Mutation of Arginine 44 of GAT-1, a (Na\(^+\) + Cl\(^-\))-coupled γ-Aminobutyric Acid Transporter from Rat Brain, Impairs Net Flux but Not Exchange*

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The γ-aminobutyric acid (GABA) transporter GAT-1 is a prototype of a large family of neurotransmitter transporters that includes those of dopamine and serotonin. GAT-1 maintains low synaptic concentrations of neurotransmitter by coupling GABA uptake to the fluxes of sodium and chloride. Here we identify a stretch of four amino acid residues predicted to lie in the juxtamembrane region prior to transmembrane domain 1 in the cytoplasmic amino-terminal tail of GAT-1, which is critical for its function. Two residues, arginine 44 and tryptophan 47, are fully conserved within the transporter family, and their deletion abolishes GABA transport in the HeLa cell expression system used. Tryptophan 47 can be replaced only by aromatic residues without loss of activity. Arginine 44 is essential for activity. Only when it is replaced by lysine, low activity levels (around 15% of those of the wild type) are observed. Using a reconstitution assay, we show that mutants in which this residue is replaced by lysine or histidine exhibit sodium- and chloride-dependent GABA exchange similar to the wild type. This indicates that these mutants are selectively impaired in the reorientation of the unloaded transporter, a step in the translocation cycle by which net flux and exchange differ. The high degree of conservation in the consensus sequence RXXXW suggests that this region may influence the reorientation step in related transporters as well.

The rat brain GABA\(^1\) transporter GAT-1 is a prototype of a large family of sodium- and chloride-dependent neurotransmitter transporters. This family includes transporters for biogenic amines, amino acids, and four different GABA transporters (for a review see Ref. 1). The transporters maintain low synaptic levels of their neurotransmitter substrates, which is necessary for effective synaptic transmission. One of the most direct pieces of evidence for this comes from studies using dopamine transporter knock-out mice; the decay of extracellular dopamine in brain slices of such mice is about 100 times longer than normal (2).

GAT-1, the first member of the family to be identified, was purified to homogeneity in a form active upon reconstitution (3) and cloned (4). The transporter catalyzes electrogenic sodium/chloride/GABA cotransport with a stoichiometry of 2:1:1 (5–7). It has been shown that the transporter undergoes extensive substrate-dependent conformational changes (8, 9). GAT-1, as well as the other members of the family, is predicted to have twelve transmembrane domains linked by hydrophilic loops, with the amino and carboxyl termini residing inside the cell (4). Many aspects of this model were verified experimentally, although the topology of the amino-terminal third of the transporter was controversial (10–13). Recent evidence suggests that the theoretical topology model (4) is correct (14).

Using site-directed mutagenesis of amino acids located in the transmembrane domains, we have identified a residue that may be involved in GABA binding (15). The hydrophilic loops connecting the transmembrane domains are also important for function. Shortening (16) or lengthening (10) the loops or changing single amino acids in them (17, 18) has been shown to affect transport activity and substrate specificity. GAT-1 transporters cleaved at the amino and carboxyl termini by proteases were fully functional and retained sodium and chloride dependence as well as electrogenicity (19). To define the exact length of the domains that are essential for transport activity, mutants truncated at both termini were constructed. Substantial activity was retained when the carboxyl tail was almost completely removed, but when part of transmembrane domain 12 was removed along with the tail, transport activity was completely abolished. In the amino-terminal, a construct in which amino acids 3–41 (∆3–42) were deleted was fully active. When the deletion was extended to include the amino acids up to residue 49 (∆3–49) the truncated transporter was completely inactive (20).

In this study we have determined which of the residues in the amino-terminal tail, between position 42 and the first transmembrane domain, are essential for GAT-1 function. We have made deletion mutants at each of the positions and substitution mutants where the deletions were inactive. We find that arginine 44 and tryptophan 47, which are fully conserved throughout the family of sodium- and chloride-dependent neurotransmitter transporters, are critical for transport activity. Many of the mutants that were severely impaired in net flux of GABA show significant activity in a partial reaction of the transport cycle, namely, the exchange of radiolabeled for unlabeled GABA.

**EXPERIMENTAL PROCEDURES**

Materials—[\(^{3}H\)]GABA (40 Ci/mmol) was obtained from PerkinElmer Life Sciences. NHS-SS-Biotin and streptavidin beads were obtained from Pierce (catalogue numbers 21331 and 20349, respectively).

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1 The abbreviations used are: GABA, γ-aminobutyric acid; wt, wild type; NHS-SS-Biotin, sulfo[35S]uccinimidyl-2-(biotinamide)ethyl-1,3-dithiopropionate.

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Prestained molecular mass markers (broad range) were from New England Biolabs, Inc. Restriction enzymes were from New England Biolabs, Inc. and Roche Molecular Biochemicals. T<sub>4</sub> polynucleotide kinase, T<sub>4</sub> DNA polymerase, T<sub>4</sub> DNA ligase, and the transfection reagent DOTAP were also from Roche Molecular Biochemicals. Kits for plasmid DNA preparation were from Roche Molecular Biochemicals and Qiagen. ECL kits were obtained from Amersham Pharmacia Biotech. Tissue culture media, sera, and antibiotics were from Biological Industries (Kibbutz Beit HaEmek, Israel). The peptide PCOOH, located in the carboxyl terminus of GAT-1, and the antibody against it, were described previously (8). Affi-Gel 15 for affinity purification of primary antibody from whole serum (19) was obtained from Bio-Rad. The affinity purified antibody was stored at 2°20 °C in the presence of bovine serum albumin (10 mg/ml). Antibody against calnexin was obtained from StressGen (catalogue number SPA-860). Secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxide) was obtained from Jackson Immunoresearch Laboratories, Inc. (catalogue number 111-035-144). All other reagents were obtained from Sigma.

Mutagenesis—All mutations were made by site-directed mutagenesis of the wild type GAT-1 according to the Kunkel method as described (21, 22). The mutations were planned to include diagnostic restriction sites and were identified by restriction analysis. All of the mutations at position 44 and all other inactive mutations were subcloned back into the wild type using pairs of restriction enzymes that cut the cDNA at both sides of the mutation. The constructs were sequenced in both directions between these two restriction sites. For all other mutants at least two independent Escherichia coli colonies carrying the mutant plasmids were characterized by transport activity. Transport—Heterologous expression of the wild type and mutant transporters was done as follows: HeLa cells plated on 24-well plates were infected with recombinant vaccinia/T7 virus vTF (23) and transfected with cDNA (pBluescript SK with the wild type or mutant transporter inserted downstream to the T7 promoter) using the transfection reagent DOTAP as described (24). Uptake of [3H]GABA into whole cells was assayed 18–20 h post transfection. The wells were washed twice.

**Fig. 1.** Transport activity of wild type and deletion mutants. A, sodium dependent uptake of [3H]GABA into HeLa cells expressing the wild type and deletion mutants. Uptake was performed as described under “Experimental Procedures.” Results are given as the percentages of transport of each mutant relative to the wild type. Each bar represents the means ± S.E. of at least three experiments. B, sodium-dependent uptake of [3H]GABA by wild type and mutants reconstituted into liposomes. Uptake was performed as described under “Experimental Procedures.” Results were normalized to protein levels and are given as the percentages of transport of each mutant relative to the wild type. Each bar represents the mean ± S.E. of three experiments.

**Fig. 2.** Cell surface biotinylation of wild type and deletion mutants at positions 44–47. HeLa cells expressing the wild type and indicated mutants were labeled and processed as described under “Experimental Procedures.” The first seven lanes show the biotinylated samples, and the subsequent lanes show the total samples. The first lanes of each group show biotinylated and total samples from HeLa cells expressing the vector alone. All samples were separated on the same SDS gel, transferred to nitrocellulose, and detected, as described under “Experimental Procedures.” Each biotinylated lane represents the lysate from one confluent well in a 12-well plate, and each total lane represents 15% of the lysate from one confluent well in a 24-well plate. The positions of the molecular mass standards are indicated at the left.
with a solution containing 150 mM choline chloride, 5 mM KPi, pH 7.4, 0.5 mM MgSO₄, and 0.3 mM CaCl₂. Each well was then incubated with 0.4 μCi of [³H]GABA (40 Ci/mmol) in a NaCl transport solution (150 mM NaCl with KPi, MgSO₄, and CaCl₂ as above). Transport reactions were carried out for 10 min at room temperature, and the assay was terminated by washing the wells twice with ice-cold NaCl transport solution. Cells were lysed with 1% SDS, and radioactivity was measured by liquid scintillation counting.

**Reconstitution**—Reconstitution of the transporters into liposomes was done as described (25). For net flux the liposomes contained 120 mM KPi, pH 7.4, 5 mM Tris-SO₄, 0.5 mM EDTA, 1 mM MgSO₄, and 1% glycerol. The external medium consisted of 150 mM NaCl supplemented with 2.8 mM valinomycin and 1 μCi of [³H]GABA (40 Ci/mmol). For exchange the internal medium of the liposomes contained 10 mM GABA, 120 mM NaCl, 20 mM NaPi, pH 7.4, and Tris-SO₄, EDTA, MgSO₄, and glycerol as above, unless indicated otherwise in the figure legends. The liposomes were passed over another spin column of identical composition but lacking the GABA. The control liposomes (without entrapped GABA) were also passed over a second spin column. The external medium contained 150 mM NaCl and 2 μCi of [³H]GABA unless stated otherwise in the figure legends. Exchange reactions were carried out for 2 min, which corresponds to the end of the linear phase of the uptake. Total protein concentration in the liposomes was determined by the Lowry method (26).

**Cell Surface Biotinylation**—Labeling of wild type and mutant transporters at the cell surface, using NHS-SS-Biotin, was done as described by Chen et al. (27) with the following changes. First, HeLa cells were plated in 12-well plates, and all volumes were increased accordingly, except the elution of the biotinylated proteins from the streptavidin beads which was kept at 100 μl. Second, for samples of total cell transporter, cells plated in 24-well plates and transfected as described above were lysed on ice for 30 min in 200 μl of 1× SDS-polyacrylamide gel electrophoresis sample buffer/well. The lysates were collected to Eppendorf test tubes, heated to 85 °C for 10 min, sonicated for 10 s, centrifuged, and frozen at −70 °C until loading together with the biotinylated samples. Samples were separated by SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to nitrocellulose. The GAT-1 protein was detected with affinity purified antibody to GAT-1 at 1:1,000 dilution, horseradish peroxidase-conjugated secondary antibody at 1:40,000 dilution, and ECL. 1% of goat serum was present in all antibody, blocking, and washing solutions to minimize the appearance of nonspecific bands. Scanning and quantification of bands on the developed films was done using a Fluor-S™ Multimager scanner with MultiAnalyist software, both from Bio-Rad.

**RESULTS**

**The Effect of Single-residue Deletions on Transport**—To understand the role that the stretch between proline 42 and the first transmembrane domain (PDRDTWKGRFDF) plays in GABA transport, we first measured the separate contribution of each of these amino acids to transport activity. We made single deletion mutations of the amino acid residues by site-directed mutagenesis of the wild type (wt) GAT-1. The mutants were expressed in HeLa cells using the vaccinia/T7 recombinant virus and uptake of [³H]GABA was measured. Constructs with deletions of arginine 44, threonine 46, and tryptophan 47 (ΔR44, ΔT46, and ΔW47) are almost totally inactive, with less than 1% of residual activity (Fig. 1). Constructs ΔP42, ΔD43, ΔK48, ΔR50, and ΔF51 are active and retain levels of activity that range from 50 to 85% of the wt activity. ΔD45 has 16% and ΔG49 has 20% of the wt activity. The first transmembrane domain is predicted to start with phenylalanine 53 (4). Deletions of residues that are, according to the model, close to or within the lipid bilayer, results basically in loss of activity; ΔD52 shows 5% of wt activity, and all deletions beyond it that we
The inactivity of the deletion mutants at positions 44, 46, and 47 is due to a defect in synthesis; when immunoprecipitation was performed, the expression levels of the mutants did not differ from the wt (data not shown, but see the right half of Fig. 2 for their steady state levels). We examined the possibility of a defect in trafficking of the mutants to the cell membrane by solubilization of HeLa cells expressing the mutants and their reconstitution into liposomes. Measuring [3H]GABA uptake into the proteoliposomes can detect activity of transporters that were trapped in intracellular membranes. Fig. 1B shows the level of radioactive GABA uptake into proteoliposomes obtained from the wt and mutants between positions 44 and 49. As can be seen, no significant elevation of activity is detected in ΔR44, ΔT46, ΔW47, ΔK48, and ΔG49. Only ΔD45 shows a significant increase in transport activity (Fig. 1). These results indicate that there is probably no defect in the targeting of ΔR44, ΔT46, and ΔW47 to the plasma membrane. To visualize the wt and mutant GABA transporters at the cell membrane directly, we labeled cell surface proteins using NHS-SS-Biotin. This nonpermeant biotinylation reagent modifies lysines located in extracellular domains of membrane proteins (14, 28). The biotinylated proteins are recovered by incubation with streptavidin-agarose beads and separated by SDS-polyacrylamide gel electrophoresis, and GAT-1 is detected using a specific antibody. For every construct tested we ran both a biotinylated sample and a total cell lysate sample that was not subjected to biotin labeling or streptavidin purification. The results are shown in Fig. 2. In the total sample of the wild type transporter, the main form is a doublet band of ~50 kDa that represents the glycosylated form of the transporter (10). The adjacent lane shows the total sample of a mutant in which all three N-linked glycosylation consensus sequences have been removed (mutant DDD; Ref. 10). Here the doublet band is observed but its mobility is increased. The reason for the appearance of a doublet band in both the wt type and the aglyco-transporter is not clear; one possible explanation is that the lower band represents a proteolytic product. In the biotinylated wild type sample an additional form is observed, which runs as a wide band of ~60 kDa. This is a form of the transporter found preferentially at the cell membrane and apparently represents the mature form of GAT-1. In addition, in both the total and the biotinylated samples, small amounts of a dimer form and sometimes also a high molecular weight aggregate form are observed. These latter forms are also seen in immunoprecipitation experiments (10). The specificity of the antibody is seen in lanes labeled SK; when the cells are transfected with the vector alone, no bands are detected in the total or the biotinylated samples. Moreover, when the antibody was preincubated with the peptide against which it was raised, these bands were not observed (data not shown). The specificity of the biotinylation reaction was seen when cells expressing the wt transporters underwent all the labeling steps without NHS-SS-Biotin present. No transporter was observed in the fraction eluted from the streptavidin beads, and this indicates that there is no nonspecific binding of GAT-1 to the beads and that all the transporter detected in the biotinylated fraction is indeed labeled with biotin (data not shown). To make sure that in the vaccinia-based expression system used here, the biotinylation reagent modifies only proteins at the plasma membrane, we have stripped some of the blots and reprobed them with an antibody against calnexin, an endoplasmic reticulum transmembrane protein. As expected, calnexin was present in the total samples but depleted in the biotinylated fractions (data not shown). The total and biotinylated fractions of the deletion mutants ΔR44, ΔD45, ΔT46, and ΔW47 are also shown in Fig. 2. The mutants do not differ from the wt in the levels of total cell transporter or in the mobility of the bands, and the same is true for the biotinylated samples. In addition to the mature form, the glycosylated form also reaches the plasma membrane, and the proportion of both bands is similar in all the constructs shown. In contrast to what is seen in the wild type and deletion mutants, the composition of the biotinylated and total samples of the aglyco-mutant is similar. Thus, although glycosylation appears to be a prerequisite for the formation of the mature form, it is not required for targeting to the membrane, at least in the expression system used here. To quantify the membrane targeting of mutants relative to the wt, we scanned between two and four developed films for each mutant, from different experiments (see “Experimental Procedures”). The ratio between the intensities of the biotinylated and the total sample of each construct was calculated, and this value was compared between the wt and the mutants. Alternatively, the intensities of the biotinylated fractions alone were compared. The intensities of the biotinylated form of the mutants relative to that of the wild type varied between experiments. For example, the biotinylated form of JR44 ranges from 50 to 140% of the wt. Other mutants range from 100 to 150% of the wild type. These values are similar for both methods of comparison. We conclude that although the mutants are not targeted to the membrane at exactly the same level as the wt, they are not expressed at levels consistently lower than the wt. Therefore, the inactivity of the mutants is not due to a targeting defect but to an intrinsic change in the transporter.
Properties of Substitution Mutants—To understand why the deletion of amino acids at positions 44–47 leads to such a pronounced drop in activity, we made a number of substitution mutations at each of these positions. Transport of $[^{3}H]$GABA into whole cells expressing these mutants is shown in Fig. 3A. As can be seen, replacement of arginine by serine or histidine at position 44 restores no transport activity. Partial activity (around 15% of the wt) is restored only when lysine substitutes for the arginine (R44K). At position 45 the negatively charged aspartic acid can be replaced by a polar residue (serine) or by a hydrophobic residue (leucine), yielding 44 and 56% of the wt activity, respectively. At position 46, within the transporter family there are either polar or positive residues. When replaced by a polar (T46N or T46S) or even hydrophobic (T46I) residue, over 85% of wt activity is seen. At position 47, replacement of tryptophan by the uncharged serine or the hydrophobic valine does not restore activity. Only in the case of W47F and W47Y, where the substitutions have an aromatic ring, activity of over 100% of wt levels is observed. The inactive substitution mutants R44S, R44H, W47S, and W47V and the partially active R44K were further examined as described above for the deletion mutants. First, these mutants were reconstituted into liposomes, and uptake of $[^{3}H]$GABA was measured. Fig. 3B shows the uptake activity for each of the mutants. R44S is inactive, whereas both R44H and R44K show a distinct gain in activity. R44H is elevated from 3.3 ± 1.2% (n = 6) to 15.4 ± 6.9% (n = 3), and R44K from 14.6 ± 3.4% (n = 11) to 34.2 ± 1.6% (n = 4) of the wt activity. In contrast, no gain in activity is seen in W47S and W47V. Transporter synthesis and targeting to the cell membrane was determined for these mutants using NHS-SS-Biotin labeling, exactly as described above for the deletion mutants. There was no difference between the wt and the mutants in the amount of total cell transporter synthesized or in the mobility of this form (data not shown). The biotinylated, mature plasma membrane form is seen in all of the mutants, and the bands were quantified as described above. Variability between experiments is seen here too, and the biotinylated form of R44S, for example, ranges from 65 to over 200% of the wt. The other mutants also show a range of expression that is close to or above wt expression. Thus, the inactivity (or partial activity, in the case of R44K) of these mutants is not due to faulty targeting to the plasma membrane but to an intrinsic change in transporter function that is caused by the mutation itself.

Functional Studies on the Active Substitution Mutants—To determine how the mutations affect transporter function, we examined the affinity of the active replacement mutants to each of the substrates of GAT-1. The sodium affinity for the mutants and the wt was determined by measuring uptake of radioactive GABA into HeLa cells with increasing concentrations of sodium. Fig. 4 shows the profiles obtained for the wt, D45L, and R44K. As can be seen, D45L gives rise to a curve that is identical in shape to that of the wt. All the other mutants, with the exception of R44K, also exhibit sodium dependences identical to the wt, but they are not shown to simplify the figure. In contrast, R44K shows a clear shift in apparent sodium affinity, which is most marked at low sodium concentrations. The chloride affinity for the wt and the mutants was also determined by measuring uptake of radioactive GABA into HeLa cells with increasing concentrations of chloride. There was no difference between the wt and the mutants (data not shown). Next, the apparent $K_m$ for GABA and the $V_{max}$ of the transport reaction were determined for the wt and one active substitution at each of the positions. The results are shown in Table I. W47F does not differ significantly from the wt in either the $K_m$ or the $V_{max}$. D45L shows a 3-fold decrease in $K_m$, with a 5-fold decrease in $V_{max}$. wt, and T46I shows a 2-fold increase in $K_m$ with a similar increase in $V_{max}$. R44K shows the most marked change of all the constructs examined; the $K_m$ is decreased almost 6-fold in comparison with the wt, and the $V_{max}$ is decreased 40-fold.

Exchange Experiments—To clarify the defect in R44K, we examined its ability to catalyze exchange, a partial reaction of the transport cycle (Scheme 1). GABA is not normally present under 0.4 mM $[^{3}H]$GABA leads to accumulation of $[^{3}H]$GABA into HeLa cells with increasing concentrations of chloride. There was no difference between the wt and the mutants (data not shown). Next, the apparent $K_m$ for GABA and the $V_{max}$ of the transport reaction were determined for the wt and one active substitution at each of the positions. The results are shown in Table I. W47F does not differ significantly from the wt in either the $K_m$ or the $V_{max}$. D45L shows a 3-fold decrease in $K_m$, with a 5-fold decrease in $V_{max}$. wt, and T46I shows a 2-fold increase in $K_m$ with a similar increase in $V_{max}$. R44K shows the most marked change of all the constructs examined; the $K_m$ is decreased almost 6-fold in comparison with the wt, and the $V_{max}$ is decreased 40-fold.

Exchange in GAT-1 Mutants

**Table I**

| Mutant     | $K_m$ (mM) | $V_{max}$ (pmol/min/mg) |
|------------|------------|-------------------------|
| Wild type  | 1.40 ± 0.16| 6.47 ± 2.28             |
| R44K       | 0.25 ± 0.01| 0.16 ± 0.07             |
| D45L       | 0.42 ± 0.14| 1.29 ± 0.14             |
| T46I       | 3.06 ± 0.35| 15.74 ± 5.16            |
| W47F       | 1.48 ± 0.14| 7.22 ± 2.55             |

**Scheme 1. The transport cycle of GAT-1.**
Further evidence that the \(^{3}H\)GABA accumulation seen in the wt is due to exchange by GAT-1 comes from the observation that a saturating concentration of GABA (200 \(\mu M\)) in the external medium reduces the accumulation of \(^{3}H\)GABA to the control level, whereas the same concentration of L-aspartate has no effect (data not shown). Although there was considerable variation between experiments, we found that exchange is dependent on both internal sodium and chloride. In the absence of sodium, exchange was only 13.4 \(\pm\) 12.5% \((n = 4)\) of that seen in its presence. In the absence of chloride exchange was 15.7 \(\pm\) 8.4% \((n = 4)\) of the maximum levels seen in its presence.

Once we established the specificity of the wt exchange reaction and the conditions required, we measured the exchange of the inactive deletion mutants, the substitution mutants at position 44, and both inactive substitution mutants at position 47. Fig. 6 shows the results, expressed as percentages of the wt activity. Mutants \(\Delta R44, R44S, \Delta T46, W47S,\) and \(W47V\) showed no more than 4% of wt net flux, when measured in whole cell and reconstitution assays (Figs. 1 and 3). In the exchange assay they all gained activity, with exchange rates ranging from 12 to 28% of wt activity. Mutant \(\Delta W47\) shows no change and remains totally inactive. The most marked change is observed in \(R44H\) and \(R44K\). Whereas these mutants have no more than 15.4 \(\pm\) 6.9 and 34.2 \(\pm\) 1.6% of wt net flux, respectively (Fig. 3B), both are as active as the wt in the exchange reaction (100.5 \(\pm\) 30.6% and 115.8 \(\pm\) 20.2%, respectively; Fig. 6). In the case of \(R44K\) we determined the dependence of exchange on internal sodium and chloride. It is not significantly different from the wt; in the absence of internal sodium or chloride the values were 10.6 \(\pm\) 9.1 and 21.1 \(\pm\) 9% \((n = 2)\) of those seen when the internal medium contained sodium chloride.

**DISCUSSION**

In the study described here we have defined a domain of GAT-1, located in the cytoplasmic amino-terminal tail, which is critical for net flux (Figs. 1 and 3). Point mutations in this domain do not appear to cause a gross misfolding of the transporter, because mutants \(R44K\) and \(R44H\) retain full capability of catalyzing exchange (Fig. 6). Exchange can be viewed as a partial reaction of the transport cycle, as shown in Scheme 1. In net influx, binding of the substrates (two sodium ions, GABA, and a chloride ion) from the extracellular medium to the transporter (step 1) is followed by the cotransport (translocation) step (step 2). After release of the substrates into the intracel-
lular medium (step 3), a new cycle can begin only after reori-
tentation of the substrate binding sites to the outside. This is
referred to as the return of the unloaded transporter (step 4).
Net efflux can be viewed as a reversal of the cycle from step 3,
in a clockwise direction. Exchange proceeds by the reversible
execution of steps 1–3 in both directions. The selective impact of
the mutations on net flux, as compared with exchange, can
be readily explained by this scheme. It appears that these
mutants are predominantly affected in step 4, the return of the
unloaded transporter. This is seen most clearly in mutants of
arginine 44. Replacement by histidine and lysine leads to net
flux of 15 and 30% of the wt in reconstituted proteoliposomes
(Fig. 3B), whereas exchange values are indistinguishable from
those of the wild type (Fig. 6). Other mutations at this position
(R44 and R44S) as well as ΔT46, W47S, and W47V show a
similar trend (Figs. 1B and 3B as compared with Fig. 6). It is
important to note that the model shown in Scheme 1 is very
simplistic. For instance, it does not specify the order in which
the three substrates are released on the inside. The currently
available models of the GAT-1 transport cycle indicate that
GABA is released before at least one of the sodium ions and the
chloride ion (29, 30). Thus, an alternative way to explain the
impact of the mutations on net flux relative to exchange, is that
the release of the sodium or chloride ions is impaired. If,
for example, chloride release were the affected step, one would
expect exchange in the mutants to be less dependent on internal
chloride than the wild type. The data indicate that this is
not so; R44K does not differ from the wt in its dependence on
internal sodium and chloride.

A defect in the return of the unloaded R44K transporter is
consistent with both the 40-fold reduction observed in \( V_{\text{max}} \)
and the almost 6-fold decrease in apparent \( K_m \). According to
the formalism of Michaelis and Menten, this constant equals
\( k_1 + k_2/k_1 \) with \( k_2 \) representing steps subsequent to GABA
binding from the outside. A decreased rate constant of step 4
could contribute to a decreased \( k_2 \), which in turn could lead to
a reduction in the apparent \( K_m \). In fact, earlier studies indicate
that the return of the unloaded GABA transporter can be a
rate-limiting step in net flux (31).

Although it appears that the domain encompassing residues
44–47 influences the return of the unloaded transporter, mu-
tation of these residues has additional effects. Solubilization
and reconstitution cause an enhancement of net flux in several
mutants (ΔD45, R44K, and R44H) relative to the whole cell
transport (Figs. 1 and 3). In principle, this stimulation could be
due to defective targeting to the plasma membrane, but the
data (Fig. 2 and data not shown) indicate normal targeting of
the mutants. It is not clear why solubilization and reconstitu-
tion enhance their activity relative to the wild type. Possible
reasons could be a minor refolding induced by the detergent
used (choleate) or by the different phospholipid composition of
the liposomes compared with the HeLa cells or a change in the
protein/lipid ratio in the liposomes compared with the cells.
Alternatively, the stimulation of activity in these mutants
could be due to a functionally negative, detergent-sensitive
association with another protein, which is stabilized by these
mutations. An example of an interaction of GAT-1 with another
protein is the case of Syntaxin-1A. This interaction leads to
decreased transport (32).

Another effect of mutations in this domain is the somewhat
decreased affinity for sodium seen in R44K (Fig. 4). Although
reproducible, this lowered affinity cannot account for the im-
paired activity of the mutant (Fig. 3A), because the assay is
performed at saturating sodium concentrations where this ef-
fect of the mutation is lost. We do not know the structural basis
for this change in sodium affinity. One possibility is that argi-
nine 44 is not far away from tryptophan 68. Mutation of this
residue to leucine (W68L) leads to decreased transport but an
increased affinity for sodium (22, 33). It has been shown that
cation–π interactions are common in proteins. Of the aromatic
residues, tryptophan is most likely to be involved and arginine
is a more probable partner than lysine (34). A change in the
interaction of arginine 44 with tryptophan 68 might influence
sodium affinity. Other explanations, such as long range effects,
are also possible, and there is no evidence as yet for the pro-
ximity of positions 44 and 68. It is perhaps more likely that
arginine 44 interacts directly with tryptophan 47. Deletion of
aspartate 45 or threonine 46 has a much larger influence on net
flux than their replacement with unrelated amino acid residues
(Figs. 1A and 3A). This implies that the distance between
arginine 44 and tryptophan 47 is a critical determinant of
activity. However, it should be noted that ΔD45 has consid-
able activity upon reconstitution (Fig. 1B). The reason for this
is not clear. This suggests that there may be multiple effects of
residues in this region, complicating interpretation. Studies of
proximity relations in GAT-1, such as those pioneered by
Kaback and co-workers (35) in the lactose permease, could
contribute to our understanding of the interactions between
specific residues in this domain and between this domain and
other parts of the transporter.

Arginine 44 and tryptophan 47 of GAT-1 are completely
conserved in the family of sodium- and chloride-dependent
transporters. This suggests that the consensus sequence
RXRW may control the return of the unloaded transporter in
the other family members as well. In the related serotonin
transporter it has been found that, unlike GAT-1, this process
exhibits a requirement for potassium or protons (36). It is
nevertheless possible that some aspects of the reorientation
of the substrate-free transporter are shared between all the sodi-
um- and chloride-dependent transporters. It should therefore
be of considerable interest to evaluate the role of this consensus
sequence in the mechanism of these transporters.

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