Proximity of Two Oppositely Oriented Reentrant Loops in the Glutamate Transporter GLT-1 Identified by Paired Cysteine Mutagenesis*

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Sodium- and potassium-coupled transporters clear the excitatory neurotransmitter glutamate from the synaptic cleft. Their function is essential for effective glutamatergic neurotransmission. Glutamate transporters have an unusual topology, containing eight membrane-spanning domains and two reentrant loops of opposite orientation. We have introduced pairwise cysteine substitutions in several structural elements of the GLT-1 transporter. A complete inhibition of transport by Cu(II)(1,10-phenanthroline)₃ is observed in the double mutants A412C/V427C and A364C/S440C, but not in the corresponding single mutants. No inhibition is observed in more than 20 other double cysteine mutants. The Cu(II)(1,10-phenanthroline)₃ inhibition can be served in more than 20 other double cysteine mutants. This paper is available online at http://www.jbc.org

Received for publication, August 13, 2001, and in revised form, November 4, 2001

Published, JBC Papers in Press, November 27, 2001, DOI 10.1074/jbc.M107735200

† Supported by a postdoctoral fellowship from the Lady Davis Foundation and by the Ministry of Absorption, Israel.
‡ Supported by guest on July 24, 2018http://www.jbc.org/Downloaded from
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* This work was supported in part by Grant 9900123 from the United States-Israel Binational Science Foundation; by the European Community Training and Mobility of Researchers Program; by Grant NS16708 from the NINDS, National Institutes of Health; by the Federal Ministry of Education, Science, Research and Technology (Germany) and its International Bureau at the Deutsches Zentrum fur Luft und Raumfahrt; and by the Bernard Katz Minerva Center for Cellular Biophysics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: TM, transmembrane; WT, wild type; CL, cysteine-less; CuPh, Cu(II)(1,10-phenanthroline)₃; DTT, dithiothreitol; MTSET, (2-trimethylammoniumethyl)methanethiosulfonate; GABA, y-aminobutyric acid; DHK, dihydrokainic acid.

Glutamate, the predominant excitatory neurotransmitter in the brain, is removed from the synaptic cleft by glutamate transporters located in the plasma membrane of nerve and glial cells. Uptake of glutamate by the glutamate transporters maintains its extracellular concentrations below the neurotoxic level (1–6). In some synapses, glutamate transporters play an important role in limiting the duration of synaptic excitation (7–10). The uptake process is electrogenic (11–13), involving co-transport of three sodium ions, a proton, and a glutamate molecule and countertransport of a potassium ion (14–16). In addition to the coupled flux, glutamate transporters mediate a thermodynamically uncoupled chloride flux, activated by two of the molecules they transport, sodium and glutamate (17, 18).

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

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

Accessibility studies have shown that cysteine residues introduced at positions 364 and 440 of GLT-1, located on reentrant loops I and II, respectively, react with the impermeant sulphydryl reagent MTSET added from the extracellular side (24–26, 29). Substrates and nontransportable analogues partially protected against the modification of cysteines introduced at these positions. Therefore, we have suggested that positions 364 and 440 may be close in the three-dimensional structure of the protein (25). To verify this prediction and to obtain the first information regarding the tertiary structure of the carboxyl-terminal half of the glutamate transporters, we have set out to determine proximity relationships between the different structural elements in this region. In this study, we have used two types of functional assays to infer proximity of engineered cysteine pairs. We report here the identification of two cysteine pairs, A412C/V427C and A364C/S440C, which behave as if they are close in space. The data provide evidence that the two oppositely oriented reentrant loops are spatially close to one another.
Experimental Procedures

Mutagenesis—Specific oligonucleotide primers containing the desired mutation and an engineered restriction site were synthesized. One or two primers were used to introduce single or double mutations on uracil-containing single-stranded DNA derived from wild type GLT-1 (WT-GLT-1) or cysteine-less GLT-1 (CL-GLT-1) constructs (30, 31). Mutated clones were selected using the engineered restriction site(s) as a diagnostic tool. The fragment of GLT-1 containing the mutation(s) was then subcloned into WT- or CL-GLT-1 using unique restriction sites flanking the mutation(s), and the mutated constructs were sequenced in both directions between these unique sites.

Whole Cell Transport—Uptake assays using D-3[H]aspartic acid were carried out essentially as described (31). Briefly, HeLa cells were cultured in 24-well plates, transfected with the indicated GLT-1 construct, and infected with the vaccinia/T7 virus vTF7-3 (32) as described (31). Mutated clones were selected using the engineered restriction site(s) as a diagnostic tool. The fragment of GLT-1 containing the mutation(s) was then subcloned into WT- or CL-GLT-1 using unique restriction sites flanking the mutation(s), and the mutated constructs were sequenced in both directions between these unique sites.

After 16–20 h, the cells were washed twice with cold choline solution (10 mM potassium phosphate, pH 7.5, 150 mM sodium chloride, 0.5 mM magnesium sulfate, and 0.3 mM calcium chloride). Next, the cells were preincubated with the indicated solution for 5 min at room temperature. Following a brief wash with the choline solution, a sodium solution (10 mM potassium phosphate, pH 7.5, 150 mM sodium chloride, 0.5 mM magnesium sulfate, and 0.3 mM calcium chloride) was added, and the uptake was carried out for 10 min at room temperature. The cells were washed twice with ice-cold sodium solution and lysed with 1% SDS solution for scintillation counting. For oxidation studies, the preincubation solution contained 100 μM Cu(II)(1,10-phenanthroline)₃ (CuPh) (or the indicated concentration). The CuPh stock solution (150 mM) was prepared for each experiment by mixing 0.4 ml of 1.25 M 1,10-phenanthroline in water:ethanol (1:1) and 0.6 ml of 250 mM CuSO₄. The experiments depicted in the figures were performed at least three times (n = 3), and values within a given experiment were the mean of triplicate or quadruplicate determinations. The values presented in the Figs. are the mean ± S.D. of the several experiments. Statistical analysis of the data was done using the Student’s t test with α = 0.01. Statistically significant differences (with p < 0.001) are marked with asterisks in Figs. 2, 3, and 6.

Reduction by DTT—After the preincubation step with CuPh, cells were rinsed once with choline solution and incubated with sodium solution containing 100 μM Cu(II)(1,10-phenanthroline)₃ (CuPh) (or the indicated concentration) for 10 min at room temperature. The cells were washed twice with ice-cold sodium solution and lysed with 1% SDS solution for scintillation counting. For oxidation studies, the preincubation solution contained 100 μM Cu(II)(1,10-phenanthroline)₃ (CuPh) (or the indicated concentration). The CuPh stock solution (150 mM) was prepared for each experiment by mixing 0.4 ml of 1.25 M 1,10-phenanthroline in water:ethanol (1:1) and 0.6 ml of 250 mM CuSO₄. The experiments depicted in the figures were performed at least three times (n = 3), and values within a given experiment were the mean of triplicate or quadruplicate determinations. The values presented in the Figs. are the mean ± S.D. of the several experiments. Statistical analysis of the data was done using the Student’s t test with α = 0.01. Statistically significant differences (with p < 0.001) are marked with asterisks in Figs. 2, 3, and 6.

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solution containing freshly prepared 12 mM DTT for 5 min at room temperature. After another washing step, the uptake activity assay was performed as above.

**Inhibition by Cadmium**—HeLa cells transfected with the indicated construct were washed twice with choline solution and preincubated with the indicated concentration of cadmium chloride in sodium solution for 5 min at room temperature. The solution was aspirated, and the cells were incubated with the transport solution and the indicated concentration of cadmium chloride. After the uptake step, the cells were processed as above.

**RESULTS**

**Activity-based Screen for Thiol Cross-linking of Double Cysteine Mutants**—The pioneering studies of Kaback and co-workers (33) to detect proximity relationships in lactose permease were based on creating, under oxidative conditions, a disulfide bond between two single cysteines, each located at a different TM domain or connecting loop. Cross-linking of two cysteines can be easily detected when each is located on a different part of a split transporter (33, 34). We have made unsuccessful attempts to generate a functional split GLT-1 transporter (25). Molecular engineering of artificial protease-sensitive sites in GLT-1, with the aim to obtain physical evidence of cross-linking of engineered cysteines, led to inactivation of the transporter (data not shown). Therefore, we have employed a functional assay to study proximity relationships. Based on the well-established observation that GLT-1 undergoes extensive conformational changes during transport (35), we reasoned that introduction of a covalent disulfide bridge may hamper these changes, leading to inhibition of transport. To increase the likelihood to detect proximal pairs of cysteines, we started our study with positions where introduced cysteines are accessible to the impermeant hydrophilic sulfhydryl reagent MTSET. Such positions are presumably facing a water-filled cavity in the transporter, possibly the permeation pathway. The positions selected for the screen are shown in Fig. 1. These include: Ala-364 (reentrant loop I; Ref. 25); Tyr-403 (TM7; Refs. 27 and 36); Ala-412 (top of TM7), Val-427 (middle of the descending limb of reentrant loop II), and Gly-437 (ascending limb of reentrant loop II); Ser-440 (ascending limb of reentrant loop II); and Val-469 (top of TM8).

**FIG. 3.** Effect of CuPh on the activity of single cysteine mutants. For each of the double cysteine mutants, A412C/V427C (A) and A364C/S440C (B), we constructed the individual corresponding cysteine mutations in the WT-GLT-1 background. Co-expression of two single cysteine mutants in HeLa cells is marked by “co.” Whole cell uptake was measured in the absence (black bars) and in the presence (white bars) of 100 μM CuPh. The results are shown as percentage activity of WT-GLT-1 activity.
Pairs of cysteines were introduced in many combinations in the WT-GLT-1, and the double mutants were expressed in HeLa cells. Transport of the nonmetabolizable substrate D-[3H]aspartate was assayed with and without preincubation with the oxidizing agent CuPh. Some of the most significant results are depicted in Fig. 2. Although WT-GLT-1 contains nine endogenous cysteine residues (see Fig. 1), its activity is only slightly decreased upon exposure to 100 μM CuPh. Two double cysteine mutants, A412C/V427C and A364C/S440C, completely lose their activity under the same conditions. Significantly, other combinations of cysteine pairs among these four positions do not lead to an increased sensitivity of the double mutants to oxidizing conditions (Fig. 2). Moreover, pairing of A412C, V427V, A364C, or S440C with a cysteine residue introduced at position 469 in TM8 results in CuPh-insensitive double mutants. Uptake activity of additional double cysteine mutants is also not affected by CuPh treatment (see legend to Fig. 2).

Although most of the double cysteine mutants retain at least 50% transport activity compared with WT-GLT-1, two (A364C/S440C and A364C/V469C) show ~20% of WT activity (Fig. 2). Analysis of the kinetic properties of the A364C/S440C transporter shows that the decrease of activity is the result of a lowering of $V_{\text{max}}$ (1.2 ± 0.6 compared with 4.7 ± 0.5 nmol/mg of protein/min for WT-GLT-1) ($n$ = 3). The apparent $K_m$ is not significantly changed (34 ± 6 μM compared with 38 ± 2 μM for the WT-GLT-1). Thus, the conformation of the double mutant is not altered, at least regarding its substrate affinity.

It is of interest to note that, in the case of the two double mutants which are inhibited by CuPh, A412C/V427C and A364C/S440C, the observed activity is lower than what would be expected from the independent impact of the single mutants. Thus, A364C and S440C exhibit 71 ± 3% ($n$ = 5) and 56 ± 13% ($n$ = 7) of WT-GLT-1 activity (Fig. 3A). However, the observed activity of A364C/S440C is only 18 ± 3% ($n$ = 8). The same is found for A412C/V427C, which has 48 ± 6% ($n$ = 5) of the wild type activity (Fig. 2), whereas A412C and V427C exhibit 96 ± 7% and 83 ± 1% ($n$ = 4) of the wild type, respectively (Fig. 3B).

Characterization of the Cysteine Pairs A412C/V427C and A364C/S440C—Because the double mutants were constructed in the WT-GLT-1 background, it is possible that the oxidation of A412C/V427C and A364C/S440C double mutants leads to the cross-linking of one of the newly introduced cysteines with an endogenous cysteine residue. To investigate this possibility, the two cysteine pairs were introduced in the background of CL-GLT-1, where all nine endogenous cysteines have been removed (23, 36). However, we could not analyze the effect of CuPh on the double mutants, because they are completely devoid of activity (Fig. 4). The single cysteine mutants in this background are active (Fig. 4). Surface biotinylation experiments indicate that this is probably a result of the fact that these double mutant transporters do not reach the plasma membrane (data not shown). In view of this result, we have continued to characterize the structural determinants leading to the loss of activity caused by oxidative conditions in the wild type background. None of the single mutants A412C, V427C, A364C, or S440C in the WT-GLT-1 background are sensitive to the oxidizing conditions (Fig. 3, A and B). Moreover, transport activity of the 412C/V427A and A412S/V427C mutants is not significantly inhibited by CuPh, as opposed to the A412C/V427C mutant (Fig. 3A). The same is shown for A364S/S440C and A364C/S440A mutants (Fig. 3B). These observations render highly unlikely the possibility that perturbation of one of the positions causes a conformational change bringing an endogenous cysteine close to one of the introduced ones.

The formation of a disulfide bond between proximal cysteines induced by CuPh would be expected to be reversed by a reducing agent such as DTT, provided it can reach the dithiol bond. Indeed, transport activity of both A412C/V427C and A364C/S440C previously treated with CuPh was significantly restored upon exposure to 12 mM DTT (Fig. 5). This concentration of DTT is similar to that used to reverse the inhibition of serotonin transporter mutants by methanethiosulfonate reagents (37).

Recent studies indicate that glutamate transporters may form oligomers (38, 39), although it is still unclear if the monomer or the oligomer is the functional unit. Therefore, the oxidative cross-linking inferred for the two cysteine pairs may be intermolecular rather than intramolecular. Our results do not support this possibility. When the single mutants A412C and V427C are coexpressed in HeLa cells, no inhibition of transport activity by CuPh is observed. On the other hand, when both mutations are present on the same cDNA molecule, a potent inhibition by CuPh is observed (Fig. 3A). The same is observed for A364C and S440C (Fig. 3B).

When the substrate analogue DHK is present during the oxidative treatment, 6- and 2.5-fold protection is observed with A412C/V427C and A364C/S440C, respectively (Fig. 6). The substrate L-glutamate has a 3-fold protective effect on A412C/V427C, whereas GABA, which is not a substrate, does not.
In A364C/S440C, the protective effect of L-glutamate is smaller but statistically significant (Fig. 6). Replacement of the other cosubstrate (sodium) by choline, has no effect on inhibition by CuPh (data not shown).

The Pairs A412C/V427C and A364C/S440C Create a High Affinity Binding Site for Cadmium (II) Ions—As a complementary approach to establish the proximity between cysteines introduced at positions 412 and 427 as well as between those at positions 364 and 440, we have examined the ability of the double mutants to form a high affinity Cd²⁺ binding site. This divalent cation interacts with cysteinyl side chains (40, 41), and the affinity of the interaction is dramatically increased if the Cd²⁺ ion can be coordinated by two cysteines (42). Exposure of the WT-GLT-1 to up to 100 μM Cd²⁺ has no significant effect on D-[³H]aspartate transport (Fig. 7A). The single cysteine mutants are also unaffected by this treatment (Fig. 7A). On the other hand, the activity of the A412C/V427C double mutant is inhibited to 50% at 100 μM Cd²⁺ (Fig. 7A). The activity of the A364C/S440C double mutant is dramatically more sensitive to the divalent cation. Half-maximal inhibition is attained at −0.5 μM and total inhibition at −10 μM Cd²⁺. In both double mutants, a partial reversal of this inhibition is observed when the Cd²⁺ ions are washed away (data not shown). It is of interest to note that the A364C/S440C transporter is also more sensitive to CuPh compared with A412C/V427C (Fig. 7B), although the difference is not as dramatic as that observed with Cd²⁺.

We have examined the impact of CuPh and Cd²⁺ on the kinetics of the double mutant transporters. Transfected cells were treated with appropriate concentrations of these reagents to achieve 50–70% inhibition of transport. Because of the relative low transport rate of A364C/S440C, no reliable data could be obtained for this double mutant. However, analysis of cells expressing the A412C/V427C with 3 μM CuPh results in a V_max of 26–40% of untreated controls, whereas the K_m remains essentially the same −40.0 ± 1.6 μM compared with 38.1 ± 3.1 μM for untreated controls (n = 3). Similarly, treatment of cells expressing A412C/V427C with 100 μM Cd²⁺ results in a V_max of 50–55% of
untreated controls, whereas the $K_m$ remains essentially unchanged $-37.7 \pm 0.7 \mu M (n = 2)$.

**DISCUSSION**

The results described in this paper provide the first information on distance constraints in the (Na$^+$ + K$^+$)-coupled glutamate transporters. We have identified two cysteine pairs, A412C/V427C and A364C/S440C, which appear to be close in space. We have used two functional assays to infer proximity relationships between the engineered cysteine pairs. One assay (Figs. 2 and 3) is based on the observation that extensive conformational changes take place during glutamate transport (35) and the idea that formation of disulfide bonds could hamper them. This idea is supported by our observations that CuPh impacts $V_{max}$ rather than $K_m$ of A412C/V427C, indicating that its effect is the result of the inactivation of modified transporters. CuPh treatment of other transporters, containing two engineered proximal cysteines, has also been shown to impair function (43, 44). In some transporters and pumps, the cross-linking of the intact molecule has been shown to result in a gel-shift (45, 46). We have attempted this experiment, but could not observe a change in mobility on SDS-PAGE upon treatment with CuPh (data not shown). This is probably a result of the fact that our observed functional impact represents inactivation of surface transporters. In the vaccinia/T$_7$ virus expression system employed, these are only a small fraction of the total protein pool made in the HeLa cells. Thus, the system used in our studies is not optimal for the detection of surface-directed cross-linking.

The second functional assay is based on the creation of a high affinity Cd$^{2+}$ binding site between vicinal cysteines (Fig. 7A). Engineering of artificial high affinity Zn$^{2+}$ binding sites has recently been used to define proximity relationships in the dopamine transporter (47, 48). The inhibitory effect of Zn$^{2+}$ on the dopamine transporter was primarily the result of a decrease in $V_{max}$ (47). Similar effects of Zn$^{2+}$ have recently been observed with the GABA transporter GAT-1 (49). This is in agreement with our results on Cd$^{2+}$ inhibition of A412C/V427C, where $V_{max}$ is selectively reduced. Although it has been reported that the affinity of the interaction with Cd$^{2+}$ is increased even when the binding site is coordinated by two cysteines.
teines (42), three cysteines have been implicated for high affinity Cd\(^{2+}\) binding in another system (50). Therefore, it is possible that the markedly higher Cd\(^{2+}\) sensitivity of A364C/S440C compared with A412C/V427C can result from the contribution of an additional (yet unknown) contact site within the A364C/S440C transporter, similar to the finding with the zinc sensitivity studies of the dopamine transporters (48). Another explanation for the higher Cd\(^{2+}\) sensitivity of A364C/S440C relative to A412C/V427C is the possibility that the bond angles of the former are more conducive to Cd\(^{2+}\) binding than the latter. Alternatively, positions 364 and 440 may be closer to each other than the other pair. Consistent with this option is the higher CuPh sensitivity of A364C/S440C compared with A412C/V427C (Fig. 7B).

The activity of the two double cysteine mutants compared with their single counterparts suggests that the impact of the individual mutations is not simply additive (Fig. 3). This is seen even more dramatically in the cysteine-less background (Fig. 4). In the latter case, it appears that the double cysteine transporters do not reach the plasma membrane, consistent with our inability to observe activity in these mutants after exposure of the cells (or reconstituted preparations derived of them) to DTT (data not shown). One possibility is that a disulfide bridge between the two cysteines is generated because of the oxidative environment of the endoplasmic reticulum, thus preventing their arrival or maintenance at the plasma membrane. The presence of one or more of the endogenous cysteines of GLT-1 somehow moderates this phenomenon (Figs. 3 and 4).

We have attempted to see whether the distance of the two pairs is changing as a function of the conformation of the transporter. For both pairs we have observed a significant expression levels in oocytes than GLT-1. Cross-linking of yet unidentified cysteine pairs would be expected to selectively block the chloride conductance, whereas that of others might hamper coupled transport alone.

Acknowledgment—We thank Beryl Levene for expert secretarial help.

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J. Biol. Chem. 2002, 277:3985-3992.
doi: 10.1074/jbc.M107735200 originally published online November 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107735200

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