The membrane topology of GAT-1, a sodium- and chloride-coupled γ-aminobutyric acid transporter from rat brain, has been probed using N-glycosylation scanning mutagenesis. Overall, the results support the theoretical 12-transmembrane segment model. This model (based on hydropathy analysis) was originally proposed for GAT-1 and adopted for all other members of the sodium- and chloride-dependent neurotransmitter transporter superfamily. However, our data indicate that the loop connecting putative transmembrane domains 2 and 3, which was predicted to be located intracellularly, can be glycosylated in vivo. Furthermore, studies with permethylated and impermeant methanesulfonate reagents suggest that cysteine 74, located in the hydrophilic loop connecting transmembrane domains 1 and 2, is intracellular rather than extracellular. We present a model in which the topology deviates from the theoretical one in the amino-terminal third of the transporter. It also contains 12 transmembrane segments, but the highly conserved domain 1 does not form a conventional transmembrane α-helix.

Sodium-coupled transporters of neurotransmitters, located in presynaptic and glial membranes, are thought to play a major role in maintaining low synaptic levels of the transmitter (for a review, see Ref. 1). Recently, this has been shown directly for the dopamine transporter using homologous mice in which the transporter was disrupted (2). Transporters of many neurotransmitters, including GABA, norepinephrine, serotonin, and glycine, belong to a large superfamily of sodium- and chloride-dependent neurotransmitter transporters (see Refs. 3 and 4 for reviews). GAT-1 is a GABA transporter that was reconstituted, purified to homogeneity (5), and cloned (6). It is the first identified member of the superfamily and catalyzes the electrogenic transport of GABA with one chloride and two sodium ions (7–9).

Knowledge of the membrane topology is one of the first steps toward the elucidation of the structural basis of transporter function. The topology of GAT-1 has been predicted using hydropathy plots (6), and this interpretation has been adopted for the other members of the superfamily. The theoretical model predicts that the transporter spans the membrane 12 times in α-helical stretches, with amino and carboxyl termini both at the intracellular side of the membrane. GAT-1 is modified by asparagine-linked glycosylation (5, 10, 11), and the model predicts a large extracellular loop between transmembrane helices 3 and 4 containing three N-linked glycosylation sites (see Fig. 1) (6). Even though experimental support has been obtained recently for a few aspects of this model and predictions by hydropathy plots usually provide a reasonable first approximation, it is imperative to verify the topology experimentally.

In this report, we describe an experimental evaluation of the topology mainly by N-glycosylation scanning mutagenesis. Since glycosylation occurs on the luminal side of the endoplasmic reticulum only, this method can be used to determine which domains of the protein are located extracellularly. After showing that all three predicted N-linked glycosylation sites are used in vivo, we have inserted such sites independently into each of the putative hydrophilic domains of an aglyco-GAT-1 mutant. Glycosylation and transport activity have been monitored in these transporter constructs upon their expression in HeLa cells. This approach has been applied recently to several transporters and channels (cf. Refs. 12–14). Our studies indicate that the predicted topology is correct in the carboxyl-terminal two-thirds of the transporter, but suggest a significant deviation from it in the remaining amino-terminal part.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—*[3H]GABA (47.6 Ci/mmol) was obtained from the Nuclear Research Center (Negev, Israel). Molecular mass markers were from Pharmacia Biotech Inc. Solutions of acrylamide/bisacrylamide were obtained from Bio-Rad. Restriction enzymes were from New England Biolabs Inc. and Boehringer Mannheim. T4 polynucleotide kinase, T4 DNA polymerase, T7 DNA ligase, and the transfection reagent DOTAP were also from Boehringer Mannheim. Sequenase Version 2.0 kits were obtained from U. S. Biochemical Corp., and kits for plasmid DNA preparation were from QIAGEN Inc. [32P]dATP, and EXPRESS™S™S™ protein-labeling mixture (1000 Ci/mmol) were from DuPont NEN. Tissue culture medium, serum, and antibiotics were from Biological Industries (Kibbutz Beit HaEmek, Israel). P$_{COOH}$, a peptide located in the carboxyl terminus of GAT-1 (residues 571–586, IQPSIDIREPENGPEQ) was synthesized by Dr. Lea Goldberg (Weizmann Institute, Rehovot, Israel) on an Applied Biosystems Model 430A peptide synthesizer. The antibody against P$_{COOH}$ was raised in a rabbit as described (15). MTSEA and MTSET were generous gifts from Dr. Arthur Karlin. All other reagents were obtained from Sigma.

**METHODS—**Removal and insertion of N-linked glycosylation sequences were performed using site-directed mutagenesis according to the method of Kunkel as described (16, 17). To alleviate the sequencing burden, the mutations were planned so as to include recognition sequence of restriction enzymes (six cutters). Mutants were identified by restriction analysis. The first step was to make the deglycosylated form of the GABA transporter. This was done by sequential removal of the three native N-glycosylation sites located in the extracellular area of the protein. The resulting aglyco construct (DDD) was then used as the basis for inserting N-linked glycosylation sequences into putative hydrophilic loops and tails. The important mutants, including all those displayed in the figures, were subcloned back into the wild type (see
Figs. 2 and 6) or into aglyco-GAT-1 (see Figs. 3–5) using unique restriction sites. The inserts were then sequenced in both directions. In the case of all other mutants (listed in Tables I and II), at least two independent Escherichia coli colonies harboring the mutant plasmids were characterized by transport activity. In two cases (out of the 44 mutants), no transporter was detected upon immunoprecipitation. However, when additional colonies were examined, immunoprecipitation showed a full-length transporter.

Heterologous expression of wild-type or mutant transporters was performed exactly as described (11). Briefly, HeLa cells were infected with the recombinant vaccinia/T7 virus vTF7-3. Subsequently, they were transfected with the desired cDNA (pBluescript SK with the transporter insert downstream of the T7 promoter) using the transfection reagent DOTAP. Immunoprecipitation was done as described (11) with the following modifications. Cells were plated on 12-well plates (2.5-cm diameter) and transfected with the cDNA encoding wild-type or mutant transporters (11). 16–20 h post-transfection, cells were incubated for 1–2 h in methionine-free Dulbecco’s medium with or without the glycosylation inhibitor tunicamycin (10 μg/ml). The subsequent [35S]methionine labeling (1–2 h) was also carried out in the presence and absence of tunicamycin, respectively. The cells were washed three times with 2 ml of ice-cold phosphate-buffered saline and lysed by the addition of 200 μl of a solution containing 150 mM NaCl, 5 mM EDTA, 10 mM sodium P2, pH 7.4, and 0.25 mM phenylmethylsulfonyl fluoride, supplemented with 1% SDS. After the mixture was gently agitated with the microfuge tip, 1 ml of the same solution, now supplemented with 1% Triton X-100, was added. All subsequent steps were done at 4 °C. After a 15-min centrifugation in a microcentrifuge, the DNA was removed, and each of the supernatants was precleared by end-over-end shaking with 10 mg of protein A-Sepharose CL-4B to which preimmune serum had been bound previously. After a 5-min centrifugation, the supernatants were incubated overnight with 10 mg of the beads, this time prebound to 10 μl of P2000 antiseraum. After extensive washing and elution (11), samples were analyzed by SDS-polyacrylamide gel electrophoresis (10% gel, 2.6% C). Each lane depicted in the figures represents the processed material from a 2.5-cm well. To obtain maximal resolution, electrophoresis was continued for 3–1.5 h after the dye front ran out. The glycosylation state of each of the mutants shown was verified in at least three independent experiments.

Reconstitution of the transporters into liposomes was done exactly as described (17). Briefly, the cells were concentrated and mixed with cholate and liposomes, and after a 10-min incubation on ice, the proteoliposomes were formed by centrifugation of the mixtures on spin columns. Transport in intact cells (11) and in proteoliposomes (17) was measured as described.

RESULTS

N-Linked Glycosylation in GAT-1—The theoretical model of GAT-1 with its putative hydrophilic domains is shown in Fig. 1. The transmembrane domains are connected by six extracellular loops (EL1–6) and five intracellular loops (IL1–5). Amino- and carboxyl-terminal tails (Fig. 1, NT, and CT, respectively) are both located in the cytoplasm. The three glycosylation consensus sequences located in the large EL2 are indicated, but an additional one, located in transmembrane domain 9 (unlikely to be used), is not. Using site-directed mutagenesis, we have eliminated these sites consecutively by converting the asparagines of each of the NX(S/T) sequences into aspartate (Fig. 2A). After heterologous expression in HeLa cells and in vivo labeling with [35S]methionine, the synthesized transporters have been immunoprecipitated with an antibody directed against an epitope located in the carboxyl-terminal tail (18). The wild-type transporter is detected in three forms: a monomer running as a band of ~70 kDa, a more abundant dimer, and a high molecular mass aggregate (Fig. 2B). The specificity of the antibody is illustrated by the fact that no bands are detected when the cells are transfected with the vector alone (Fig. 2B, SK). Upon removal of one (NDN), two (NDD and DDN), and three (DDD) of the consensus sites, a stepwise increase in the mobility of the monomer forms of the mutant transporters is observed (Fig. 2B). In some extent, this can also be seen for the dimer forms, but the effect is less pronounced due to the lower resolution of the gel at higher molecular masses (Fig. 2B). When cells expressing the transporters are incubated with the N-linked glycosylation inhibitor tunicamycin (Fig. 2B, + lanes), an increased mobility is detected for the wild type, NDN, NDD, and DDN, which is identical to that for DDD (Fig. 2B). The mobility of DDD is not affected by preincubation with tunicamycin. This indicates that all three N-glycosylation consensus sites are used in vivo and that there are no additional ones. The removal of one or two glycosylation sites has little effect on the expression of transport activity (Fig. 2C). When all three sites are removed, there is a reduction of transport activity. This seems to be due at least in part to
(indicate the positions of 94- and 67-kDa standards on the gel.)

sodium-dependent [3H]GABA uptake in HeLa cells infected with recombinant vaccinia/T7 virus and transfected with pBluescript containing the wild type, the indicated mutants lacking consensus glycosylation sites (see Fig. 2A), or the vector without insert (SK). The cells were preincubated with (+) or without (−) tunicamycin, labeled with [35S]methionine, lysed, and immunoprecipitated with anti-PCOOH antibody as described under “Experimental Procedures.” The arrowheads indicate the positions of 94- and 67-kDa standards on the gel. C. sodium-dependent [3H]GABA uptake in HeLa cells infected with recombinant vaccinia/T7 virus and transfected with the above cDNA constructs. Results are given as percent of transport of the mutants relative to that of the wild type (NNN). Each bar is the mean ± S.E. of three to five different experiments.

**FIG. 2. Analysis of GAT-1 transporters from which the N-glycosylation consensus sites in EL2 have been removed sequentially.** A, nucleotide and amino acid sequences of the mutations. The number on the right-hand side of the protein sequence indicates the position of the last amino acid shown. WT, wild type. Italics indicate the mutations. B, immunoprecipitation of the wild-type (NNN) and mutant transporters synthesized in HeLa cells. HeLa cells were infected with recombinant vaccinia/T7 virus and transfected with pBluescript containing the wild type, the indicated mutants lacking consensus N-glycosylation sites (see A), or the vector without insert (SK). The cells were preincubated with (+) or without (−) tunicamycin, labeled with [35S]methionine, lysed, and immunoprecipitated with anti-PCOOH antibody as described under “Experimental Procedures.” The arrowheads indicate the positions of 94- and 67-kDa standards on the gel. C. sodium-dependent [3H]GABA uptake in HeLa cells infected with recombinant vaccinia/T7 virus and transfected with the above cDNA constructs. Results are given as percent of transport of the mutants relative to that of the wild type (NNN). Each bar is the mean ± S.E. of three to five different experiments.

**FIG. 3.** Documents results on transporters with glycosylation sites inserted at different positions in EL3. Replacement of the two amino acids phenylalanine 276 and arginine 277 with isoleucine and threonine creates, together with asparagine 275, a glycosylation site (Fig. 3A, 275). In the absence of tunicamycin, this transporter has a lower mobility than in its presence (Fig. 3B). This suggests that the created site is in fact glycosylated. The reproducibility of the method is demonstrated in Fig. 3B. It shows the results of separate immunoprecipitations of this transporter from HeLa cells transfected in parallel with the 275 construct. The transport activity of this construct is similar to that of the DDD transporter (Fig. 3D). It should be noted that the activity in this and in all subsequent figures is expressed as percent of wild-type (NNN) activity rather than of DDD activity. The latter, of course, will result in a much larger percentage of activity. For comparison, the activity of the DDD construct is also included in all figures. The functionality of the 275 transporter indicates that the mutation does not result in a transporter with an altered topology. The above data prove that EL3 is in fact an extracellular loop, in harmony with the theoretical model. When a double glycosylation site is created by insertion of four amino acid residues (NSSR) after asparagine 275 (Fig. 3C), no glycosylation is detected (Fig. 3C) even though transport activity is not compromised (Fig. 3D). Insertion of a consensus site at position 265 (Fig. 3A) also yields a glycosylated transporter (Fig. 3C). In this case, the glycosylated transporter is inactive, both in intact cells (Fig. 3D) and in reconstituted proteoliposomes (data not shown). No glycosylation is observed when a site is inserted at position 281 of EL3 (Fig. 3A, C), and this transporter is inactive (Fig. 3D). Similar results are seen when sites are inserted at positions 276 and 283 (data not shown). This indicates that some of the domains in EL3 are important for the expression of transport activity.

The results shown in Fig. 4 indicate that EL6 is also an extracellular loop. Thus, consensus sites generated by insertion of two (NA) or five (NNNSSR) amino acid residues at position 525 (Fig. 4A) are glycosylated in vivo (Fig. 4B), and these glycosylated transporters are functional (Fig. 4C).

**FIG. 4.** Indicates that EL6 is also an extracellular loop. Thus, consensus sites generated by insertion of two (NA) or five (NNNSSR) amino acid residues at position 525 (Fig. 4A) are glycosylated in vivo (Fig. 4B), and these glycosylated transporters are functional (Fig. 4C).
many positions in IL1, none of the mutants was active (Table I).

Probing of the topology of the remaining putative hydrophilic domains is documented in Table II. The insertion of an N-linked glycosylation site into EL1 at position 78 does not compromise the transport activity, but the site is not glycosylated. Creation of sites in EL4 and EL5 does not compromise activity in many cases, but glycosylation could not be detected (Table II). In all cases, creation of N-linked glycosylation sites at IL2, IL4, and IL5 yields nonfunctional, nonglycosylated transporters. Creation of a site at position 316 in IL3 yields a functional, albeit nonglycosylated, transporter.

Reactivity of Cysteine 74 toward Permeant and Nonpermeant Methylthiosulfonate Reagents—Because of the indication of an external location for IL1, it was of special interest to determine on which side of the membrane EL1 is located. The lack of glycosylation in this loop may be due to accessibility problems of the N-oligosaccharyltransferase. Therefore, we have used small, very hydrophilic methylthiosulfonate derivatives, which react covalently with free cysteines (19–24) and are expected to be very accessible to short and possibly buried loops.

In preliminary experiments, we have observed that MTSEA inhibits GABA transport in HeLa cells expressing GAT-1. This inhibition can be reversed by dithiothreitol (data not shown). According to the theoretical model, three cysteine residues are exposed to the outside. These are cysteine 74, located in EL1, and cysteines 164 and 173, both located in EL2. In the dopamine transporter, the two cysteines at comparable positions to Cys164 and Cys173 are important for targeting the transporter to the plasma membrane (25). They probably form a disulfide bond, as has recently been demonstrated in the related serotonin transporter. Thus, according to the theoretical model, cysteine 74 is the only free cysteine exposed to the outside and is a prime candidate for modification by MTSEA. Evidence consistent with this idea is presented in Fig. 6A.

2 G. Rudnick, personal communication.
transporter becomes more resistant to inhibition by MTSEA. It is of interest to note that C74S is still sensitive to the sulfhydryl reagent, suggesting that other cysteines located on the transporter may also participate in the inhibition. However, the remaining 13 cysteine residues in GAT-1 are predicted to be in the membrane or to face the interior of the cell. A possible explanation for this is that MTSEA can permeate the membrane. While this work was in progress, several groups have obtained experimental evidence for the membrane permeability of MTSEA (26, 39). In the same studies, it was found that MTSET is impermeant. This sulfhydryl reagent does not inhibit GABA transport either in the wild-type transporter or in the C74S transporter (Fig. 6A), even though it is more reactive toward sulfhydryl groups than MTSEA (23). Reactivity of our MTSET reagent was ascertained by checking its ability to react with reduced 5,5′-dithiobis(2-nitrobenzoic acid) (Fig. 6B). Furthermore, when the permeability barrier is taken away by solubilization, MTSET is able to inactivate the transporter as monitored after reconstitution (Fig. 6C). Thus, cysteine 74 appears to be located intracellularly.

**DISCUSSION**

The data presented in this paper show unambiguously that the three hydrophilic stretches EL2, EL3, and EL6 are extracellular. The extracellular location of EL2 was anticipated since it is the only loop throughout the superfamily of (Na+ +
Cl\(^-\)-coupled transporters (excluding the so-called orphan transporters) containing N-glycosylation consensus sites. This is also in harmony with similar data obtained with the transporters for serotonin (27) and norepinephrine (28) as well as the glycine transporter GLYT-1 (29). No experimental evidence on the location of EL3 and EL6 in any transporter of the superfamily has been presented in the literature to date.

Earlier studies using this approach on other transporting proteins had indicated that insertion of glycosylation sites into hydrophilic linker loops is very well tolerated (12, 13). However, our results (Figs. 2–5 and Tables I and II) indicate that the approach is not straightforward, as is also seen in the case of the Na\(^+\)-coupled glucose transporter (14). The ability to undergo glycosylation is critically dependent on the position of

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**TABLE I**

*Mutations in intracellular loop 1 (Glu\(^{101}\)–Lys\(^{223}\))*

Sodium-dependent \(^3\)H[GABA uptake was measured in HeLa cells expressing the wild-type transporter (NNN) and the indicated mutants. Activity of the mutants is given as the percentage of wild-type activity. Values are the mean ± S.E. of three independent experiments. Each independent experiment was performed in triplicate.

| Location | Mutation | Activity |
|----------|----------|----------|
| 106      | 106QYT108→106NYT108 | 0        |
| 109      | 109NSG111→109NGS111 | 0        |
| 110      | 110GGG112→110NGS112 | 3.5 ± 0.2 |
| 112      | 112GLG114→112NIT114 | 0        |
| 114      | 114GVW116→114NGS116 | 0        |
| 116      | Insertion of NNSS | 0        |
| 117      | 117KAP120→117NGS120 | 0        |

\(^a\) WT, wild type.

**TABLE II**

*Transport activity of additional nonglycosylated mutants*

Activity of the mutants listed below is given as the percentage of wild-type activity. Values are the mean ± S.E. of three to four independent experiments, each performed in triplicate, except for mutant 316 (n = 2).

| Location | Mutation | Activity |
|----------|----------|----------|
| EL1 (Cys\(^{74}\)–Gly\(^{79}\)) | Insertion of NNSS | 21.5 ± 6.4 |
| 78       | Insertion of NNSS | 6.3 ± 1.2 |
| EL4 (Val\(^{342}\)–Thr\(^{373}\)) | Insertion of NNSS | 8.7 ± 0.8 |
| 350      | Insertion of NNSS | 25.0 ± 3.0 |
| 351      | Insertion of NNSS | 6.0 ± 1.7 |
| 357      | Insertion of NNSS | 24.5 ± 3.5 |
| 365      | Insertion of NNSS | 25.5 ± 5.2 |
| EL5 (Gly\(^{443}\)–Ser\(^{456}\)) | Insertion of NNSS | 0        |
| 448      | Insertion of NNSS | 0        |
| 449      | Insertion of NNSS | 22.7 ± 6.1 |
| 450      | Insertion of NNSS | 21.3 ± 7.8 |
| 455      | Insertion of NNSS | 0        |
| IL2 (Trp\(^{235}\)–Lys\(^{238}\)) | Insertion of NNSS | 0        |
| 235      | Insertion of NNSS | 27.5 ± 12.0 |
| IL3 (His\(^{313}\)–Ser\(^{320}\)) | Insertion of NNSS | 0        |
| 316      | Insertion of NNSS | 0        |
| IL4 (Asp\(^{395}\)–Glu\(^{421}\)) | Insertion of NNSS | 0        |
| 400      | Insertion of NNSS | 0        |
| 411      | Insertion of NNSS | 0        |
| 415      | Insertion of NNSS | 0.8 ± 0.4 |
| IL5 (Asn\(^{478}\)–Cys\(^{493}\)) | Insertion of NNSS | 0        |
| 483      | Insertion of NNSS | 0        |
| 485      | Insertion of NNSS | 0        |
| 487      | Insertion of NNSS | 0        |

\(^a\) WT, wild type.

**FIG. 6.** Effects of MTSEA and MTSET on wild-type and C74S transporters. A, HeLa cells expressing wild-type (WT) GAT-1 (filled symbols) and C74S-GAT-1 (open symbols) were incubated for 5 min in a solution containing 150 mM NaCl, 0.5 mM MgSO\(_4\), 0.3 mM CaCl\(_2\), and 5 mM potassium P\(_2\), pH 7.4, supplemented with 0, 0.8, and 1.6 mM concentrations of either MTSEA (circles) or MTSET (squares). Sodium-dependent \(^3\)H[GABA transport was assayed as described under “Experimental Procedures.” Data are the means ± S.E. of triplicate samples from a representative experiment. B, several incubations were set up

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**Topology of a GABA Transporter**

Cl\(^-\)-coupled transporters (excluding the so-called orphan transporters) containing N-glycosylation consensus sites. This is also in harmony with similar data obtained with the transporters for serotonin (27) and norepinephrine (28) as well as the glycine transporter GLYT-1 (29). No experimental evidence on the location of EL3 and EL6 in any transporter of the superfamily has been presented in the literature to date. Earlier studies using this approach on other transporting proteins had indicated that insertion of glycosylation sites into hydrophilic linker loops is very well tolerated (12, 13). However, our results (Figs. 2–5 and Tables I and II) indicate that the approach is not straightforward, as is also seen in the case of the Na\(^+\)-coupled glucose transporter (14). The ability to undergo glycosylation is critically dependent on the position of
the site in the loop and on the nature of the insert (Fig. 3). In some cases, transport is abolished, but glycosylation is not (Figs. 3 and 5). In other cases, the opposite is true (Table II). The accessibility of an external loop to the glycosylating enzymes may be limited because it may participate in the formation of the transporter's pore. This would also explain loss of function upon insertion and/or replacement of amino acids. Also, from the importance of hydrophilic loops for transport and substrate specificity (30, 31), it can be predicted that any perturbation may result in impaired transport. It is therefore not surprising that we were unable to obtain active and glycosylated mutants in EL4 and EL5. Glycosylation in these two loops has been observed in the accompanying paper on the topology of GLYT-1, although this resulted in impaired function (32). Good supporting evidence for an external location of EL4 has been obtained in the case of the human norepinephrine transporter (28).

With the method of N-glycosylation scanning mutagenesis, internal loops score negative. Because of the problem of steric hindrance of glycosylation, negative results may also be observed for external loops. Thus, there is an inherent problem in proving the location of internal loops by this method. This is perhaps less critical in the amino- and carboxyl-terminal tails as they are much more hydrophilic than the loops. They do not participate in the transport process (15, 33) and therefore probably do not intercalate between the transmembrane domains. Were they located on the outside, they would probably be accessible to N-oligosaccharyltransferase. The lack of glycosylation observed in both tails (Fig. 5) is consistent with their predicted internal localization. Independent evidence supporting this is available for transporters of glycine, GLYT-1, and GLYT-2 (34) and of dopamine (35). We conclude that the theoretical model is by and large correct between EL2 and the carboxyl-terminal tail.

The glycosylation in IL1 at position 110 results in a nonfunctional transporter. We cannot rule out the explanation that the insertion has scrambled the topology. However, in the case of the sodium-coupled glucose transporter, this problem has been observed with large inserts (48 amino acids), but not with small ones like we have used here. The most straightforward explanation is that this glycosylation is due to the external location of IL1. The loss of transport activity probably means that this loop is critical for GABA transport. In fact, all the modifications in IL1 (even those without a change in the number of amino acids) impair activity (Table I). It should also be noted that glutamate 101, located in this loop, has been found to be essential for transport activity (36). As shown in the accompanying paper (32), glycosylation of IL1 has also been observed in the related transporter GLYT-1.

An external location of IL1 and an internal location of the amino-terminal tail implies that one of the first two transmembrane domains does actually not span the membrane. Inspection of the hydropathy plots throughout the superfamily indicates that the first domain does not score very well as a transmembrane a-helix. In fact, our results with permeant and impermeant methylthiosulfonate reagents (Fig. 6) suggest that cysteine 74 in EL1 is located intracellularly. The lack of glycosylation of this loop (Table II) is consistent with this. These findings can be accommodated in the topological model shown in Fig. 7. The former transmembrane a-helix 1 (Fig. 1) does not cross the membrane. Since it is highly conserved, has considerable hydrophobicity, and contains the critical arginine 69
(37), we have depicted it as a “pore loop” associated with the membrane. Such pore loops are involved in ion permeation through voltage- or ligand-dependent ion channels (see Ref. 38 for a review). Evidence for a membrane association of domain 1 is presented in the accompanying paper (32). However, there are other possibilities. For instance, the domain could be cytoplasmic and might serve as an intracellular plug on the transporter’s pore. Transmembrane α-helix 2 in the theoretical model (Fig. 1) is now the first true transmembrane domain. EL2 has been shortened at the amino-terminal side to make a place for an additional transmembrane domain (domain 3*).

This is required so that the natural glycosylation sites still face outside. Theoretically, this is feasible, as around the former transmembrane helix 3, there is a hydrophobic stretch of 44 amino acids, from residues 118 to 161 (legend to Fig. 7) (6). This could easily accommodate two transmembrane domains. Moreover, experimental evidence for such an additional transmembrane domain has been obtained in the case of GLYT-1, as reported in the accompanying paper (32). It should be emphasized that although the model is consistent with the experimental data obtained, it will be important to carry out studies with independent methods to test if all of its aspects are accurate.

We anticipate that the modified topological model presented here will be relevant not only for GAT-1 and GLYT-1, but for all members of the superfamily. The results reported here are a first step toward further experiments designed to clarify the structural basis of the transport mechanism.

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