The Reactivity of the γ-Aminobutyric Acid Transporter GAT-1 toward Sulfhydryl Reagents Is Conformationally Sensitive

IDENTIFICATION OF A MAJOR TARGET RESIDUE*

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The γ-aminobutyric acid (GABA) transporter GAT-1 is a prototype of neurotransmitter transporters that maintain low synaptic levels of the transmitter. Transport by GAT-1 is sensitive to the polar sulfhydryl reagent 2-aminomethyl methanethiosulfonate. Following replacement of endogenous cysteines to other residues by site-directed mutagenesis, we have identified cysteine 399 as the major determinant of the sensitivity of the transporter to sulfhydryl modification. Cysteine-399 is located in the intracellular loop connecting putative transmembrane domains eight and nine. Binding of both sodium and chloride leads to a reduced sensitivity to sulfhydryl reagents, whereas subsequent binding of GABA increases it. Strikingly binding of the nontransportable GABA analogue SKF100330A gives rise to a marked protection against sulfhydryl modification.

These effects were not observed in C399S transporters. Under standard conditions GAT-1 is almost insensitive toward the impermeant 2-(trimethylammonium)ethyl methanethiosulfonate. However, in a chloride-free medium, addition of SKF100330A renders wild type GAT-1, but not C399S, very sensitive to this impermeant reagent. These observations indicate that the accessibility of cysteine 399 is highly dependent on the conformation of GAT-1. Consequently, topological assignments based on accessibility of endogenous or engineered cysteines to small polar sulfhydryl reagents need to be interpreted with extreme caution.

GAT-1 is a GABA1 transporter that was reconstituted, purified to homogeneity (1), and cloned (2). It is the prototype of a large family of sodium- and chloride-dependent transporters of neurotransmitters. This includes transporters for norepinephrine, dopamine, serotonin, and glycine as well as several additional GABA transporters (for review see Ref. 3). The role of these transporters is to maintain low synaptic levels of their respective transmitter substrates. Direct proof of this has been obtained using knockout mice; the decay of extracellular dopamine in brain slices from homozygous mice in which the dopamine transporter was disrupted is ~100 times longer than normal (4).

GAT-1 catalyzes the electrogenic transport of GABA with one chloride and two sodium ions (5–7). Using site-directed mutagenesis, we have attempted to identify amino acid residues of this transporter involved in substrate binding. Although we have identified several of its residues that are critical for transport (8–11), only in one case have we been able to pinpoint the reason underlying the transport defect. Tyrosine 140 appears to be involved in the binding of GABA, possibly in the liganding of the amino group, the moiety common to all neurotransmitters (11). GAT-1 is sensitive to sulphydryl reagents (12). This suggests that one or more cysteines may play a critical role in the transport process or else are located in the vicinity of other critical residues. One of these cysteines, located at position 74, has recently been identified (12). According to the originally predicted topology (2), which recently has received strong experimental support (13), this residue faces the extracellular medium (Fig. 1). However, transporters in which this cysteine was replaced by serine were still sensitive to the sulphydryl reagent MTSEA (12). This suggests that there are additional cysteines that are targets for the covalent modification by this reagent. In this study we have identified the predominant target residue, cysteine 399. Furthermore, we have observed that inactivation of the transporters by sulphydryl reagents is very sensitive to the conformation of the transporter. Under all conditions, even those where the reactivity of wild type GAT-1 toward MTSEA is potentiated, C399S transporters remain relatively resistant to the reagent. It appears therefore that cysteine 399 is a highly conformationally sensitive residue.

EXPERIMENTAL PROCEDURES

Materials

[35S]dATP was from NEN Life Science Products. Tissue culture media, serum, and antibiotics were from Biological Industries (Kibbutz Beit Ha’Emek, Israel).

Methods

Site-directed Mutagenesis—This was performed using the method of Kunkel et al. (14) as described (10). Mutations were confirmed by DNA sequencing and were subcloned into wild type using pairs of unique restriction enzymes cutting the cDNA on either side of the mutation. The subcloned mutant cDNAs were then sequenced from each direction.
between the two 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 (15) and subsequent transfection with plasmid DNA as well as GABA transport (16) were done as published previously. Protein was determined as described by Bradford (17).

Inhibition Studies with Sulfhydryl Reagents—Before the transport measurements, the cells adhering to 24-well plates were washed with the 150 mM NaCl-containing transport medium. Each well was then incubated at room temperature with 200 μl of this solution (cases of different compositions are indicated in the figure legends) and the indicated concentration of reagent under study. After 5 min, the medium was aspirated, and the cells were washed twice with 1 ml of the transport solution. Subsequently they were assayed for [3H]GABA transport using the solution to which the labeled amino acid was added (16). The hydrophilic methanethiosulfonate reagents used were purchased from Toronto Research Chemicals, Inc. Positively charged MT-SEA and MTSET are approximately 1 nm in length, differing only at the charged head group, which measures 0.58 nm in MTSET and 0.36 nm in MTSEA (18). Although MTSEA is membrane-permeant to some extent, MTSET is impermeant (19).

RESULTS

Identification of a Major Determinant of Sensitivity of GAT-1 to Sulfhydryl Reagents—Two of the 15 cysteines of the GABA transporter GAT-1, cysteines 164 and 173 (Fig. 1), are at equivalent positions to those proposed to form a disulfide bond in dopamine and serotonin transporters (20, 21). Disulfide-bonded cysteines are unavailable to sulfhydryl reagents. To identify those residues involved in rendering GAT-1 sensitive to sulfhydryl reagents, we have therefore mutated each of the 13 remaining cysteines to serine: 11 individually and residues 493 and 499 (Fig. 1) simultaneously. Each of these replacement mutants retains significant GABA transport activity (Fig. 2). A comparison of transport measurements after a 5-min preincubation in the absence or presence of 0.75 mM hydrophilic sulfhydryl reagent MTSEA shows that most of the replacement mutants exhibited a similar sensitivity to MTSEA as the wild type GAT-1 (Fig. 3A). Consistent with our previous observations (12), substitution of cysteine 74 by serine (C74S) resulted in reduced inhibition by MTSEA (Fig. 3A), indicating that part of the effect is contributed by this residue. Strikingly, replacement of cysteine 399 by serine resulted in an almost complete resistance of GAT-1 to inhibition by this concentration of MTSEA (Fig. 3A). The resistance to inhibition is unlikely to be because of the introduction of a serine residue at this position rather than the elimination of cysteine 399, as similar results were obtained when cysteine 399 was replaced by either alanine or glycine (Fig. 3B). Transport of [3H]GABA by C399S-GAT-1 is 48 ± 9% that of the wild type based on all comparative experiments performed (n = 21). It is also sodium- and chloride-dependent just like wild type (data not shown). This indicates

![Fig. 1. Hypothetical secondary structure of the GAT-1 transport protein. Putative transmembrane segments are depicted as rectangles. The position of each of the endogenous cysteines is indicated by an ellipse with the position number inside. EL, predicted external loop; IL, predicted internal loop; Y, N-linked glycosylation site.](image-url)

![Fig. 2. Transport activity of GAT-1 and mutant transporters in which cysteine has been changed to serine. HeLa cells were infected with recombinant virus and transfected with pBluescript SK-containing wild type (WT) or the indicated single cysteine replacement mutants. *C493S represents the double mutant C493S/C499S. Uptake was measured as described under "Experimental Procedures." Values are the average (±S.E.) of quadruplicate determinations from a typical experiment where all mutants have been compared simultaneously and are expressed as percent of wild type values.](image-url)
that elimination of cysteine 399 does not cause an overall change of the conformation of the transporter. The related nonpermeant MTSET does not inhibit GAT-1 very much (12). Therefore we have used N-ethylmaleimide to test whether C399S-GAT-1 is resistant to sulfhydryl reagents other than MTSEA. The data presented in Fig. 4 indicate that this is indeed the case.

**Reactivity of GAT-1 toward MTSEA Is Dependent on Its Conformation**—Under our standard conditions, preincubation with MTSEA is performed in the simultaneous presence of sodium and chloride, both of which are driving ions for GABA transport. When either ion is omitted during the preincubation using choline chloride or sodium glucuronate instead of sodium chloride, the inhibitory potency of the reagent is increased in the case of the wild type and also in C74S-GAT-1 (Fig. 5). Again, not much inhibition is observed with C399S-GAT-1, but the small increase of inhibition upon preincubation of MTSEA in the presence of choline chloride or sodium glucuronate seen in the experiment depicted in Fig. 5 is not significant. The percent activity remaining after exposure to MTSEA in the different media was NaCl 92 ± 5% (n = 7), sodium glucuronate 88 ± 7% (n = 7), and choline chloride 92 ± 6% (n = 5).
MTSEA is observed in the presence of GABA (Fig. 6A). This increase is dependent on the presence of both cosubstrates; no potentiation by GABA is observed when either ion is omitted (Fig. 6B). The potentiation by GABA is specific, because it is not observed with several compounds that are not substrates for the transporter such as L-aspartate, L-glutamate, and D-glucose (Fig. 6A).

The potentiation of sensitivity toward MTSEA is also observed with C74S-GAT-1 and to a similar extent as wild type GAT-1 (Fig. 7). On the other hand, no such potentiation is observed with C399S-GAT-1 or a double mutant in which both these cysteines have been replaced by serine (Fig. 7). These observations indicate that the potentiation by GABA is exerted by a conformational change causing cysteine 399 to become more exposed.

**Effect of the Nontransportable Blocker SKF100330A on Re-**activity toward MTSEA—SKF100330A is a derivative of the GABA analogue guvacine to which, via the nitrogen atom, a diphenylbutenyl group has been attached (22). It is a potent inhibitor of GABA transport (22), but is not transported (7, 11). Therefore, this compound may allow us to distinguish between the possibility that the effect of GABA on MTSEA reactivity of GAT-1 is because of the mere binding of the substrate or one invoking a conformational change of the transporter, subsequent to the binding step. The experiment depicted in Fig. 8A shows that 30 μM SKF100330A almost completely blocks GABA transport in HeLa cells expressing wild type GAT-1. When the cells are preincubated with the blocker at this concentration and subsequently washed with medium lacking it, around 50% of the transport activity is recovered when assayed subsequently in the absence of SKF100330A. This activity is blockable when the reagent is added again to the transport assay (Fig. 8A). The inability to obtain a full recovery of transport activity is probably because of the hydrophobic nature of SKF100330A. We could not improve this by adding liposomes to the washing medium in an attempt to partition out the blocker (data not shown). However, enough activity is recovered to enable the determination of the effect of the blocker on inhibition of GAT-1 by MTSEA. In contrast to the potentiation by GABA (Figs. 6 and 7), SKF100330A provides a strong protection against the inhibition of wild type GAT-1 by MTSEA in an NaCl-containing medium (Fig. 8B). This protection is not observed when the sodium is replaced by either choline or lithium (Fig. 8B). In fact, the blocker exerts a modest potentiation by MTSEA in these media. Strikingly, a dramatic potentiation by SKF100330A of the inhibition by this sulfhydryl reagent is observed in chloride-free media (Fig. 8B). Under these conditions there is not much of an effect of SKF100330A on the already very low susceptibility of C399S-GAT-1 transporters toward MTSEA (Fig. 8C). On the other hand although the wild type GAT-1 is almost not inhibited by the membrane-impermeant MTSET, exposure to SKF100330A in chloride-free media results in a marked inhibition by it (Fig. 8D). This potentiation is much smaller with C399S transporters (Fig. 8D) as well as with C399A and C399G transporters (data not shown).
DISCUSSION

By replacing individual cysteines to serine, we have identified the major site for inhibition of GAT-1 by the sulfhydryl reagents MTSEA and \( N \)-ethylmaleimide. This is cysteine 399, which is located on an intracellular loop, according to all published topological models (2, 12, 13, 23–25). Direct experimental evidence for the internal location of this loop has been obtained with the related norepinephrine transporter (26). The ideal way to demonstrate this would be to replace all 15 endogenous cysteines with other residues in such a manner that a cysteine-less transporter exhibiting considerable transport activity will be obtained and to introduce a single cysteine at position 399. The data depicted in Fig. 2 show that replacement of several individual cysteines, each results in loss of around 75% of the transport activity. There might be a variety of reasons for this: a reduction in the rate of conformational changes intrinsic in the transport cycle, reduced biosynthesis or increased degradation, or a reduced targeting of mutant transporters to the membrane. Regardless of the reasons, the above, together with observations on the critical nature of the two cysteines thought to form a disulfide bond in the related dopamine and serotonin transporters (20, 21), makes it virtually impossible to obtain an active cysteine-less GAT-1. However, the observation that regardless of the nature of the replacement at the 399 position, resistance to MTSEA was obtained (Fig. 3B) indicates that cysteine 399 is the major site...
of action by sulfhydryl reagents. Transport of C399S is also somewhat reduced, 48 ± 9% that of the wild type. However, it retains the hallmarks of GABA transport catalyzed by GAT-1, namely sodium and chloride dependence. Therefore, it appears that replacement of cysteine 399 by serine does not cause an overall change in the conformation of the C399S transporters.

The sensitivity of GAT-1 to MTSEA is influenced by the presence of its substrates, sodium, chloride, and GABA. This is in harmony with our earlier observations that GAT-1 undergoes extensive conformational changes upon substrate binding (27). It is of interest to note that in the related serotonin transporter, replacement of sodium by lithium renders this transporter more sensitive to methanethiosulfonate reagents (21). Wild type GAT-1 becomes more sensitive to MTSEA in the absence of either sodium or chloride (Fig. 5). It also becomes more sensitive to the sulfhydryl reagents in the presence of GABA in NaCl-containing media (Fig. 6). These effects are largely absent in C399S transporters (Figs. 5 and 7) implying that these effects are largely because of effects of substrate binding on the accessibility of cysteine 399. This is further illustrated in Fig. 9. In the presence of chloride but in the absence of sodium, cysteine 399 is readily accessible to covalent modification by sulfhydryl reagents from the inside. Sodium binding to this form of the transporter causes a conformational change, resulting in decreased sensitivity to MTSEA (Figs. 5 and 9) and also to an improved ability of GABA to bind to the transporter (Ref. 28, Fig. 9). In the presence of sodium and chloride, GABA and its nontransportable analogue SKF100330A have opposing effects. Whereas GABA potentiates the inhibition by MTSEA (Fig. 6A), SKF100330A protects (Fig. 8B). Because both of them bind to the GABA binding site, we infer that this binding leads to a markedly decreased accessibility of cysteine 399 (Fig. 9). In the presence of SKF100330A, the transporter remains locked in this mode, but in the presence of GABA, translocation takes place, and this exposes cysteine 399 again (Fig. 9). The protective effect of SKF100330A is sodium-dependent (Fig. 8B). Surprisingly, in the absence of chloride, SKF100330A markedly potentiates the inhibition by MTSEA (Fig. 8B), whereas GABA has no effect (Fig. 6B). One explanation for this may be that in the absence of chloride, GABA is not capable of binding to its site, whereas SKF100330A still can do so, perhaps by virtue of its hydrophobic tail, which could anchor it to additional residues in the vicinity of the binding site. Although not indicated in Fig. 9, this somehow results in a conformation where GAT-1 is rendered supersensitive to MTSEA. This supersensitivity is not observed with C399S transporters (Fig. 8C), again indicating that cysteine 399 contributes to this effect.

In contrast to MTSEA, the permanently charged MTSET is not permeant (19). The very low sensitivity of GAT-1 to MTSET (12) is in harmony with the idea of an intracellular location of cysteine 399 (Fig. 1 and Ref. 26). Interestingly, in the absence of chloride the blocker SKF100330A renders GAT-1 sensitive to the impermeant sulfhydryl reagent MTSET, and cysteine 399 contributes to this sensitivity (Fig. 8D). Thus, the accessibility of this residue to MTSET, which is not very different in size to the transported GABA, is highly dependent on the conformational state of the transporter. Thus, experiments using accessibility of cysteines to small sulfhydryl reagents as a criterion for topological assignments need to be interpreted with extreme caution. This accessibility should at least be tested under a variety of conditions. Moreover, externally added MTSET may permeate into a water-filled pore representing part of the translocation pathway of the transporter. Thus, it is possible that it can react with a cysteine located closer to the cytoplasm than the polar head-groups of the external or even the internal phospholipid leaflet. Even though the loop on which cysteine 399 is located faces the cytoplasm, in one of the conformations of the transporter, externally added MTSET may permeate through the translocation pathway and react with cysteine 399.

Only in the four GABA transporters of the superfamily is there a cysteine residue in the loop connecting transmembrane domains 8 and 9. Bacterial GABA transporters belong to the distinct family of Afc transporters. They also have a cysteine at a similar position, and this cysteine is critical for function (29). It has been proposed that this might be a general feature extending to the GABA transporters of the family of sodium- and chloride-dependent neurotransmitter transporters (29). It appears that this is not the case, as C399S transporters have substantial activity, around 50% that of wild type. Rather, it appears that the properties of cysteine 399 in GAT-1 are reminiscent of those of cysteine 148 of lac permease. This cysteine has been shown to be the site of inhibition of lac permease by N-ethylmaleimide (30), but it is not required for function (31).
It appears that cysteine 148 is close to the sugar binding site of this permease. Making its side chain more bulky by the covalent modification apparently perturbs sugar binding (31). It seems likely therefore that cysteine 399 of GAT-1 is located in the vicinity of an important functional domain of the transporter. Covalent modification would perturb the function exerted by this domain. Although we do not know the proximity relations in GAT-1, it is tempting to speculate that determinants of this domain may be located in transmembrane domains 8 and 9 or perhaps in the loop connecting them. Therefore, it will be of special interest to examine these regions using the scanning cysteine accessibility method (see for instance Refs. 18 and 32–35).

Even though it seems impossible to design a cysteine-less GAT-1 construct, the low sensitivity of the C399S transporters to sulfhydryl reagents indicates that by elimination of selected cysteines we could produce a transporter that is virtually insensitive to sulfhydryl reagents. The double mutant C74SC399S is even less sensitive to sulfhydryl reagents than C399S (data not shown). In the related dopamine transporter, C74SC399S is even less sensitive to sulfhydryl reagents than sensitive to sulfhydryl reagents. The double mutant to sulfhydryl reagents indicates that by elimination of selected GAT-1 construct, the low sensitivity of the C399S transporters Refs. 18 and 32–35).

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