Selective Amino Acid Substitutions Convert the Creatine Transporter to a γ-Aminobutyric Acid Transporter*

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The creatine transporter (CRT) is a member of a large family of sodium-dependent neurotransmitter and amino acid transporters. The CRT is most closely related to the γ-aminobutyric acid (GABA) transporter, GAT-1, yet GABA is not an effective substrate for the CRT. The high resolution structure of a prokaryotic homologue, LeuT has revealed precise details of the substrate binding site for leucine (Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Nature 437, 215–223). We have now designed mutations based on sequence comparisons of the CRT with GABA transporters and the LeuT structural template in an attempt to alter the substrate specificity of the CRT. Combinations of two or three amino acid substitutions at four selected positions resulted in the loss of creatine transport activity and gain of a specific GABA transport function. GABA transport by the “gain of function” mutants was sensitive to nipecotic acid, a competitive inhibitor of GABA transporters. Our results show LeuT to be a good structural model to identify amino acid residues involved in the substrate and inhibitor selectivity of eukaryotic sodium-dependent neurotransmitter and amino acid transporters. However, modification of the binding site alone appears to be insufficient for efficient substrate translocation. Additional residues must mediate the conformational changes required for the diffusion of substrate from the binding site to the cytoplasm.

The creatine transporter (CRT)\(^2\) is required for the uptake of creatine, which participates in the creatine kinase reaction, providing a high energy buffering system to maintain ATP levels in tissues with high energy demands (1, 2). Deficiencies in the CRT result in the absence of creatine in the brain and a novel form of X-linked mental retardation (3, 4).

The CRT is a member of the neurotransmitter/sodium symporter (NSS) family (5) also known as the Na\(^+\)/Cl\(^−\)-dependent neurotransmitter transporter (SLC6) family (6). These transporters utilize Na\(^+\) gradients to drive the accumulation of a wide array of substrates across the cell membrane, including the biogenic amines (5-hydroxytryptamine, dopamine, and norepinephrine), γ-aminobutyric acid (GABA), betaine, taurine, and amino acids (6, 7). Members of the NSS family were first characterized from eukaryotic organisms, although now genes encoding >200 putative transport proteins have been identified in prokaryotes (8). TnAT from Symbio bacterium thermophilum and Tyt1 from Fusobacterium nucleatum have been shown to function as tryptophan and tyrosine transporters, respectively (9, 10).

Recently, the structure of a leucine transporter LeuT from Aquifex aeolicus was determined to very high resolution (1.65 Å) by x-ray crystallography (11). This structure not only revealed the protein folds of the transporter but also provided clear details of the substrate binding site, with leucine and two Na\(^+\) ions bound. Leucine is bound near the middle of TM3 and TM8 and adjacent to the unwound portions of TM1 and TM6. The aliphatic side chain of leucine interacts with the side chains of residues Val-104 and Tyr-108 in TM3, Phe-253 and -259 in TM6, and Ser-355 and Ile-359 in TM8 (11). Two of the corresponding residues in TM3 of the CRT, Cys-144 and Tyr-148, have previously been shown to be close to the substrate binding site and essential for CRT activity, respectively (12, 13).

The CRT is most closely related to GAT-1 within the family of NSS/SLC6 transporters (6, 7, 14, 15). The CRT recognizes substrates with a carboxyl group and a guanidino group separated by 2–3 carbon atoms (16, 17). GABA is not an effective substrate for the CRT (16), although GPA, an analogue of creatine, is a competitive inhibitor of some subtypes of GABA transporters (17, 18).

In the present paper, we have engineered amino acid substitutions in the CRT at positions predicted to be involved in substrate binding. We found that combinations of two or three of the selected amino acid substitutions can switch the substrate and inhibitor selectivity of the CRT to that of a GABA transporter.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Preparation of Plasmid Constructs**—Mutations were introduced singly by site-directed mutagenesis of the wild-type bovine CRT cDNA using the QuikChange™ kit (Stratagene, La Jolla, CA) as described previously (12, 13). The mutated cDNA was excised from pBluescript(KS+®) and subcloned into pcDNA3.1(+) (Invitrogen). CRT cDNAs with two or more substitutions were prepared by ligation of BamHI, BstEI, SacII, and CclI restriction fragments from cDNAs encoding the single amino acid

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2. The abbreviations used are: CRT, creatine transporter; GABA, γ-aminobutyric acid; GAT-1, γ-aminobutyric acid transporter 1; GPA, β-guanidinopropionic acid; LeuT, leucine transporter from Aquifex aeolicus; NSS, neurotransmitter/sodium symporter family; SLC6, solute carrier family 6; TM, transmembrane domain; wt, wild-type.

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substitutions. The exception was for the generation of the F68Y substitution in combination with the C144L substitution, which required that the second mutation be introduced by another round of site-directed mutagenesis. The cDNA sequence of all CRT mutants was confirmed by DNA sequencing. Wild-type rat GAT-1 cDNA in pBluescript(SK−) was received from Prof. Baruch Kanner. The cDNA was subcloned into pcDNA3.1(+) using the restriction enzymes HindIII and XbaI.

Creatine and [3H]GABA Uptake Experiments in COS-7 Cells—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, streptomycin, and penicillin and seeded at a density of 1.2 × 10^5 cells/well in 12-well (3.8 cm^2 area) plastic culture dishes (Falcon) 24 h prior to transfection. The cells were transfected with 0.4 μg of plasmid and 4 μl of Lipofectamine 2000/well following procedures recommended by the manufacturer (Invitrogen).

Cells were assayed for either GABA or creatine uptake activity 24 h after transfection. The initial rate of creatine uptake was determined by incubation with 20 μM [14C]creatin (American Radiolabeled Chemicals, ARC-176, 55 μCi/mmole, diluted to a specific activity of 1 μCi/mmole) for 3 min (13). The initial rate of GABA uptake was determined by incubation of the cells with 20 μM [2,3-3H]GABA (Amersham Biosciences, TRK527, 96 Ci/mmole, diluted to a specific activity of 24 μCi/mmole) for 5 min. Otherwise, cell transport assays were carried out using buffers and conditions described previously (13).

Assays for creatine and GABA transport were also carried out in the presence of competing substrates and inhibitors. These experiments used 20 μM [14C]creatin (American Radiolabeled Chemicals, ARC-176, 55 μCi/mmole, diluted to a specific activity of 1 μCi/mmole) or 5 nM [2,3-3H]GABA (Amersham Biosciences, TRK527, 96 Ci/mmole) in the presence of the concentrations of substrates/inhibitors indicated in the text. All experiments were carried out in triplicate. Curve fitting to data was by GraphPad Prism, version 4.0 (GraphPad Software, San Diego, CA) using the nonlinear one-site binding (hyperbola) analysis (see Fig. 2A) or one-site competition analysis (see Fig. 4).

### FIGURE 1. Schematic diagram of an alignment of the amino acid sequences of TM domains 1, 3, 6, and 8 from the CRT, GABA transporters, and the bacterial homologue LeuT.

![Schematic diagram of an alignment of the amino acid sequences of TM domains 1, 3, 6, and 8 from the CRT, GABA transporters, and the bacterial homologue LeuT.](image)

### TABLE 1

| Substitutions | Creatine uptake | Relative creatine uptake | GABA uptake | Relative GABA uptake | Selectivity (GABA/creatine uptake) |
|---------------|-----------------|--------------------------|-------------|----------------------|-----------------------------------|
|               | pmol/min/mg protein | pmol/min/mg protein |            |                      |                                   |
| Plasmid control | 56.8 ± 3.5      | 1.0                      | 19.8 ± 1.4 | 1.0                  | 1.0                               |
| wt CRT        | 1729.1 ± 179.2  | 30.5                     | 27.6 ± 2.6 | 1.4<sup>a</sup>      | 229.3<sup>a</sup>                 |
| wt GAT-1      | 60.0 ± 3.3      | 1.1                      | 4531.7 ± 230.6 | 2217.1              |                                   |
| FY            | 943.2 ± 144.7   | 16.6                     | 30.2 ± 2.1 | 1.5<sup>b</sup>      | 0.1                               |
| CL            | 157.9 ± 16.4    | 2.8                      | 36.6 ± 2.8 | 1.9<sup>c</sup>      | 0.7                               |
| AG            | 676.1 ± 101.3   | 11.9                     | 38.2 ± 2.6 | 1.9<sup>c</sup>      | 0.2                               |
| GT            | 429.6 ± 39.3    | 7.6                      | 19.7 ± 1.7 | 1.0                  | 0.1                               |
| FY/CL         | 75.3 ± 2.9      | 1.3                      | 42.3 ± 3.3 | 2.1<sup>c</sup>      | 1.6                               |
| FY/AG         | 154.3 ± 16.8    | 2.7                      | 79.0 ± 8.0 | 4.0<sup>c</sup>      | 1.5                               |
| CL/AG         | 79.0 ± 3.1      | 1.4                      | 82.4 ± 8.7 | 4.2<sup>c</sup>      | 3.0                               |
| CL/GT         | 1049. ± 9.2     | 18.0                     | 203.8 ± 13.3 | 1.0                  | 0.6                               |
| AG/GT         | 482.3 ± 79.9    | 8.5                      | 25.1 ± 2.0 | 1.3                  | 0.1                               |
| FY/CL/GT      | 55.4 ± 2.6      | 1.0                      | 205.1 ± 15.0 | 10.2<sup>c</sup>    |                                    |
| FY/AG/GT      | 172.0 ± 17.1    | 3.0                      | 35.9 ± 3.4 | 1.8<sup>c</sup>      | 0.6                               |
| CL/AG/GT      | 78.7 ± 3.2      | 1.4                      | 46.5 ± 3.7 | 2.4<sup>c</sup>      | 1.7                               |
| FY/CL/AG/GT   | 57.5 ± 3.4      | 1.0                      | 54.3 ± 5.2 | 2.7<sup>c</sup>      | 2.7                               |

<sup>a</sup>Level of significance, p < 0.05.
<sup>b</sup>Level of significance, p < 0.001.
<sup>c</sup>Level of significance, p < 0.001.


**RESULTS**

The amino acid sequence of transmembrane domains 1, 3, 6, and 8 of the CRT, GAT-1, -2, -3, and -4 were aligned with the equivalent regions of LeuT to predict residues that may be important for substrate recognition (Fig. 1). Only 4 of the 12 positions known to be involved in substrate binding in LeuT (11) contained amino acid differences between the CRT and GAT-1. To investigate the importance of these residues for substrate specificity, the following CRT residues were substituted with their counterpart in GAT-1: Phe-68 → Tyr in TM1, Cys-144 → Leu in TM3, Ala-318 → Gly in TM6, and Gly-421 → Thr in TM8. These substitution mutants are referred to as FY, CL, AG, and GT, respectively.

The Na⁺-dependent creatine and GABA transport activity of FY, CL, AG, GT, and mutants containing combinations of two, three, or four of the amino acid substitutions was determined in transfected COS-7 cells. Transport activity rates of these mutants and the wild-type CRT and GAT-1 are shown in Table 1. Uptake activity was determined in the presence of 20 μM creatine or GABA, and the data are presented in absolute terms (pmol/min/mg protein) and normalized relative to the background cells transfected with the empty plasmid.

Single amino acid substitutions resulted in a substantial decrease (46–91%) in creatine transport compared with wt CRT. The CL mutant showed the largest decrease (retained 9% of wt CRT activity). Creatine uptake of mutants with two amino acid substitutions was reduced by 91–96% apart from the AG/GT double mutant (retained 28% of wt CRT activity). The activity of triple mutants containing the CL substitution and the quadruple mutant were almost identical to the plasmid control.

Interestingly, for some substitution mutants, the loss of creatine uptake was associated with a significant gain of GABA transport. The GABA transport rates of FY/CL, FY/AG, and CL/AG were 2–4 times greater than the background for the plasmid control (Table 1). FY/CL/AG showed a 3-fold greater than determined for GABA. FY/CL/AG/GT mutant was 702 pmol/min/mg protein.

### Table 2

| Substitutions | $V_{max}$ | $K_m$ | $V_{max}/K_m$ |
|---------------|----------|-------|--------------|
| wt GAT-1      | 12,560 ± 473 | 40 ± 3 | 314          |
| FY/CL         | 1,743 ± 252 | 1,437 ± 258 | 1           |
| FY/AG         | 4,959 ± 780 | 1,934 ± 385 | 3           |
| CL/AG         | 7,231 ± 334 | 2,826 ± 157 | 3           |
| FY/CL/AG      | 1,570 ± 75  | 175 ± 15  | 9           |
| FY/CL/AG/GT   | 702 ± 40    | 377 ± 32  | 2           |

Western blotting with affinity-purified C-terminally directed antibodies against the CRT was carried out as described previously (12, 13). Chemiluminescence was detected using a Fuji LAS-1000 digital imaging system.

**FIGURE 2. Saturation kinetics of gain-of-GABA-transport amino acid substitution mutants.** COS-7 cells were transfected with FY/CL, FY/AG, CL/AG, FY/CL/AG, FY/CL/AG/GT, or the empty pcDNA3.1 plasmid and the uptake of [3H]GABA determined at varying concentrations of GABA (10 μM to 5 mM). A, Michaelis-Menten plot of transport velocity (V) versus substrate (S) concentration. Each point represents the mean ± S.E. of three independent determinations, each in triplicate. The GABA transport for control cells (empty plasmid) was subtracted for each GABA concentration. Curve fitting was carried out by non-linear one-site binding (competition) analysis. B, Eadie-Hofstee plot of the data presented in A. C, cell surface biotinylation of COS-7 cells transfected with empty pcDNA3.1+ (lane 1), wild-type CRT (lane 2), FY/CL (lane 3), FY/AG (lane 4), CL/AG (lane 5), FY/CL/AG (lane 6), and FY/CL/AG/GT (lane 7). Samples of biotinylated (cell surface) proteins were run on SDS-PAGE and subjected to Western blotting with CRT antibodies. The positions of protein markers of known molecular mass (kDa) are shown on the left. Arrows indicate the 80- and 160-kDa immunoreactive bands that correspond to monomer and dimer forms of the CRT.

Studies of saturation kinetics (Fig. 2, Table 2) showed these “gain-of-GABA-transport” mutants to have $K_m$ values for GABA from 175 to 2,826 μM. Double mutants had lower affinity for GABA. FY/CL/AG was the mutant with the lowest $K_m$ (175 μM) for GABA. This value was 4-fold higher than determined for GAT-1 in COS-7 cells (40 μM). The FY/CL/AG/GT mutant was the mutant with the highest $V_{max}$ (702 pmol/min/mg protein) and the lowest $K_m$ (377 ± 32 μM) for GABA. This value was 4-fold higher than determined for GAT-1 in COS-7 cells (40 μM).
had the lowest $V_{\text{max}}$ value. Surface biotinylation showed that surface expression of FY/CL/AG/GT was similar to the other substitution mutants (Fig. 2C), suggesting this mutant to have a reduced capacity to transport GABA than FY/CL/AG. The $V_{\text{max}}/K_m$ values of the substitution mutants, used as an estimate of the transport efficiency, were $\sim 30–170$-fold lower than for GAT-1 (Table 2).

The loss of creatine and the gain of GABA transport function correlated with altered sensitivity to inhibitors. Initially, we tested the effect of 1 mM creatine or GPA, 0.1 mM nipecotic acid, and 10 $\mu$M SKF89976A on $[^{14}\text{C}]$creatine and $[^{3}\text{H}]$GABA transport. The single substitution mutants FY, CL, and AG retained properties similar to wt CRT (Fig. 3A). Creatine transport was inhibited 60–90% by creatine and GPA, whereas the GABA transport inhibitors nipecotic acid and SKF89976A had little effect at the concentrations tested. The five gain-of-GABA-transport mutants were found to differ in their sensitivity to the inhibitors (Fig. 3B). FY/CL and FY/AG retained the greatest sensitivity to 1 mM creatine. $[^{3}\text{H}]$GABA transport of all mutants and GAT-1 was inhibited by 1 mM GPA. CL/AG, FY/CL/AG, and FY/CL/AG/GT were sensitive to nipecotic acid. Unlike GAT-1, no mutants were inhibited by 10 $\mu$M SKF89976A (Fig. 3B).

To further evaluate the effect of the amino acid substitutions, $[^{3}\text{H}]$GABA uptake was determined in the presence of varying concentrations of substrates and inhibitors for the five gain-of-GABA-transport function mutants and GAT-1 (Fig. 4, Table 2). Creatine was a very weak inhibitor ($K_i$ values in the mM range) of CL/AG, FY/CL/AG, and FY/CL/AG/GT. All mutants, except FY/CL, were more sensitive to inhibition by GPA than creatine. FY/AG had the lowest $K_i$ value for GPA.

Remarkably, the inhibition curves and $K_i$ values indicated that nipecotic acid was as potent an inhibitor for CL/AG, FY/CL/AG, and FY/CL/AG/GT as for GAT-1. Of these, CL/AG and FY/CL/AG could also be inhibited by SKF89976A, although the $K_i$ values were $\sim 20$-fold higher than for GAT-1 (Fig. 4). SKF89976A was a very weak inhibitor for FY/CL and FY/CL/AG/GT.
TABLE 3

| Substitutions | Creatine | GPA | Nippecotic acid | SKF89976A |
|---------------|----------|-----|----------------|-----------|
|               | $K_i$    | 95% CI | $K_i$     | 95% CI  | $K_i$    | 95% CI | $K_i$     | 95% CI  |
| wt GAT-1      | ND       | ND   | 692       | 497–964 | 60       | 53–67 | 3.5       | 2.3–5.1 |
| FY/CL         | 740      | 565–969 | 908     | 557–1,480 | 1,627   | 1,136–2,332 | ND       | ND     |
| FY/AG         | 543      | 473–622 | 88      | 80–97    | 643     | 338–1,222 | 78       | 25–244 |
| CL/AG         | 5,204    | 2,863–9,460 | 597     | 383–929 | 79      | 44–142 | 58       | 23–145 |
| FY/CL/AG      | 14,830   | 8,415–26,150 | 967     | 622–1,504 | 83      | 54–128 | 67       | 19–241 |
| FY/CL/AG/GT   | 4,912    | 3,341–7,223 | 1,147   | 758–1,736 | 32      | 20–51  | ND       | ND     |

DISCUSSION

The recent high resolution structure of LeuT from *A. aeolicus* (11) provided a major advance in our understanding of the structure and function of the NSS/SLC6 family of transporters (for reviews, see Refs. 19–21). Despite only 20–25% sequence identity between LeuT and its eukaryotic counterparts, there is high conservation in the TMs 1, 3, 6, and 8 that surround the leucine binding site (11). Biochemical studies on mammalian transporters had identified, previously, that residues in TM1 (22, 23) and TM3 (12, 13, 24, 25) were important for substrate binding and transporter function.

Our most interesting finding was that 2–3 selected amino acid substitutions, based on the LeuT structural template and sequence alignment with GABA transporters, were sufficient to generate a gain of specific GABA transport function. In particular, the triple substitution mutant FY/CL/AG demonstrated a striking change in substrate specificity. This mutant did not demonstrate creatine uptake above background levels. The $K_m$ value of this mutant for GABA transport of 170 $\mu M$ is similar to $K_m$ values (180–200 $\mu M$) determined for the wt CRT for creatine (11, 12). The $K_m$ value of FY/CL/AG for GABA was ~4-fold higher than that determined for GAT-1 expressed in COS-7 cells (40 $\mu M$). These compare with $K_m$ values of ~7 $\mu M$ for GAT-1 expressed in oocytes (14), 79 $\mu M$ for GAT-2, (27), and 18 and ~0.9 $\mu M$ for GAT-3 and -4, respectively (18).

The CL substitution appeared to contribute significantly toward the gain of GABA and loss of creatine transport. The substituted cysteine (Cys-144) is unique to creatine transporters (8) and is close to the substrate binding site of the CRT (12).

The position equivalent to Phe-68 in the CRT is an important determinant of substrate specificity for GABA transporters (28). A Glu → Gly substitution in GAT-4 was markedly more sensitive to inhibition by taurine (28). Also, substitution of a Glu for Tyr in GAT-1 leads to loss of activity (29). In the present study, the FY substitution was reasonably well tolerated with respect to the preservation of creatine transport activity but was important, in combination with the CL and AG mutations, to the gain-of-GABA-transport function (Table 1).

More than one combination of amino acid substitutions was able to generate a gain-of-GABA-transport function. The occurrence of mutants with differing affinities for GABA (Table 2) indicates that the combination of substituted residues is likely to affect the size and shape of the substrate binding site. CL/AG acted as a low affinity GABA transporter and was the only substitution mutant with GABA transport inhibited by β-alanine (data not shown), a characteristic of mouse GAT-3 and GAT-4 (18).

The data from the inhibitor kinetics provided additional insight regarding the relationship between amino acid changes and substrate selectivity. GPA contains one more carbon atom between the guanidino group and the carboxylic acid groups than creatine. The ~10-fold greater sensitivity of FY/AG to GPA compared with FY/CL suggests that the longer side chain of leucine may interfere with the binding of the larger substrate.

Nippecotic acid is a potent inhibitor of GABA transport and is considered to bind to the same site as GABA (30, 31). The $K_i$ values of nippecotic acid for CL/AG, FY/CL/AG, and GAT-1 were very similar. Nippecotic acid was a more potent inhibitor of the quadruple mutant FY/CL/AG/GT than GAT-1 (Table 3). This indicates that the amino acid substitutions were very effective at altering the specificity of the CRT substrate binding site.

It is interesting to consider why CL/AG, FY/CL/AG, and FY/CL/AG/GT, which share similar affinities for nippecotic acid, show such a wide range of $K_m$ values for GABA. One obvious possibility is that the GABA and nippecotic acid are interacting with different or overlapping sites in the mutants. It also appears that GAT-1 must contain additional sites for interaction with SKF89976A. Despite the presence of the nippecotic acid moiety in SKF89976A, this high affinity GAT-1 inhibitor has ~20-fold reduced affinity for the gain-of-GABA-transport mutants. The highest concentration of SKF89976A tested was not able to maximally inhibit FY/CL or FY/CL/AG/GT (Fig. 4).

Notwithstanding their remarkable change in substrate and inhibitor selectivity, gain-of-GABA-transport mutants did not exhibit high rates of GABA transport. This was apparent from comparison of the initial rates and maximal rates of activity to GAT-1 (Fig. 2 and Table 1). Further, comparison of $V_{max}/K_m$ values indicates that the transport of GABA by the mutants was substantially less efficient than for GAT-1. This indicates a clear distinction between a transporter being able to bind the substrate and efficiently translocate it across the membrane.

The LeuT structure gives exquisite detail regarding how the transporter binds its substrate but is uninformative in terms of how the substrate passes from the binding site to the cytoplasm. According to the alternating access model, a transporter contains a binding site for substrate that is exposed alternatively to each side of the membrane (32, 33). Hence, once the substrate is bound, the transporter protein must undergo a conformational
change enabling the substrate to pass from the binding site to the cytoplasm. Recent studies on the serotonin transporter provide the first identification of residues involved in a cytoplasmic permeation pathway (34). The accessibility of residues in the cytoplasmic half of TM5 is altered by the presence of the substrate or inhibitor on the extracellular side of the membrane. Specifically, the inhibitor cocaine decreases accessibility, whereas the substrate 5-hydroxytryptamine increases accessibility at these positions. Given this evidence for substrate- and inhibitor-dependent conformational changes, it is conceivable that mutations at the substrate binding site may have distal effects, altering the conformation of residues that form part of the substrate permeation pathway. This would explain the decrease in the maximal rate of substrate transport by CRT mutants with three or more substitutions. Substrate translocation appeared to provide the first identification of residues involved in a cytoplasmic permeation pathway (34). The accessibility of residues in the cytoplasmic half of TM5 is altered by the presence of the substrate or inhibitor on the extracellular side of the membrane. Recent studies on the serotonin transporter provide the first identification of residues involved in a cytoplasmic permeation pathway (34). The accessibility of residues in the cytoplasmic half of TM5 is altered by the presence of the substrate or inhibitor on the extracellular side of the membrane. Specifically, the inhibitor cocaine decreases accessibility, whereas the substrate 5-hydroxytryptamine increases accessibility at these positions. Given this evidence for substrate- and inhibitor-dependent conformational changes, it is conceivable that mutations at the substrate binding site may have distal effects, altering the conformation of residues that form part of the substrate permeation pathway. This would explain the decrease in the maximal rate of substrate transport by CRT mutants with three or more substitutions. Substrate translocation appeared to have been particularly constrained by the addition of the GT substitution to FY/CL/AG.

In summary, the LeuT structural template has enabled us to change amino acid residues in the CRT to alter substrate and inhibitor selectivity. These findings have biological relevance regarding the evolution of substrate specificity for this important family of Na+-dependent neurotransmitter and amino acid transporters. However, modification of the binding site on its own appeared to be insufficient for efficient substrate translocation. There must be additional residues that play an important role in allowing the conformation changes required for the diffusion of substrate from the binding site to the cytoplasm.

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