA can prevent disulfide bond formation when co-incubated with CuPh. This may be the result of both substrates and inhibitors inducing conformational changes and thus altering the interaction between these two regions.

In contrast to A395C/A367C, disulfide bond formation in the A395C/A440C mutant results in a transporter with much lower apparent affinity for glutamate (Table 1) and DL-TBOA (Table 2) but, quite strikingly, with the same maximal transport velocity as the untreated transporter (Fig. 4A), suggesting that the cross-link alters the binding step for substrates and inhibitors without effect on subsequent steps in the transport process. This is further supported by data showing that the presence of glutamate and DL-TBOA in the substrate-binding site could reduce disulfide bond formation when co-applied with CuPh. In the context of an alternating access model for unidirectional transport, the outer gate opens to provide access for binding of external substrate and then closes to proceed through subsequent steps, including substrate release. Surprisingly, because the A395C/A440C carrier retains a maximal rate of transport even when constrained by cross-linking, these data suggest that no large movements between the center of TM7 and the tip of HP2 take place during a complete transport cycle. Indeed, it was recently proposed using fluorescence resonance energy transfer analysis that small conformational changes are required during transport (43). Alternatively, if HP2 does undergo sig-

FIGURE 7. Representation of structure and disulfide bond formation between cysteines substituted for residues in EAAT1. Model of the EAAT tertiary structure (A) was based on the crystal structure of the P. horikoshii GltPh bacterial carrier. Spheres depict Ala-395 in TM7, Ala-367 in HP1, and Ala-440 in HP2. Disulfide cross-links formed between A395C and A367C only in the presence of sodium (B) and between A395C and A440C in the presence and absence of sodium (C) are depicted as dotted lines. The colors of the N-terminal scaffold (gray) and the C-terminal translocation core (HP1, yellow; HP2, red; TM7, orange; TM8, magenta) are the same as those used to illustrate these domains in the structural model in Fig. 1.
significant conformational changes during the transport cycle, the first helix of TM7 may move with it and not disrupt transport. In this case, HP2 and TM7 may move together during the transport cycle. As these residues have been modeled to be close to the glutamate-binding site, it is possible that cross-linking of HP2 and TM7 may move together during the translocation process. Moreover, by examining the proximity between these pairs of residues under different transport conditions, we provide insight into the dynamics of the tertiary structure of the C-terminal domain involved in substrate translocation, in particular, the relationship between the two re-entrant loops and the highly conserved region of TM7, which resides between the two gates within the translocation pore. Further work to elucidate the structural basis of transport will come from studies employing inter- and intra-molecular cross-linking, voltage-clamp fluorometry measurements, as well as additional crystal structures representing specific conformational states of the transport cycle.

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