Cysteine-scanning Mutagenesis of Serotonin Transporter Intracellular Loop 2 Suggests an α-Helical Conformation*

Yuan-Wei Zhang and Gary Rudnick‡

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06620-8066

Like other proteins involved in neurotransmitter transport, serotonin transporter (SERT) activity is regulated by multiple intracellular signal transduction pathways. The second intracellular loop (IL2) of SERT contains consensus sequences for cGMP-dependent protein kinase and protein kinase C. A 24-residue region of SERT including IL2, from Ile-270 through Ser-293, was analyzed by cysteine-scanning mutagenesis and chemical modification. 2-(Aminomethyl)ethanethiosulfonate hydrobromide (MTSEA) failed to inhibit serotonin transport or binding of the cocaine analog 2β-carbomethoxy-3β-(4-[125I]iodophenyl)tropane (β-CIT) in intact cells expressing these mutants, but it inactivated β-CIT binding in membrane preparations. From the pattern of sensitivity, IL2 appears to extend from Trp-271 through Trp-275, and the pattern of the more reactive mutations suggested that IL2 is in an α-helical conformation. Some of the mutants had significantly elevated transport rates, suggesting a possible mechanism for the regulation of SERT activity.

The serotonin transporter (SERT,1 SLC6A4) is a presynaptic plasma membrane protein responsible for the reuptake of serotonin (5-hydroxytryptamine, abbreviated 5-HT) after its release by neurons. It is a member of a large family of amine and amino acid transporters (SLC6, NSS) distributed throughout the prokaryotic and animal kingdoms. Together with norepinephrine and dopamine transporters (NET and DAT), SERT is part of a subgroup of transporters that mediate the transport of biogenic amine neurotransmitters by symport with Na+ and Cl− ions (1).

SERT is of particular interest in neurobiology because it is the molecular target of several drugs of abuse and many therapeutic agents used to treat psychiatric disorders. Along with NET and DAT, SERT is inhibited by cocaine, and cocaine analogs bind to SERT with high affinity (2–4). Amphetamine and its derivatives, including 3,4-methylenedioxyamphetamine (MDMA, also known as ecstasy), interact with SERT, DAT, and NET as substrates (5, 6). Inhibitors that prevent 5-HT reuptake into serotonergic neurons have been used to treat a variety of neuropsychiatric disorders, including affective disorder, anxiety disorder, obsessive-compulsive disorder, and autism (7–9). Furthermore, the incidence of some psychiatric disorders and the effectiveness of antidepressant drugs has been linked to polymorphisms in the promoter of the gene encoding SERT (10–13).

A naturally occurring mutation in human SERT, I425V, was found to be associated with obsessive-compulsive disorder and several other 5-HT-related disorders (14). Further investigation revealed that this mutation caused SERT to be in an activated state that is reached in the wild type through the action of cGMP-dependent protein kinase (15).

Hydropathy analysis of SERT predicted 12 transmembrane (TM) domains connected by intracellular and extracellular loops (16, 17). This analysis predicted that the second intracellular loop (IL2) was a short cytoplasmic loop containing nine residues connecting TM domains 4 and 5. Further analysis suggested that this loop contained consensus phosphorylation sites for cGMP-dependent protein kinase and protein kinase C (18). From the location of glycosylation sites in the second extracellular loop, the NH2 and COOH termini were predicted to be cytoplasmic (19). Subsequent experiments using mutagenesis and chemical modification in intact cells and disrupted membranes have verified the overall topology (20, 21). However, the extent of most of the loops remains unknown, with the exception of extracellular loops 4 (22) and 5 (23).

Previous studies of intracellular and extracellular loop residues by cysteine-substitution have revealed changes in conformation of these loops in response to substrate and inhibitor binding (21, 22, 24, 25). To further examine the structure of SERT IL2, we have studied IL2 by cysteine-scanning mutagenesis. The results reported here suggest that it is longer than predicted and contains an α-helical structure.

EXPERIMENTAL PROCEDURES

Mutagenesis—Mutant transporters were generated by site-directed mutagenesis using the QuikChange™ kit (Stratagene). The mutated region was excised by digestion with appropriate restriction enzymes and subcloned into the X5C mutant of rat SERT, which contains sequences encoding a c-Myc epitope tag at the NH2 terminus and a FLAG epitope tag at the COOH terminus (19). X5C (C15A/C21A/C109A/C357I/C622A) is lacking all of the endogenous cysteine residues known to react with MTS reagents (25). All mutations were screened by restriction mapping and confirmed by DNA sequencing.

Expression—The expression system used has been described in detail elsewhere (26, 27). Briefly, HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 incubator. Cells were plated in 96-well culture plates and allowed to grow overnight. The confluent cells were
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infected with recombinant VTF7-3 virus and transfected with a plasmid containing rat SERT cDNA under the control of the T7 promoter. Transfected cells were incubated for 20–22 h at 37 °C with 5% CO₂ and then assayed for transport. Protein concentration was determined with the Micro BCA protein assay reagent kit ( Pierce).

Cell Surface Biotinylation—Surface expression of SERT mutants was determined using the membrane-impermeant biotinylation reagent sulfo-NHS-SS-biotin (Pierce) as described previously (20). HeLa cells expressing SERT mutants were treated twice with NHS-SS-biotin (Pierce) as described previously (20). HeLa cells were then lysed, and the biotinylated proteins were recovered using streptavidin-agarose beads (Pierce) in an overnight incubation at 4 °C with gentle agitation. The beads were washed, and the biotinylated proteins were eluted with 100 μl of SDS-PAGE wash buffer. Portions of each sample were applied to a 10% SDS-polyacrylamide gel and visualized by Western blotting. The transporters were detected using anti-FLAG polyclonal antibody (Affinity Bioreagents, Inc.) (1:1000) against the FLAG epitope tag at the COOH terminus of rat SERT (19). A horseradish peroxidase-conjugated anti-rabbit IgG (Pierce) was detected using anti-FLAG polyclonal antibody (Affinity Bioreagents, Inc.) (1:1000) against the FLAG epitope tag at the COOH terminus of rat SERT (19).

Data Analysis—Nonlinear regression fits of experimental and calculated data were performed with Origin (Microcal Software, Northampton, MA) using the Matignon-Levenberg-Marquardt-Levenberg least squares curve-fitting algorithm. The statistical analysis given was from multipe-ple experiments. Data with error bars represent the mean S.D. for triplicate measurements. Statistical analysis was performed using Student’s paired t tests.

RESULTS

Transport Activity of IL2 Mutants—Initial predictions based on hydropathy profiles suggested that IL2 is a nine-residue loop between TM4 and TM5 (from Trp-271 through Lys-279) (16, 17, 28). Amino acid alignment indicates the residues in IL2 are highly conserved within the neurotransmitter transporter family and especially so within the biogenic amine transporters (Fig. 1). To investigate the structural and functional properties of this region, we substituted the native residue at each position from Ile-270 to Ser-293, one at a time, with cysteine. The cysteine mutants were individually subcloned into a background construct designated X5C (C15A/C21A/C109A/C357I/C622A), which is relatively insensitive to cysteine MTS reagents (25). X5C has ~25–30% of wild type transport activity. Fig. 2 shows the transport activity of each cysteine substitution mutant relative to the parental X5C construct. Of the twenty-four mutants, most retained transport activity comparable with or greater than that of X5C with the exception of V291C and L292C, which retained <50% of X5C activity. Seven mutants (W271C, K272C, K275C, T276C, A285C, P287C, and I290C) had levels of 5-HT uptake similar to that of X5C, and in the remaining mutants transport activity was increased from 150 to 400% that of X5C. Full kinetic analysis was performed on each mutant, yielding the data for Kₘ and Vₘₐₓ shown in Table I. Actual surface expression levels are shown in Fig. 3. The majority of the cysteine mutants were expressed at lower levels than X5C on the cell surface. One of the mutants, L292C, was almost undetectable. Analysis of the surface expression of about one-third of the mutants was not significantly different from that of X5C (Fig. 3, A and B). From the Vₘₐₓ values, and the levels of surface expression, we calculated a normalized Vₘₐₓ for each mutant (Fig. 3C). Several of the mutants had maximal rates of transport higher than both the parent X5C construct (Fig. 3C, white dashes) and the wild type transporter (black dashes).

The Effect of Extracellular MTSEA on Transport and Biotinylation of IL2 Cysteine Mutants—For each of the cysteine replacement mutants we tested the effect of incubating intact

FIG. 1. Amino acid sequence alignment of the putative second intracellular loops of rat NSS transporters. Each line represents a different sequence with the abbreviated name and the residue number preceding the sequence. Residues different from SERT are in white text on black backgrounds. CAT, γ-aminobutyric acid transporter; GLYT, glycine transporter; ProT, proline transporter; Chop, creatine transporter.

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The results represent data from three experiments. Asterisks indicate values significantly different (p < 0.05) from that of X5C.

Table 1

| Mutant | $K_{\text{m}}$ | $V_{\text{max}}$ |
|--------|---------------|----------------|
| X5C    | 0.21 ± 0.03   | 1.51 ± 0.06    |
| I270C  | 0.23 ± 0.02   | 2.69 ± 0.07    |
| W271C  | 0.19 ± 0.03   | 1.77 ± 0.10    |
| K272C  | 0.19 ± 0.01   | 1.52 ± 0.05    |
| G273C  | 0.10 ± 0.01a  | 1.94 ± 0.04a   |
| V274C  | 0.21 ± 0.01   | 2.88 ± 0.05b   |
| K275C  | 0.19 ± 0.02   | 1.61 ± 0.06    |
| T276C  | 0.23 ± 0.04   | 1.38 ± 0.10    |
| S277C  | 0.23 ± 0.03   | 4.05 ± 0.17    |
| G278C  | 0.10 ± 0.01b  | 2.40 ± 0.06b   |
| K279C  | 0.16 ± 0.01a  | 4.97 ± 0.21b   |
| V280C  | 0.13 ± 0.02b  | 4.57 ± 0.25b   |
| V281C  | 0.12 ± 0.01b  | 3.34 ± 0.09b   |
| W282C  | 0.15 ± 0.02a  | 1.75 ± 0.07    |
| V283C  | 0.19 ± 0.02   | 2.36 ± 0.09b   |
| T284C  | 0.13 ± 0.01b  | 3.71 ± 0.10b   |
| A285C  | 0.16 ± 0.01a  | 1.65 ± 0.08    |
| T286C  | 0.13 ± 0.01b  | 1.77 ± 0.07    |
| F287C  | 0.19 ± 0.03   | 1.30 ± 0.11    |
| V288C  | 0.22 ± 0.01   | 3.27 ± 0.08b   |
| V289C  | 0.40 ± 0.02b  | 7.60 ± 0.07b   |
| I290C  | 0.18 ± 0.01   | 0.79 ± 0.07b   |
| V291C  | 0.18 ± 0.01   | 0.45 ± 0.02b   |
| L292C  | 0.40 ± 0.03b  | 0.60 ± 0.08b   |
| S293C  | 0.18 ± 0.01   | 1.63 ± 0.09    |

a $p < 0.05$.
b $p < 0.01$.

Fig. 3. Cell surface expression of SERT IL2 cysteine substitution mutants. A, cells expressing the indicated mutants were treated with sulfo-NHS-SS-biotin to label cell surface proteins. Cells were lysed, proteins were solubilized, and the biotinylated fraction was extracted with streptavidin beads. Transports were detected by immunoblotting with a polyclonal antibody against the FLAG epitope tag. B and C, from the relative integrated density of each 96-kDa band, which represents the mature, fully glycosylated form of SERT, the expression levels of IL2 mutants were estimated as a percentage of X5C expression. (B). Asterisks in panel B indicate values significantly different (p < 0.05) from that of control (X5C). Using the $V_{\text{max}}$ values for these mutants from Table I and the surface expression levels from panel B, we calculated a normalized $V_{\text{max}}$ for each mutant (C). These normalized values are expressed relative to the activity of SERT C109A (black dashes). The activity of X5C is shown by the white dashed lines. Asterisks in panel C indicate values significantly higher (p < 0.05) than C109A activity. The results represent data combined from three experiments, each with triplicate measurements for each mutant.

MTSEA Inactivation of β-CIT Binding—In previous studies (21, 24), intact cells expressing cysteine substitution mutants in putative intracellular domains were insensitive to MTS reagents in the external medium. However, in membrane preparations from those cells, binding of the cocaine analog β-CIT was inactivated by the same reagents, presumably by inducing an indirect conformational change at the binding site. We infer that the cytoplasmic face of the plasma membrane became accessible to MTS reagents in the membrane preparations. For this reason, we tested the effect of MTSEA concentration on β-CIT binding to membrane preparations from cells expressing each mutant.

Representative data for four of the mutants and X5C are shown in Fig. 4. These inactivation profiles show that some mutants, for example, G273C and V274C, were completely inactivated at low concentrations of MTSEA, whereas others, such as K272C and K275C, retained partial activity even at the highest MTSEA concentrations used. Each of the curves in Fig. 4 and similar curves from the 20 other mutants were fit to estimate the MTSEA concentration that led to half-maximal inhibition for each mutant and also the maximal extent of MTSEA inhibition.

Fig. 5 shows the level of β-CIT binding remaining after maximal inhibition, which was estimated from fits of MTSEA-dependent inactivation. Remarkably, MTSEA either partially or completely inactivated almost all of the mutants with cysteines at positions between 271 and 290. Significant binding activity was retained in W271C, K272C, K275C, K279C,
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TABLE II
Effect of MTSEA on 5-HT transport and β-CIT binding activities of IL2 cysteine mutants in intact cells

HeLa cells expressing each of the IL2 cysteine mutants were treated with 1 mM MTSEA at 20 °C for 10 min then washed three times to quench unreacted MTSEA. Transport and binding activity were assayed as described under "Experimental Procedures." Data are from triplicate experiments and are expressed as the percentage of the activity of each mutant in the absence of MTSEA.

| Mutant  | Transport activity | Binding activity |
|---------|--------------------|------------------|
| X5C     | 82.4 ± 5.7         | 85.5 ± 1.7       |
| K272C   | 78.3 ± 5.1         | 83.5 ± 2.3       |
| G273C   | 78.6 ± 5.2         | 78.9 ± 9.9       |
| V274C   | 76.1 ± 14.0        | 73.4 ± 8.5       |
| K275C   | 84.9 ± 2.6         | 83.5 ± 6.5       |
| T276C   | 84.2 ± 11.1        | 80.7 ± 7.4       |
| S277C   | 85.4 ± 2.8         | 84.6 ± 6.2       |
| G278C   | 79.3 ± 10.5        | 79.2 ± 3.6       |
| K279C   | 86.1 ± 3.1         | 81.1 ± 6.0       |
| V280C   | 87.6 ± 1.1         | 86.0 ± 2.9       |
| V281C   | 81.7 ± 6.2         | 85.5 ± 1.7       |
| W282C   | 90.9 ± 3.0         | 83.5 ± 2.2       |
| V283C   | 87.9 ± 2.9         | 85.9 ± 11.6      |
| T284C   | 74.6 ± 12.5        | 91.8 ± 5.1       |
| A285C   | 89.1 ± 9.3         | 78.9 ± 9.9       |
| T286C   | 86.6 ± 3.6         | 75.4 ± 8.5       |
| F287C   | 89.9 ± 3.9         | 83.5 ± 6.5       |
| P288C   | 89.2 ± 2.5         | 80.7 ± 7.4       |
| Y289C   | 89.9 ± 4.4         | 84.6 ± 6.2       |
| I290C   | 87.3 ± 4.8         | 79.2 ± 3.6       |
| V291C   | 87.5 ± 7.5         | 81.1 ± 6.0       |
| L292C   | 97. ± 5.8          | 86.0 ± 2.9       |

FIG. 4. Concentration dependence of MTSEA inactivation of IL2 cysteine mutants. Membranes prepared from HeLa cells expressing each of the IL2 cysteine mutants were treated with the indicated concentrations of MTSEA for 15 min, washed, and assayed for the residual β-CIT binding as described under "Experimental Procedures." Shown are representative data for four of the mutants (K272C, G273C, V274C, and K275C) and the parental X5C. Data are from triplicate experiments and are expressed as the percentage of the binding activity of each mutant remaining after treatment with MTSEA.

Y289C, and I290C. Mutants with cysteines at positions proximal to 270 and distal to 290 retained levels of β-CIT binding similar to that of the parental construct X5C after treatment with MTSEA. These results suggest that IL2 functionally extends from Trp-271 to Ile-290.

In addition to MTSEA, the negatively charged MTSES was also used to treat some of the mutants. Although MTSES failed to inactivate X5C over this concentration range, Fig. 6 shows that it was quite effective at inactivating mutants at positions 272–279 including the three lysine-to-cysteine mutants K272C, K275C, and K279C.

The differences in sensitivity of K272C, K275C, and K279C to treatment with MTSES and MTSEA were apparently due to their inability to react with MTSEA. Treatment with MTSEA up to 1 mM failed to protect these mutants from subsequent inactivation by MTSES as shown in Fig. 7. In this experiment, membranes were treated with various concentrations of MTSEA prior to MTSES.

The EC50 concentration of MTSEA, sufficient to half-maximally inactivate each mutant, was used to calculate rate constants with the assumption of bimolecular kinetics and a first-order time course of activity loss (29). In the 15-min inactivation reaction, half-maximal inactivation gives a t1⁄2 of 15 min and a pseudo first-order rate constant of 0.046 min⁻¹. From this value and the concentrations of MTSEA required for half-maximal inactivation, the rate constants shown in Fig. 8
were calculated. We interpret a higher inactivation rate as greater accessibility to MTSEA. Six cysteine mutants, G273C, V274C, S277C, V280C, V281C, and T284C, reacted with rate constants over 20 s^{-1} M^{-1}, which is >75 times faster than the background X5C, whereas the remaining mutants reacted with rate constants below 1 s^{-1} M^{-1}.

**DISCUSSION**

The data presented in Fig. 5 clearly define a region of SERT from approximately Trp-271 through Ile-290, where the residues are likely to be in contact with the cytoplasm. A significantly shorter region (271–279) was initially predicted from hydropathy profiles to constitute the IL2 of SERT (16, 17, 28). The region is likely intracellular, because mutants with cysteine at these positions were inactivated by MTSEA in membrane preparations (Figs. 4, 5, and 8) but did not react with extracellular MTSEA in intact cells (Table II). It is possible that IL2 extends even beyond this range, because residues proximal to Trp-271 or distal to Ile-290 might react with MTSEA with no consequence for transport or binding activity. However, it is problematic to measure reactivity in the absence of a change in activity.

There were two mutations within the reactive region, K275C and K279C, where substitution with cysteine did not adversely affect transport or binding activity and which retained a considerable fraction of activity even at high MTSEA concentrations (Fig. 5), although the negatively charged MTSEA was able to totally inactivate each of the lysine-to-cysteine mutants (Fig. 6). Cysteine mutants at neighboring positions were mostly inactivated by the same treatment. We considered the possibility that the modified cysteine residue, a disulfide with 2-thio-ethylamine, was sufficiently similar in structure to the endogenous lysine residues at these positions so that the modification did not disrupt function. An example in which an MTSEA-modified cysteine mimicked a lysine residue was described for the dopamine D2 receptor (30). An alternative possibility was that MTSEA did not fully modify K275C or K279C, possibly because of electrostatic repulsion from neighboring residues. Consistent with this latter hypothesis, pre-treatment with MTSEA failed to protect these mutants against subsequent inactivation by MTSES, as would be expected if MTSEA modified these positions (Fig. 7). Thus, it is most likely that MTSEA did not react with K275C and K279C.

At six positions, cysteine substitution mutants were inactivated with dramatically higher rate constants than those of the remaining positions tested (Fig. 8). These six positions appeared to be in a repeating pattern, every 3–4 residues, within IL2. When modeled as an α-helix (Fig. 9), the more reactive positions mapped to a vertical stripe along the side of the helix encompassing four helical turns. This was somewhat surprising, because structure prediction algorithms did not identify this region as α-helical. Neither the Garnier-Osguthorpe-Robson algorithm, nor the Agadir algorithm (31), nor the Predict-Protein algorithm (32) predicted helical structure in this region. The pattern or reactivity, however, strongly suggests that at least part of this region is in an α-helical conformation. To our knowledge, this is the first observation of secondary structure in the cytoplasmic domain of a neurotransmitter transporter in the NSS family.

We assume that the increased reactivity of G273C, V274C, S277C, V280C, V281C, and T284C results from increased accessibility of the substituted cysteine residue to aqueous MTSEA. Other possibilities are that the microenvironments of these positions lead either to greater ionization of the cysteine sulfhydryl group (pK_a = 8.33) or to a higher local concentration of MTSEA. We consider the first of these possibilities unlikely, because at pH 7.3 we expect ~10% of the cysteine sulfhydryl groups to be ionized, and the maximum increase from a decrease in pK_a would be 10-fold. The more reactive mutants are up to 100-fold more reactive than the less reactive ones (Fig. 8). It is also possible that limited aqueous accessibility increases the pK_a of cysteine at the less reactive positions. In the absence of any evidence in favor of an increased local MTSEA concentration, we consider that accessibility is the most likely determinant of reactivity in this region.

Our estimates of first-order rate constants for inactivation were based on measurements over a range of MTSEA or MTSES concentration rather than time courses at a single concentration. As a consequence, all these estimates were based on the amount of inactivation in a 15-min incubation. Because
hydrolysis of the MTS reagents during this time competes with inactivation, the concentrations are only estimates of the true rates. However, by using the same time point for each measurement we minimize the variability in the fraction of reagent hydrolyzed between mutants that reacted at different rates.

Because the positions studied were accessible only in disrupted plasma membrane preparations that expose residues facing the cytoplasm, we have used β-CIT binding rather than transport as an assay to follow modification of these mutants. In the remaining positions, cysteine substitution mutants were 10- to 100-fold less reactive. A shown is the same helical net with the positions highlighted where substitution with cysteine increased activity above that of wild type.

The dramatic change in reactivity from one side of the helix to the other suggests that other parts of the protein or associated proteins must restrict access to some of the residues but not to others. The helical region could be an extension of one of the adjacent transmembrane domains as seen in the structures of Ca²⁺-ATPase (33) and a glutamate transporter homologue (34) and postulated for SERT extracellular loop 4 (22). Alternatively, the IL2 helix could be independent of the transmembrane domains as in the mitochondrial adenine nucleotide transporter (35). We must also consider the possibility that a major part of the accessible region represents part of TM5 and that the accessibility of this region indicates that TM5 lines part of the permeation pathway for substrates and ions.

On the opposite side of the putative helix from the reactive residues are three lysine residues. These residues are highly conserved within the animal members of the NSS family (Fig. 1). Even among the prokaryotic members of the family there are usually at least two lysine residues separated by 2–3 residues, suggesting that this basic stripe along the IL2 helix is an important structural or functional element. When one of these lysines was replaced with cysteine (as in K275C or K279C), MTSEA (but not MTSES) reactivity was reduced dramatically (Figs. 4–6), suggesting that MTSEA was repelled by the electrostatic influence of the remaining lysine residues.

Cells expressing many of the cysteine mutants in this region transported 5-HT more rapidly than the parental X5C construct (Fig. 2). Our results (Figs. 3 and 9) suggest that the increased activity of these mutants was not due to higher levels of surface expression. Moreover, several of these mutants had greater maximal transport activity when normalized for surface expression than wild type SERT. The pattern of positions where cysteine substitution elevated $V_{\text{max}}$ over that of wild type was not a typical α-helical pattern (Fig. 9B) but rather delineated a set of positions at an acute angle to the putative helical axis. In the middle of this region is Pro-288. If a kink was formed in the helix at this point, then the residues where cysteine replacement increased activity could potentially define a contiguous region.

The ability of mutations in this region to increase transport activity suggests the possibility that the region between TM4 and TM5 may fulfill a conformationally active role in the catalytic cycle of SERT. Disruption of the native conformation, evidently an α-helix, may allow greater flexibility in this region and a consequently increased turnover. In the related GABA transporter, GAT-1, Hansra et al. (36) suggested that IL4 acts similarly as a negative regulator of transport rate, presumably by forming a barrier near the substrate permeation path. If IL2 represents an inhibitory element by decreasing SERT activity through intramolecular interactions, then mutations in SERT IL2 might disrupt its inhibitory influence, possibly by destabilizing the α-helix, and increase the intrinsic activity of the transporter. We are currently investigating this possibility that mutations or other modifications in this region affect activity by disrupting the apparently helical structure between Gly-273 and Thr-284.

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