The Substrates of the γ-Aminobutyric Acid Transporter GAT-1 Induce Structural Rearrangements around the Interface of Transmembrane Domains 1 and 6*

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The sodium- and chloride-coupled γ-aminobutyric acid (GABA) transporter GAT-1 is essential for efficient synaptic transmission by this neurotransmitter. GAT-1 is the first cloned member of the neurotransmitter-sodium-symporter family. Here we address the idea that during transport the extracellular halves of transmembrane domains (TM) 1 and 6, TM 1b/TM 6a, move relative to the binding pocket. Therefore, we have probed the aqueous accessibility of TM 6a and its proximity to TM 1b in the presence and absence of its substrates. Cysteines were introduced, one by one, at all TM 6a positions. In several mutants, transport activity was inhibited by the impermeant sulfhydryl reagent (2-trimethylammonium)methanethiosulfonate, whereas wild type GAT-1 was basically insensitive. This inhibition was potentiated by sodium, whereas GABA was protective. Moreover, we used paired cysteine mutagenesis in conjunction with treatments with copper(II)(1,10-phenanthroline)3 (CuPh). CuPh did not affect the activity of wild type GAT-1 but potently inhibited transport by the TM 6a mutant D287C. Such inhibition was not observed with D287C/C74A, indicating that Asp-287 is close to Cys-74 of TM 1b. Inhibition of transport of D287C by CuPh, but not by (2-trimethylammonium)methanethiosulfonate, was potentiated when sodium and GABA were both removed. Thus, the degree of inhibition by CuPh is not a simple function of the accessibility of the individual cysteines but also involves structural rearrangements around the TM 1b/TM 6a interface, which is electrogenic and has a stoichiometry of 2:1:1 (5–8). Although the precise order of events during a transport cycle is not yet established, it is clear that the uptake process is initiated by the binding of at least one sodium ion (7, 9, 10). The cycle consists of different conformational transitions, involving outward and inward facing conformations. Moreover, by analogy with the recent high resolution crystal structure of the bacterial homologue LeuT (11), conformations with the binding pocket occluded from both sides of the membrane are also likely to be involved in the cycle (see Fig. 1A).

The recent high resolution crystal structure of the bacterial homologue LeuT (11) appears to be an excellent model for the NSS neurotransmitter transporters (12–15). LeuT consists of 12 TMs with TMs 1–5 related to TMs 6–10 by a pseudo-2-fold axis in the membrane plane. TMs 1 and 6 have breaks in their helical structure approximately halfway across the membrane. These breaks expose main-chain carbonyl oxygen and nitrogen atoms for the binding of leucine and the two sodium ions, Na1 and Na2. The sodium ions in the binding pocket are both close to the substrate, which is in direct contact, through its carboxyl group, with Na1 (11). In contrast to NSS neurotransmitter transporters, LeuT and other bacterial homologues do not require chloride for transport (11, 16, 17), and indeed no chloride was observed in the binding pocket of LeuT (11). Recently, the chloride binding site of the NSS neurotransmitter transporters was identified (18, 19), and it appears that the role of chloride is mainly to compensate for the multiple positive charges provided by the sodium ions during the GABA translocation step.

In the absence of structures of conformations other than the one seen in the LeuT structure, it is important to obtain dynamic information in order to get further insights into the mechanism of transport of NSS transporters. It has been proposed that the extracellular and cytoplasmic helical segments, TM 1b/TM 6a and TM 1a/TM 6b, respectively, may move, each as one unit, relative to TM 3 and TM 8 (Fig. 1B), to allow or deny access to the binding pocket from the extra- and intracellular media (11). However, there are other possibilities for opening up the binding pocket to the outside, such as for instance by increasing the distance between TM 1b and TM 6a. Earlier findings indicate that TM 1b of GAT-1 and of the serotonin transporter SERT are conformationally sensitive (20, 21). Therefore, we have studied the impact of the impermeant sulfhydryl reagent MTSET on cysteine residues, introduced into all positions of TM 6a of GAT-1. This was done in the presence and absence of substrate and cotransported ions. Furthermore,
we have used paired cysteine mutagenesis in conjunction with treatment with CuPh. In this paper, we have identified proximal amino acid residues, on TM 1b and TM 6a (Fig. 1B). Analysis of the effects of GAT-1 ligands on the reactivity of the cysteine mutants toward MTSET and CuPh are indicative of structural rearrangements around the TM 1b/TM 6a interface during transport.

EXPERIMENTAL PROCEDURES

Generation and Subcloning of Mutants—Mutations were made by site-directed mutagenesis of the wild type (WT) GAT-1 in the vector pBluescript SK(−) (Stratagene) using single-stranded uracil-containing DNA as described previously (22, 23). Briefly, the GAT-1-containing plasmid was used to transform Escherichia coli CJ236 (dut−, ung−). From one of the transformants, single-stranded uracil-containing DNA was isolated upon growth in uridine-containing medium, according to the standard protocol from Stratagene, using helper phage R408. This yields the sense strand, and consequently mutagenic primers were designed to be antisense. The mutants were subcloned into constructs containing either WT or C74A GAT-1 in the vector pBluescript SK(−), using the unique restriction enzymes NheI and AgeI. The coding and noncoding strands were sequenced between the above restriction sites.

Cell Growth and Expression—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 (24) and subsequent transfection with plasmid DNA, as well as GABA transport, was done according to the equivalent cysteine residue has been shown to be the major determinant of sensitivity of transport toward membrane-impermeant sulfhydryl reagents, such as MTSET (29, 30), although GAT-1 is relatively insensitive to this reagent (31, 32).

Several of the cysteine mutants in the domain corresponding to TM 6a retained significant sodium-dependent [3H]GABA transport activity (Fig. 2A), but W285C, D287C, A288C, and S295C exhibited only 5–10% of the transport activity of C74A. The Y296C mutant was completely devoid of any activity (Fig. 2A). Mutants Q291C and F294C had only around 1% of the activity of C74A; therefore, these mutants were not studied further. The low activity of these two mutants and of W285C, D287C, and S295C was not due to a defective targeting to the plasma membrane, as probed by surface biotinylation (data not shown). Although many of the cysteine mutants in TM 6a exhibited low activity, our assay is sensitive enough to quantitatively detect even a few percent of C74A activity. This enabled us to study the impact of impermeant MTSET on the low activity mutants W285C, D287C, A288C, and S295C, in addition to that on the mutants with higher activity. The activity of mutants S282C, W285C, L286C, D287C, T290C, and S295C was potently inhibited by 1 mM MTSET (Fig. 2B). Although the inhibited mutants are in the background of C74A, one possibility to explain this inhibition is that the mutants at one or more of these six positions might expose a previously inaccessible endogenous cysteine residue. However, the introduction of an alanine or a serine at these positions did not render the transporters sensitive to MTSET (Fig. 2B, open bars). This strongly suggests that the introduced cysteines themselves reacted with the sulfhydryl reagent.

Effect of Substrate and Coupling Ions on the Aqueous Accessibility of TM 6a—The inhibition by MTSET in five out of the six above mentioned cysteine mutants was dependent on the composition of the external medium. This is shown for D287C...
in Fig. 3 and mutants W285C, L286C, T290C, and S295C in Fig. 4. The only exception was S282C, where similar inhibition by MTSET was observed in the presence or absence of sodium (choline substitution), and this was also true when GABA was present during the preincubation with the sulfhydryl reagent (data not shown). We also reexamined the other active mutants, which were not inhibited by 1 mM of MTSET in the presence of sodium, to see if they became sensitive to the sulfhydryl reagent under different preincubation conditions. However, V284C, A288C, A289C, I292C, and F293C were similarly insensitive in the absence of sodium or in the presence of GABA (data not shown). Transport by D287C was extremely sensitive to MTSET in the presence of sodium (Fig. 3A, half-maximal inhibition at around 4 μM). When sodium was replaced by choline, this sensitivity was markedly reduced (Fig. 3A). When GABA (1 mM) was added together with sodium, this also resulted in a reduced inhibition by MTSET, so that the sensitivity of D287C to the sulfhydryl reagent was similar to that in the absence of sodium (Fig. 3A). This protection by GABA was not seen by either glycine or L-aspartate, both of which are not transported by GAT-1 (data not shown). The protection by GABA required sodium, because when sodium was replaced with choline, no effect of GABA was observed (data not shown). The other co-substrate, chloride, was also required for the protection by GABA (Fig. 3B). However, in the absence of GABA, removal of chloride did not influence the sensitivity of D287C to MTSET (Fig. 3B).

To determine whether the protection by GABA against the inhibition of transport of D287C by MTSET is a consequence of the mere binding of the substrate to the transporter or to its ability to be transported, we examined the effect of SKF100330A on this inhibition. SKF100330A is a bulky nontransportable hydrophobic GABA analogue, which competitively inhibits GABA transport by GAT-1 with a high apparent affinity. In contrast to GABA, SKF100330A did not protect D287C against the inhibition by the same effects by sodium, chloride, GABA, and SKF100330A on the sensitivity of D287C to MTSET were also observed in the absence of the additional C74A mutation (WT background) (data not shown). The above four different conditions had generally similar effects on the sensitivity of mutants W285C, L286C, T290C, and S295C to MTSET (Fig. 4). GABA protected in all four mutants (Fig. 4, A–D), and this protection required the
simultaneous presence of sodium and of chloride (data not shown). In all four mutants, the sensitivity was larger in the presence of sodium than in its absence (Fig. 4, A–D). The lack of effect by SKF100330A on D287C in the presence of sodium (Fig. 3A) was also seen with L286C (Fig. 4B). In the case of T290C, a slight protection was observed with the blocker (Fig. 4C). The blocker provided substantial protection of W285C (Fig. 4A) and of S295C (Fig. 4D), although not as much as GABA.

Effects of Thiol Cross-linking and Cd$^{2+}$ on Transport by D287C—To identify positions in TM 1b and TM 6a, which are potentially close to each other, we used the LeuT structure (11) and a model where the LeuT residues were replaced by their GAT-1 counterparts (using the SPDB Viewer downloaded from the ExPaSy Proteomics server). We identified the positions Cys-74 in TM 1a and Asp-287 in TM 6a, which correspond to Ala-35 and Ala-246 of LeuT (Fig. 1B). These residues are likely to be close in space, at least in the substrate-occluded form of the transporter (11). When the aspartate at position 287 is replaced by a cysteine, the distance between its sulfur atom and that of the endogenous cysteine at position 74 is 7.63 Å according to the model.

Using CuPh, which can oxidize two cysteine residues close in space (forming a disulfide bond), we obtained experimental evidence for the proximity of these two positions. This oxidizing agent is not only capable of inhibiting transport when the two cysteines are basically in direct contact (26, 33) but also when they are farther away, although then higher concentrations are required to inhibit transport (26, 27). Inhibition of paired cysteine mutants by CuPh is presumably due to restrictions imposed by the cross-link on the conformational changes that the transporter undergoes during a transport cycle. Preincubation of cells, expressing wild type GAT-1, with 30 μM CuPh did not have much effect on transport, and the same was true for the double mutant D287C/C74A (Fig. 5A). However, under the same condition, a potent inhibition of D287C in the background of wild type GAT-1 (containing the endogenous cysteine at position 74) was observed (Fig. 5A). This indicates that the inhibition of transport requires cysteine residues both at positions 74 and 287. This inhibition could be reversed by subsequent incubation of the cells with 12 mM of DTT (data not shown), in agreement with the idea that the inhibition of transport by CuPh was due to oxidative cross-linking of the cysteines at positions 74 and 287. No inhibition of transport by 30 μM CuPh was observed with the alanine replacement mutant D287A, intro-

FIGURE 3. Effect of the composition of the external medium on the inhibition of D287C/C74A by MTSET. HeLa cells expressing D287C/C74A were preincubated for 5 min in the presence of the indicated MTSET concentrations in media containing NaCl (■) or NaCl plus 1 mM GABA (○) (A and B), choline chloride (ChCl) (▲), or NaCl plus 30 μM SKF 100330A (▲) (A) or sodium gluconate (NaGlu) (▲) or sodium gluconate plus 1 mM GABA (△) (B). Values are given as a percentage of control (preincubation without MTSET) and represent the mean ± S.E. of at least three different experiments done in triplicate.

FIGURE 4. Effect of the composition of the external medium on the inhibition of cysteine mutants by MTSET. Inhibition of transport of the indicated cysteine mutants (W285C/C74A (A), L286C/C74A (B), T290C/C74A (C), and S295C/C74A (D)) by MTSET was done as described in the legend to Fig. 3.
the interface of two transporter monomers. Independent evidence for proximity of the two cysteines came from their ability to form a Cd$^{2+}$ binding site (Fig. 5B). This divalent cation interacts with cysteiny1 side chains (34, 35), and the affinity of the interaction is dramatically increased when the Cd$^{2+}$ ion is coordinated by two cysteines (36). Again, only when the two cysteines were present on the same polypeptide, was inhibition by 500 μM Cd$^{2+}$ observed (Fig. 5B).

In contrast to treatment with Cd$^{2+}$, in the case of CuPh a covalent bond is formed. Therefore, it was possible to determine the effect of the composition of the external medium on the cross-linking during the treatment with CuPh, after washing of the treated cells and the addition of transport medium (which requires, of course, both sodium and chloride). Similar to the effects of GABA and SKF100330A on inhibition of D287C by MTSET (Fig. 3A), the substrate also afforded significant protection against the cross-linking of the cysteines at positions 74 and 287, whereas the blocker did not have any effect (Fig. 6A). Removal of chloride had a similar effect on the cross-linking and the inhibition of D287C by MTSET (Figs. 3B and 6B). However, the removal of sodium had no effect on the cross-linking (Fig. 6A), in contrast to the oxidative cross-linking of cysteines at positions 74 and 287, the accessibility of the cysteine introduced at position 287 to MTSET was increased in the presence of sodium (Fig. 3A). It was therefore interesting to examine the effects of sodium and GABA on the accessibility of the endogenous cysteine at position 74. However, as noted previously, in GAT-1 this cysteine is relatively unreactive (31), and no inhibition of transport by wild type GAT-1 was observed, even if the MTSET concentration was increased to 3 mM (data not shown). Studies on the glycine transporters GlyT1 and GlyT2 indicated that the presence of an arginine, rather than a glutamine, at position +2 (corresponding to position 76 of GAT-1) reduced the reactivity of the cysteine corresponding to Cys-74 (30). In GAT-1, the

Reduced in WT background (Fig. 5A). Therefore, it is unlikely that mutation of aspartate 287 to a smaller residue, such as cysteine, has caused the cysteine at position 74 to become closer to one of the endogenous cysteines.

The inhibition by CuPh was only observed when the cysteines at positions 74 and 287 were present on the same polypeptide and not when the two cysteines resided on two different polypeptides. This was demonstrated by the lack of inhibition of transport by CuPh in cells cotransfected with wild type GAT-1 and the double mutant D287C/C74A (Fig. 5A). This suggests that the cysteines at positions 74 and 287 come into close proximity within the transporter monomer but not at
other positively charged residue, lysine, occupies this +2-position. Indeed, when the point mutation K76Q was introduced into wild type GAT-1, a small but significant inhibition of transport by 3 mM MTSET was observed (Fig. 7). This inhibition is due to the modification of the cysteine at position 74, because it was not observed with the double mutant K76Q/C74A (Fig. 7). Inhibition was most pronounced in the presence of sodium; removal of sodium or the addition of GABA protected (Fig. 7).

Effects of Thiol Cross-linking and Ca2+ on Transport by W285C—We probed the sensitivity of the other TM 6a cysteine mutants in WT background to CuPh (30 μM). W285C was the only mutant other than D287C that was sensitive to treatment with the oxidizing reagent (data not shown). However, much higher concentrations of CuPh were required to observe significant inhibition (Fig. 8A). The W285A mutant was not inhibited under these conditions, but this was not the case for the double mutant W285C/C74A, although this inhibition was significantly less than with W285C (Fig. 8A). These observations indicated that part of the inhibition of W285C by CuPh could be due to the cross-linking of the cysteine at position 285 with one or more of the cysteine residues endogenous to GAT-1. Nevertheless, the cysteines at positions 74 and 285 were able to form a Ca2+ binding site (Fig. 8B) and thus could be close to each other, at least in one of the conformations of the transporter. Also in this case, the inhibition by Ca2+ was only observed when the cysteine pairs were introduced in the same polypeptide (Fig. 8B).

**DISCUSSION**

The results described in this paper show that when cysteines are introduced into each of the TM 6a positions of GAT-1, 12 of 15 of the mutants retain measurable transport activity. Only three mutants are basically inactive, namely Q291C and F294C (Fig. 2A) as well as Y296C. Tyr-296 is not conserved in the NSS family, but Gln-291 and Phe-294 correspond to Gln-250 and Phe-253 of LeuT (11). The glutamine residue is strictly conserved within the NSS family, and the phenylalanine residue is almost fully conserved. Regarding Phe-294, the phenylalanine is replaced only in a few NSS members, but even in these cases, the substitution is by the related tyrosine Gln-250 forms part of the hydrogen bond network involved in the extracellular gate seen in the LeuT structure, and this explains the critical role of its GAT-1 counterpart in transport. In fact, our result is in excellent agreement with that of Mari et al. (37), who also showed that Gln-291 of GAT-1 was irreplaceable for transport. The LeuT counterpart of Phe-294 forms part of its binding pocket, and its side chain makes direct contact with the aliphatic side chain of the bound leucine (11). This suggests that the equivalent Phe-294 of GAT-1 may also make contact with GABA, perhaps with one or more of its methylene groups.

The LeuT counterpart of Ser-295 is Thr-254, which participates in the liganding of the transported substrate and Na1 (11). However, even S295C, where the conserved side chain hydroxyl group is replaced by a thiol group, has measurable uptake of [3H]GABA (Fig. 2), which is in good agreement with results from another recent study (37). Recently, we found that transport currents could not be measured in oocytes expressing S295C (12). These currents were measured at saturating GABA concentrations; therefore, it appears that the Vmax of S295C is strongly impaired. Tracer flux measurements are usually carried out at very low substrate con-

**FIGURE 7.** Effect of the composition of the external medium on the inactivation of K76Q and K76Q/C74A mutants by MTSET. HeLa cells expressing the two mutants were preincubated for 5 min with the indicated solutions in the presence or absence of 3 mM MTSET, and subsequently [3H]GABA transport was assayed as described under “Experimental Procedures.” Data are expressed as percentage of untreated control and represent mean ± S.E. for three separate experiments done in triplicate. For each mutant, the mean values obtained after preincubation with choline or NaCl plus 1 mM GABA were compared with those after preincubation in NaCl using one-way ANOVA with a post hoc Dunnett multiple comparison test:**p < 0.01; *, p < 0.05. The activity of K76Q and K76Q/C74A was 49.1 ± 5.1 and 69.3 ± 4.3% of that of C74A (n = 3).

**FIGURE 8.** Inhibition of transport of W285C by CuPh and Cd2+. The conditions of the experiments were the same as described in the legend to Fig. 5, except that CuPh was used at 800 μM. Values represent the mean ± S.E. of at least three separate experiments done in triplicate. Comparison of the values of W285C (A and B) and of W285C/C74A (B) with those of WT, using one-way ANOVA with a post hoc Dunnett multiple comparison test, revealed statistically significant differences (**, p < 0.01), and the same was true for the comparison of W285C with W285C/C74A in A. The activity of W285C and W285A was 8.4 ± 0.8 and 10.6 ± 1.0% of that of WT, respectively (n = 3).
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centations and are therefore a function of both \( K_m \) and \( V_{\text{max}} \). It is thus likely that the low \( V_{\text{max}} \) of this mutant may be partly compensated for by a lowered \( K_m \). Because of the low transport activity of this mutant, this issue was not further investigated.

Of the 12 mutants tested for inhibition by MTSET, six were sensitive (Fig. 2B). This includes the three consecutive positions 285, 286, and 287. Such an aqueous accessibility can be compatible with an \( \alpha \)-helical structure, provided that a substantial part of the helix is surrounded by water rather than embedded in the membrane bilayer. Such an exposure of TM 6 to the aqueous medium is consistent with the LeuT structure (11). The reactivity of five of the six mutants, which are sensitive to MTSET, is markedly affected by ligands of GAT-1 (Figs. 3 and 4). We found that in all of these five cysteine mutants, the reactivity to MTSET was diminished by GABA, provided that the two cosubstrates, sodium and chloride, were present as well (Figs. 3 and 4). This suggests that reduction in reactivity of these five mutants is transport-dependent. Consistent with this idea are our observations that the nontransportable substrate analogue SKF-100330A does not diminish this reactivity (Figs. 3 and 4). This bulky analogue can bind instead of GABA (Fig. 1A, step 2) but cannot be transported, and thus the transporter gets locked in the outward facing conformation. Because GABA is much smaller than the blocker, protection by the substrate against inhibition by MTSET is probably not due to a steric blockade of the access of the sulfhydryl reagent to the engineered cysteines in the outward-facing conformation of the transporter. This is consistent with the fact that the reactive cysteines were introduced at positions in the LeuT structure located on the extracellular side of the binding pocket (11). It should be noted that some of the cysteine mutants used have a severely decreased catalytic activity. Therefore, we cannot rule out the possibility that some of the changes in sensitivity to MTSET seen in Figs. 3 and 4 could be affected by the mutations themselves. Significantly, these changes were rather similar for all five cysteine mutants, including L286C and T290C. The reactivity of the C74A parent construct, and it is therefore likely that the effects of transporter ligands on the reactivity of all five mutants are due to similar conformational changes at or around TM 6a.

In the absence of sodium, the reactivity of cysteine residues engineered into TM 6a toward extracellular MTSET is similar to that in the presence of sodium and GABA (Figs. 3 and 4). This suggests that in the absence of sodium, as well as in the presence of sodium, chloride, and GABA, a substantial portion of the transporters resides in inward facing and/or in substrate-free occluded states (Fig. 1A). Extracellular sodium is expected to bind to the empty outward facing transporters (Fig. 1A, step 1), thereby shifting the equilibrium between outward and inward facing transporters (Fig. 1A). As a consequence, the extracellular aqueous accessibility of the introduced cysteine residues is probably increased. The increased reactivity to MTSET in the presence of sodium is observed even in the absence of chloride (Fig. 3B). This is presumably due to the fact that, in contrast to transport of GABA, the binding of sodium to GAT-1 can also proceed in the absence of chloride (9). After the subsequent binding of GABA and chloride, transport occurs, and this results in a relative increase of the occluded and inward facing states (Fig. 1A). As a consequence, the extracellular accessibility of the introduced cysteines is decreased again (Figs. 3 and 4). The faster reactivity of cysteine residues introduced at many TM 6a positions under conditions favoring the conformation in which the substrate site is accessible from the extracellular medium and less rapidly under conditions favoring the release of substrate to the cytoplasm has also been observed at other positions of GAT-1 (20) and other NSS transporters, such as the norepinephrine transporter NET (38) and the serotonin transporter SERT (21).

It is of interest to note that, whereas the reactivity of the cysteine residues introduced at TM 6a is similarly affected by the transporter ligands, this is not the case for cysteines introduced at different positions of TM 1b, where the same ligands often exert the opposite effect at different positions (20). These differences in the effects of ligands could be due to a movement of TM 1b relative to TM 6a during one of the steps of the transport cycle. Alternatively, it is possible that the complex accessibility changes on TM 1b may be due to a relative movement of other TMs toward TM 1b. Whatever the scenario, the results thus far are consistent with a movement of TM 1b and TM 6a during transport. The cross-linking of Cys-74 of TM 1b with the cysteine introduced at position 287 of TM 6a (Figs. 5 and 6) is in excellent agreement with predictions from the LeuT structure (11) and provides an additional piece of evidence for the idea that the LeuT structure is an excellent model for GAT-1 (and other NSS transporters). Cys-74 of TM 1b has an extremely low reactivity toward MTSET (Fig. 7), yet this cysteine is efficiently cross-linked to the cysteine introduced at position 287 (Figs. 5 and 6). These observations suggest that the reactive oxidizing species generated by CuPh can readily penetrate into crevasses that are difficult to reach by MTSET. It is therefore likely that the protection by GABA against inhibition by CuPh in D287C reflects an increased distance between the two cysteine residues and/or a change in their side chain orientations, resulting in decreased cross-linking of the Cys-74/Cys-287 pair. Thus, it is possible that CuPh inhibits transport, because TMs 1b and 6a need to separate during the transport cycle or because the process of forming the cross-link distorts their relationship so as to prevent an important conformational change.

The inhibition of transport of D287C by CuPh was not affected when sodium was removed (Fig. 6A), although the reactivity to MTSET of the cysteines at positions 74 and 287 was diminished under these conditions (Figs. 3A and 7). Again, this indicates that the changes in sensitivity to CuPh are not...
merely due to changes in the aqueous accessibility. At the present time, it is difficult to provide a structural interpretation of these results. One possibility is that in one or more of the states increasingly populated in the presence of GABA, such as the occluded or inward facing loaded transporters (Fig. 1A), TM 1b and TM 6a get so close that even the oxidizing species cannot reach the cysteine pair anymore. This would not be the case for the occluded and inward facing empty states, expected to accumulate in the absence of sodium (Fig. 1A). A variant of this scenario is that another structural element, such as external loop IV, could move closer and obstruct the access to the TM pocket during transport, although not necessarily as one unit as suggested previously (11). It appears that dynamic information be determined. In the LeuT structure and the GAT-1 model, the cross-linking of cysteines at positions 74 and 285 could not calculated by sodium and GABA (41). An alternative scenario for the changes in CuPh sensitivity could be that in the presence of GABA, but not in the absence of sodium, the distance between the two cysteines actually increases or the bond angles become less favorable for cross-linking.

At some point in the translocation cycle, positions 74 and 285 may also come close. This was demonstrated using inhibition by Cd$^{2+}$ of the W285C mutant, which has cysteines at both of these positions (Fig. 8B). Because we could not use inhibition by CuPh for this pair (Fig. 8A), the effect of ligands of GAT-1 on the cross-linking of cysteines at positions 74 and 285 could not be determined. In the LeuT structure and the GAT-1 model, the side chain of Asp-287 is pointing toward that of cysteine 74, whereas the side chain of Trp-285 is pointing in the opposite direction (Fig. 1B). In order for Cys-74 and Cys-285 to create a Cd$^{2+}$ binding site, a rotation of TM 6a or an unwinding event should occur at an as yet undefined step of the transport cycle. Altogether, our results support the idea that the extracellular half of TM 1b and of TM 6a move relative to the binding pocket during transport, although not necessarily as one unit as suggested previously (11). It appears that dynamic information on GAT-1 and other NSS transporters is a valuable tool to gain further insights into the mechanism of this class of transporters.

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