Critical Amino Acid Residues in Transmembrane Span 7 of the Serotonin Transporter Identified by Random Mutagenesis*

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The serotonin transporter (SERT)1 is responsible for the reuptake of serotonin from the synaptic cleft following neurotransmission. It is a member of a large superfamily of homologous transporters that use the transmembrane Na+ and Cl− gradients as their source of energy (for reviews see Refs. 1–4). This superfamily includes transporters specific for serotonin, dopamine, norepinephrine, glycine, γ-aminobutyric acid, proline, creatine, and betaine. The members of this family are predicted by hydropathy analysis to contain 12 transmembrane-spanning domains (TMs). These hydrophobic domains are thought to be α-helical in structure; however, there is little experimental evidence for this hypothesis. The serotonin transporter is most closely related to transporters specific for the other biogenic amines dopamine and norepinephrine. The three biogenic amine transporters are the molecular targets of important psychoactive drugs, including cocaine, antidepressants, and amphetamines (5–8). Detailed knowledge of these transporters’ structures and functions could allow the rational design of improved therapeutics for the treatment of depression and drug abuse.

The serotonin transporter binds serotonin and transports it into the cell by a mechanism that is thus far largely unknown. However, it can be inferred that certain steps must occur. The process most likely begins with the binding of serotonin, Na+, and Cl− ions. The energy of this binding is then transduced into conformational changes that result in translocation of both serotonin and ions across the membrane into the cell. Finally, the transporter returns to its original state, ready to receive a new substrate molecule from outside the cell. This final step is thought to be facilitated by the outward movement of a K+ ion (2). In order to elucidate this mechanism, it will be necessary to determine which amino acid residues of the transporter play specific roles. For example, we need to know which specific residues interact with serotonin; which interact with Na+, Cl−, and K+; and which residues participate in the conformational changes associated with translocation.

This information is gradually being gathered by expression and site-directed mutagenesis of the cDNA clones encoding SERT and other members of the superfamily. Since each transporter contains about 600 amino acid residues, a choice must be made of which residues to mutate first, based on hypothetical considerations. Certain types of residues, for example charged residues residing in the membrane, are thought likely to play important functional roles. Residues whose identities are highly conserved in the superfamily are also thought likely to be functionally important. These considerations, while they have proven useful, have certain limitations. Lack of information makes it difficult to predict exactly what types of residues are likely to make up a serotonin binding site or are likely to participate in conformational changes. Mutagenesis studies that are limited to conserved residues will not detect residues that are responsible for individual differences among the transporters, such as in substrate specificity or drug sensitivity.

In this study, we have taken another approach. We have chosen a specific region of SERT, predicted transmembrane span 7 (TM7), and randomly mutated all of the residues within that region without presuming that any specific residue or type of residue will be important. This span was chosen for several reasons. Data from numerous studies on chimeric transporters have suggested that maintaining proper interactions between TM7 and other nearby spans is critical for transporter function (9–11). Previous mutagenesis of TM7 in the dopamine transporter has implicated two serine residues as important for transport activity (12). TM7 is also a region where the dopamine and norepinephrine transporters show a particularly close homology that is not shared by the serotonin transporter. This suggests a possible role in substrate specificity, since the dopamine and norepinephrine transporters share the ability to transport dopamine, while SERT does not. Using random mu-
tagenesis, we have been able to identify a group of functionally important residues in TM7, based on their sensitivity to non-conservative mutations. These residues form a stripe that runs down one side of the putative α-helix, lending support to this structural prediction. Studies of transport by these mutants under low Na⁺ conditions suggest that at least some of them play a role in the interactions of the transporter with Na⁺.

**Experimental Procedures**

**Materials**—Plasmid DNA was prepared using Qiagen midiprep kits (Qiagen, Inc., Valencia, CA). Restriction and PCR fragments were purified from agarose gels using the GeneClean kit (Bio 101, Inc., Vista, CA). All other reagents were obtained from Sigma unless otherwise indicated.

**Mutagenesis**—We used the PCR-based “megaprimer” method to prepare all random and specific site-directed mutants in this study (13–15). This method uses three primers: one mutagenic primer and two non-mutagenic primers, which lie outside the region to be mutated and are used solely for amplification. In the first round of PCR, the mutagenic primer and one outside primer are used to amplify a partial fragment of the region where the mutation will be created. This mutagenized fragment is then purified and used as a “megaprimer,” together with the second outside primer, in a second round of PCR to amplify the entire region. Conditions for the first round of PCR were 30 cycles: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Conditions for the second round of PCR were 25 cycles: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. PCR was performed in an MJ Research Minicycler (MJ Research, Inc., Watertown, MA) for 26 cycles of 95 °C for 2 min, 55 °C for 2 min, and 72 °C for 3 min. Conditions for the second round of PCR were as follows: 1) 25 cycles of 94 °C for 2 min, 50 °C for 2 min, and 72 °C for 3 min. This PCR reaction ended with an elongation step at 72 °C for 7 min.

The plasmid pRSTag carries a cDNA encoding the rat brain serotonin transporter, with the addition of sequences coding for a c-Myc epitope tag at the N terminus and a FLAG epitope tag at the C terminus (16). We used site-directed mutagenesis to add a silent mutation at base pair 1273, creating a new, unique MfeI restriction site (plasmid pRSTagM). This site and a native, unique Nhel site at base pair 1077 flank the sequence coding for predicted TM7. These sites were used to subclone all subsequent mutants back into the full-length wild type cDNA. All mutants created for this study were sequenced in both subcloning vectors. The outside primers used for all of the mutants in this study were as follows: S1, 5′-CGCTCAGATCTTCTTCTCTCTTGG-3′; S2, 5′-CCAGGCGTGAATTAACATGG-3′ (complementary). These primers were also used to sequence the subcloned region. Three mutagenic primers (called A, B, and C) were used, corresponding to three segments of predicted TM7: A, 5′-GGCTTGCAGAATGGTGCATGGTGACAAGCAGCTTCG-3′; B, 5′-GCCATGACAACTTCTTCTGCTTGTAAATATTCACGCTGTCG-3′; and C, 5′-CCGTTCGGATGTCAATTTGCTACGCCTGCTGCTGC-3′. The underlined bases in each primer were randomly substituted by “doped bottle” synthesis, in which the synthesizer bottle corresponding to each base was intentionally contaminated with small amounts of the other three bases. For these experiments, we used 4% contamination (see “Results” for an explanation of how this level was chosen). Each doped bottle synthesis produced a pool of oligonucleotides, each member of which contained one or more random base changes. Use of these primers in PCR thus created a fragment that also represented a pool of randomly mutagenized sequences. The nonrandomized part of each primer either removed or created a new restriction site for screening (A removed a HindIII site; B created an SspI site; C created a BspEI site). As described above, the randomly mutagenized PCR fragments were cut with MfeI and Nhel and subcloned back into the wild type cDNA. Colonies were picked, minipreps were prepared using the boiling water lysis method, and the plasmids were sequenced for the presence of any aberrant sites, indicating incorporation of the mutagenic primer. A large plasmid preparation of each positive colony was then made and retested for the presence or absence of the screening sites. Plasmid preparations were also tested with the diagnostic enzymes PvuII and PvuI to detect any major rearrangements, insertions, or deletions in the SERT cDNA. Mutant colonies that passed these screens were sequenced and assayed for transport activity. This mutagenesis method was 90% successful in incorporating the mutagenic primer, as determined by the number of colonies carrying the new restriction pattern (163 out of 182 tested).

**Expression of Mutant SERTs**—The expression system used has been described in detail elsewhere (17, 18). Briefly, the plasmid pRSTagM contains a promoter for T7 RNA polymerase under control of the rat brain SERT cDNA. This promoter was used to express wild type and mutant SERTs in the vaccinia/T7 polymerase/HeLa cell system. HeLa cells were plated in 48-well plates at 50% confluency and allowed to grow overnight in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA), 100 units/ml penicillin G, and 100 µg/ml streptomycin (Life Technologies, Inc.). The next day they were infected with a vaccinia virus strain, VTF-7 (17), which makes T7 RNA polymerase (added to the cells in 40 µl/well Dulbecco’s modified Eagle’s medium without serum). After a 15-min incubation with the virus at 37 °C, the cells were transiently transfected with wild type and mutant plasmids (400 ng of plasmid DNA and 1.2 µl of Lipofectin (Life Technologies) per well in 80 µl of Dulbecco’s modified Eagle’s medium without serum). Wild type SERT, no plasmid (mock-transfected) controls, and mutants were each transfected in duplicate wells.

**Transport Assays**—[3H]Serotonin transport assays were carried out the next day between 19 and 24 h postinfection. The cells were washed with 250 µl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.3) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ per ml of PBSCM (dulbecco’s modified Eagle’s medium without serum). Wild type SERT, no plasmid (mock-transfected) control, and each mutant was tested at least three different times, in duplicate wells each time, and the results were averaged.

**Low Na⁺ Transport Assays**—The standard transport assay described above measures activity in the presence of saturating levels of Na⁺ (18, 19). We also tested the ability of each mutant to transport serotonin under low, nonsaturating Na⁺ conditions. For these experiments, transport was measured in 4.3 mM KCl, 150 mM NaCl, and either 0.1 mM CaCl₂ or 1 mM MgCl₂, plus either 15 or 150 mM NaCl. The low Na⁺ buffer also included 135 mM LiCl to maintain the same osmolality as the high Na⁺ buffer. Each mutant was again tested at least three times, in duplicate, in low versus high Na⁺, and the results were averaged.

**Molecular Modeling**—The 27 amino acid residues mutated in this study were modeled as an α-helix using the Chem-3D molecular modeling program (CambridgeSoft, Cambridge, MA). The α-helix was built using φ, ψ angles of −60 and −40°, respectively. These angles, which are the same as those predominantly found in soluble proteins, were also found in the transmembrane α-helices of the photosynthetic reaction center by crystallography (20). The resulting helix was energy-mimized with Chem-3D using the MM2 protocol.

**Results**

**Random Mutagenesis Method**—It was first necessary to optimize the level of contamination used in the doped bottle synthesis of the mutagenic primers. If bases were substituted too frequently, mostly multiple mutants would be isolated, producing many inactive mutants and complicating their analysis. If bases were substituted too infrequently, a large percentage of mutants with wild type sequence would be isolated, increasing the workload of colony isolation, DNA preparation, and sequencing. We developed a computer program to help us predict the results for each of the three oligonucleotides needed to mutate TM7. The program predicted, for any given level of bottle contamination, what percentage of oligonucleotides in the final product mixture would contain zero, one, two, three, or more substitutions. It also predicted the frequency with which stop codons would arise for each individual sequence. We used the program to empirically test various levels of contamination until we had optimized the mutagenesis to give us a high percentage of single, double, and triple mutants and the lowest possible percentages of mutants containing no base.
Mutants in TM7 of SERT were obtained using a set of three mutagenic primers (A, B, and C) that contained random base changes due to doped bottle synthesis. A computer program was developed to determine the appropriate level of contamination in each bottle to give a high percentage of single, double, and triple base changes. The program also predicted the percentage of stop codons that would be obtained.

### TABLE I

**Comparison of computer predictions and mutants obtained from random mutagenesis**

| Base changes | Primer A | Primer B | Primer C | Overall |
|--------------|----------|----------|----------|---------|
|              | Pred. %  | Obtained % | Pred. %  | Obtained % | Pred. %  | Obtained % | Pred. %  | Obtained % |
| None         | 9        | 14       | 9        | 18       | 8        | 10       | 9        | 14       |
| Single       | 24       | 31       | 26       | 31       | 27       | 33       | 23       | 6        | 22       | 22       | 14       | 24       | 37       |
| Double       | 31       | 28       | 31       | 33       | 10       | 23       | 6        | 22       | 14       | 22       | 14       | 24       | 37       |
| Triple       | 22       | 27       | 10       | 23       | 9        | 11       | 6        | 10       | 5        | 22       | 14       | 24       | 37       |
| Quadruple    | 10       | 0        | 9        | 9        | 11       | 6        | 10       | 5        |
| Quintuple or higher | 4    | 0        | 3        | 3        | 5        | 0        | 4        | 1        |
| Stop codons  | 4        | 0        | 2        | 0        | 7        | 0        | 4        | 0        |

*Pred., percentage of each type of mutant predicted by computer program.
 Obtained, percentage of each type of mutant actually obtained.*

Changes or stop codons.

Table I shows the predicted results at the chosen contamination level of 4% for the three mutagenic primers A, B, and C. The computer predictions for each mutagenic primer and for the overall project are shown in the left-hand columns. The actual percentages obtained are shown in the right-hand columns. We obtained a total of 80% single, double, and triple mutants for the whole project. The computer prediction was 77%, which agrees well. The results were slightly skewed in favor of single mutants and wild types. This may represent a slight disadvantage for highly substituted primers in the PCR reaction, since the increased number of mismatches would tend to make them bind less tightly to the wild type template.

**Sequencing**—One hundred five colonies that were positive for the incorporation of a mutagenic oligonucleotide by restriction digest (see “Experimental Procedures”) were sequenced. Of these, 74 were amenable to further analysis. The other 31 colonies included 13 wild types, 4 containing only silent mutations and 14 containing small insertions or deletions not detectable by restriction digest. Of the 74 usable mutants, 16 were duplicates and thus were not further analyzed.

**Transport Activities**—Table II shows the serotonin transport activity of the 58 remaining random mutants. This table also includes the results from 10 random mutants isolated in an earlier, pilot phase of the project. Another 34 mutants were added by site-directed, nonrandom mutagenesis to fill in gaps in the analysis, such as residues where no random mutations were isolated or where the only mutations that were isolated were highly conservative. A total of 102 mutants was analyzed for the 27-amino acid TM7 region. The activity of each mutant is shown as a percentage of the activity of the parent construct, pRSTagM. The serotonin transporter expressed from pRSTagM, which includes epitope tags at each end (see “Experimental Procedures”), has previously been shown by several groups to possess transport ability nearly identical to wild type SERT in this and other expression systems (16, 21).

Fig. 1 shows the transport activity of the mutants in relation to their positions in TM7. Each symbol represents a single mutation. Symbols connected by lines indicate multiple mutants. The mutants can be grouped into four activity classes as indicated by the shapes of the symbols. Fifty-three mutants had largely unimpaired transport activity, ranging from 61 to 100% of wild type activity (*white rectangles*). At the other end of the spectrum, 23 mutants had less than 5% wild type activity (*black rectangles with white letters*). In between, 14 mutants had severely impaired but detectable activity between 6 and 30% of wild type (*white ovals*) and 12 mutants were mildly impaired with 31–60% wild type activity (*white hexagons*). The majority of mutants (65%) showed either wild type or only mildly impaired activity. This finding is interesting in light of the fact that this sequence is highly conserved within the biogenic amine transporter family (56% exact homology and 89% functional conservation). All of the mutants expressed levels of protein similar to wild type as shown by Western blotting (results not shown).

**Sensitivity to Mutation**—Fig. 2 shows a summary of the acceptable (31–100% activity; *white rectangles and hexagons*) or unacceptable (30% or less activity; *white ovals and black rectangles*) amino acid substitutions for each position. For the single mutants, the data are identical to that in Fig. 1. For the multiple mutants, deductive reasoning has been applied wherever possible to determine which particular mutations are associated with loss of transport activity. For example, the mutant V367M,N368K had no detectable activity. However, another mutant, V367M,V382T, had nearly wild type activity. It is thus likely that the loss of activity in the first mutant is due to the N368K mutation, and this is shown as an unacceptable substitution. In many cases, these deductions could be corroborated by results from other mutants. Only mutations with very clear cut interpretations were included in Fig. 2. This chart also shows for comparison the corresponding sequences in the closely related dopamine transporter (DAT) and norepinephrine transporter (NET) as well as the range of substitutions found in the entire superfamily.

Six residues showed a high sensitivity to mutation, especially when functionally nonconservative residues were substituted: Asn-368, Gly-376, Phe-380, Gly-384, Tyr-385, and Met-386. Three of these residues are conserved throughout the superfamily: Asn-368, Phe-380, and Gly-384. Only one of these residues, Gly-384, was irreplaceable even by relatively conservative substitutions to alanine, serine, or cysteine. The other glycine in the span, Gly-376, was also very sensitive to mutation, but in this case the mutants did retain a low level of activity. This glycine is replaced by threonine in some members of the superfamily. One position, Phe-380, showed a preference for aromatic substitution, accepting tyrosine but not other, similarly hydrophobic residues such as valine or leucine. Mutants in residues Asn-368 and Tyr-385 retained activity only with certain hydrophilic substitutions. Met-386 required a hydrophilic substitution. Without more detailed knowledge of the transporter's structure, it is impossible to say why these preferences exist. In the cases of Asn-368 and Tyr-385, the ability to accept or donate hydrogen bonds may be the important functional characteristic that must be preserved. In all cases, is likely that a specific geometry and/or specific contacts with other regions of the transporter are preserved in the allowed substitutions, resulting in nearly wild type transport activity.

Transmembrane spans are thought to be α-helical in structure, and this idea has been confirmed in several membrane
TABLE II

Transport activity of random and site-directed mutant SERTs

Transiently transfected HeLa cells were incubated with $[^3]H$serotonin for 15 min to measure the initial rate of uptake for each mutant. Transport activity is shown as a percentage of wild type activity. Each value represents the mean and S.D. of at least three separate experiments, each of which was performed in duplicate wells.

| Mutants | Transport activity |
|---------|-------------------|
| **Single mutants** | % wild type |
| A361S   | 60 ± 4 |
| L362Q   | 86 ± 12 |
| V363T   | 65 ± 12 |
| T364I   | 103 ± 7 |
| S365I   | 89 ± 12 |
| V366L   | 98 ± 10 |
| V367T   | 82 ± 12 |
| N368D   | 76 ± 10 |
| N368I   | 19 ± 5 |
| N368L   | 0 ± 0 |
| C369W   | 92 ± 7 |
| T371I   | 64 ± 5 |
| S372T   | 52 ± 4 |
| S372A   | 46 ± 4 |
| S372C   | 8 ± 4 |
| F373C   | 92 ± 4 |
| F373Y   | 85 ± 17 |
| F373V   | 82 ± 8 |
| V374T   | 103 ± 3 |
| V374A   | 92 ± 6 |
| V374G   | 92 ± 13 |
| V374D   | 10 ± 3 |
| S375A   | 86 ± 6 |
| S375T   | 78 ± 14 |
| S375V   | 41 ± 12 |
| G376A   | 28 ± 7 |
| G376S   | 21 ± 5 |
| G376C   | 8 ± 2 |
| F377C   | 100 ± 21 |
| F377L   | 98 ± 5 |
| F377S   | 65 ± 13 |
| F377Y   | 56 ± 12 |
| V378T   | 88 ± 7 |
| V378G   | 72 ± 16 |
| E379V   | 96 ± 2 |
| P379T   | 95 ± 11 |
| I379L   | 79 ± 12 |
| I379S   | 77 ± 7 |
| I379F   | 68 ± 6 |
| I379N   | 66 ± 13 |
| F380Y   | 90 ± 9 |
| F380C   | 22 ± 8 |
| F380L   | 5 ± 1 |
| F380V   | 4 ± 1 |
| F380G   | 1 ± 1 |
| T381A   | 100 ± 9 |
| T381P   | 45 ± 5 |
| T381R   | 11 ± 2 |
| V382L   | 102 ± 13 |
| V382P   | 0 ± 2 |
| L383M   | 85 ± 4 |
| L383V   | 67 ± 24 |
| L383T   | 66 ± 13 |
| L383N   | 7 ± 3 |
| L383H   | 0 ± 1 |
| G384A   | 3 ± 2 |
| G384S   | 1 ± 1 |
| Y385N   | 74 ± 15 |
| M386L   | 58 ± 6 |
| M386T   | 5 ± 0 |
| A387S   | 90 ± 14 |
| **Double mutants** | % wild type |
| A361V,A401T<sup>a</sup> | 95 ± 9 |
| A361P,N368S | 46 ± 14 |
| L362V,T364A | 87 ± 25 |
| L362R,N368K | 1 ± 1 |
| L362R,N368I | 0 ± 0 |
| V363G,T364S | 85 ± 6 |
| T364S,V366E | 30 ± 9 |
| S365G,E386G<sup>a</sup> | 82 ± 16 |
| S365C,C398G | 72 ± 7 |

<sup>a</sup> These mutations are shown in the single mutant portion of Fig. 1 because, in each case, the second mutation is outside the mutagenized region. These secondary mutations are due to processing errors by the Taq polymerase during amplification.

proteins for which three-dimensional structures have been solved, including the photosynthetic reaction center (20), mammalian cytochrome oxidase (22, 23), bacteriorhodopsin (24), and the potassium channel of Streptomyces lividans (25). We used computer modeling to draw predicted TM7 as an α-helix. As shown in Fig. 3A, the positions of four of the six residues that were found to be highly sensitive to mutation fall on a stripe that runs at an angle down one side of the predicted α-helix. Another residue, Ser-372, also falls within the stripe by virtue of its physical position alone. This residue did not meet the criteria for inclusion as a critical residue because it can accept a functionally nonconservative substitution (alanine) while retaining a relatively high level of transport activity (46 ± 4%). However, it does show some functional significance as one of the positions where mutations affect Na<sup>+</sup> dependence (see below). It is also more sensitive to mutation than the other serines in TM7, Ser-365 and Ser-375 (see Fig. 2). The pattern formed by these five residues (Asn-368, Ser-372, Gly-376, Phe-380, and Gly-384) clearly suggests that this region of TM7 is α-helical. The helical pattern breaks down at the C-terminal (outside) end of the helix, where three residues in a row (Gly-384, Tyr-385, and Met-386) are sensitive to mutation. This region may perhaps be at the boundary where TM7 leaves the membrane, the α-helix ends, and the structure of the hydrophilic loop begins.

By contrast with the six sensitive residues, each of the other 21 positions in TM7 accepted at least one functionally nonconservative substitution without serious loss of activity (Fig. 2). In this group, the hydrophilic, potentially hydrogen bonding residues (threonine and serine) were all replaceable with hydrophobic substitutions (isoleucine, valine, or alanine) with
FIG. 1. Random and site-directed mutations made in TM7. Each symbol represents a single mutation. Symbols connected by lines indicate multiple mutants. The activity of each mutant is indicated by the shape or color of the box as follows: white rectangles, 61–100% of wild type activity; white hexagons, 31–60% of wild type activity; white ovals, 5–30% of wild type activity; and black rectangles, <5% of wild type activity. [\(^{3}H\)]serotonin uptake was measured as described under “Experimental Procedures.” The wild type SERT sequence is shown at the top and bottom.

FIG. 2. Summary of the effects of mutation at each position in TM7. The single mutants are shown as in Fig. 1, but deductive reasoning has been applied to the multiple mutants (see “Results”). The sequences above the mutations show the analogous regions from the DAT and the norepinephrine transporter (NET), as well as the wild type SERT sequence. The numbers refer to amino acid residue positions in SERT. Below the mutations, a list of the entire range of substitutions in the superfamily is given for each position. The activity of each mutant is indicated by the shape or color of the box as follows: white rectangles, 61–100% of wild type activity; white hexagons, 31–60% of wild type activity; white ovals, 5–30% of wild type activity; and black rectangles, <5% of wild type activity.
Critical Residues in TM7 of the Serotonin Transporter

Fig. 3. Positions of functionally important residues in the TM7 α-helix. A, results of mutagenesis shown on a space-filling computer model of TM7 as a putative α-helix (see "Experimental Procedures"). Side chains of residues with increased sensitivity to mutation are shown in dark gray. Labels are boxed for the six critical residues, and Ser-372 is circled (see "Results"). B, end on view of the putative TM7 helix, shown as a ball and stick model for greater clarity. Polar residues are shown in black, nonpolar in white, and aromatic in dark gray. Again, labels are boxed for the six critical residues, and Ser-372 is circled.

Little loss of activity. The hydrophobic residues (alanine, leucine, valine, methionine, phenylalanine, and isoleucine) could each be replaced with at least one hydrophilic residue. This group also contains a residue that is conserved throughout the superfamily, Thr-371. Here, substitution with a larger, hydrophobic residue, isoleucine, had no effect on activity, but substitution with proline, which tends to disrupt α-helices, completely abolished activity. This residue may be important for maintaining helical structure, but the hydroxyl side chain of threonine does not appear to be important despite its high conservation. It is also possible that this residue is important for some aspect of function that we do not measure under our transport assay conditions.

This position was one of four at which prolines were substituted (Fig. 2). Proline was accepted without loss of activity at position 361, which lies near the beginning (the cytoplasmic side) of the putative TM7 helix, perhaps indicating that helical structure begins after this residue. As mentioned above, position 371, which most likely lies in the middle of the helix, did not accept proline. A proline was also deleterious at position 382, but since a proline at the adjacent position 381 was well tolerated, this effect may not be due to its helix-breaking properties.

Very few mutagenesis studies have looked at the effects of adding novel charged residues to highly hydrophobic, predicted membrane-spanning regions. We isolated nine mutants containing aspartate, glutamate, lysine, or arginine and one containing histidine. Surprisingly, the presence of a novel, potentially charged residue did not invariably lead to complete loss of function, even for some positions predicted to lie buried deep within the membrane. In particular, mutant N368D retained full wild type activity. Aside from the addition of the acidic, potentially negatively charged, carboxyl group, the asparagine to aspartate mutation is very conservative structurally. Tolerance of the carboxyl group may indicate that this residue lies in a hydrophilic environment. Substitution of an arginine at position Leu-362 also allowed nearly wild type activity. This residue lies next to Ala-361, where a proline is tolerated. These residues, which lie at the N-terminal end of TM7, may not be part of the membrane-spanning α-helix at all but rather mark its cytoplasmic boundary. Other mutants containing V366E, V374D, and T381R retained 30 ± 9, 10 ± 3, and 11 ± 2% of wild type activity, respectively. These three residues are all predicted to lie on the same face of the α-helix but on the opposite side from Asn-368 (Fig. 3B). These residues are represented by serines in other members of the biogenic amine transporter family, perhaps indicating that the helix faces a relatively polar environment on this side as well. The remaining charged substitutions, at 368 (Lys), 369 (Arg), 372 (Arg), 383 (His), and 385 (Asp), all resulted in completely inactive transporters.

These results support the functional organization of TM7 shown in Fig. 3B. Here the predicted α-helix is viewed from the cytoplasmic side up through the membrane. Polar side chains are shown in black, nonpolar side chains in white, and aromatic side chains in gray. TM7 appears to be organized into three functional faces: a wide polar face where Asn-368 lies, another wide, aromatic face bounded by Phe-380 and Tyr-385, and a relatively narrow hydrophobic face that could be an area of contact with the membrane. The stripe of critical residues identified by sensitivity to mutation lies where the polar and aromatic faces meet (residue side chains are labeled). The aromatic face also contains a number of polar residues, as well as the positions where potentially charged residues could be substituted without completely abolishing activity (Val-366, Val-374, and Thr-381, also labeled in Fig. 3B). Both the polar and aromatic faces may thus face relatively polar environments, perhaps represented by the hydrophilic faces of neighboring TMs.

Effects on Na⁺ Dependence—Our standard transport assay uses a relatively high concentration of Na⁺ (137 mM), which is saturating for transport (18, 19). Under these conditions, defects in the ability to bind Na⁺ or in the coupling of Na⁺ to transport might be hidden. We therefore measured the transport activity of a representative set of mutants under low, nonsaturating Na⁺ conditions. Since the mutants varied in their overall levels of transport activity, we compared the transport activity of each mutant in 15 mM Na⁺ to its own activity in 150 mM Na⁺. For the wild type transporter, the ratio between its activity in 15 and 150 mM Na⁺ was 0.62 ± 0.10. Mutants whose Na⁺ dependence is similar to wild type would also have ratios of about 0.6, while mutants with defects in Na⁺ dependence would have different ratios. Fig. 4 shows this ratio
Critical Residues in TM7 of the Serotonin Transporter

**Fig. 4.** Transport activity of TM7 mutants in low Na⁺ versus high Na⁺, compared with wild type. [³H]Serotonin uptake was measured in low (15 mM) Na⁺ and high (150 mM) Na⁺ (see "Experimental Procedures"). Results are shown as the ratio between transport activity in low and high Na⁺ conditions. Results are given for the most nonconservative, but still sufficiently active, mutant at each position. No data are shown for Gly-384, where no active mutants were isolated, and for Met-386, where no active nonconservative mutants were isolated. *, mutants with 0.18 or lower ratios. **, mutations that are part of multiple mutants, as follows: V366T, V378I; N368C, C369G; M370T, F377G; V382T, V367M; and Y385C, I379V.

DISCUSSION

We have used random mutagenesis to identify six critical residues in predicted TM7 that are highly sensitive to nonconservative mutations (Table II; Figs. 1 and 2). The pattern of allowable substitutions at these residue positions suggests that, in each case, certain structural features must be preserved, such as a phenyl ring (Phe-380) or the ability to form hydrogen bonds (Asn-368 and Tyr-385). Other residue positions must remain as glycine (Gly-376 and Gly-384), and one position requires a hydrophobic side chain (Met-386). Mutations that do not preserve these features lead to serious loss of transport activity. Four of these six functionally important residues form a stripe that runs at an angle down the face of the predicted α-helix (Fig. 3A). Another residue, Ser-372, also falls within the stripe but was not as sensitive to mutation as the six critical residues. The presence of this pattern of sensitivity to mutation suggests that TM7 is indeed α-helical in structure. The angle of the stripe indicates that TM7 may be tilted, with respect to the membrane or with respect to other TMs that it may contact in the three-dimensional structure of the transporter. Alternatively, the angle might indicate the presence of a bend or kink in the α-helix.

Mutations at three of the six critical residues (Asn-368, Gly-376, and Phe-380) were found to affect Na⁺ dependence (Figs. 4 and 5). Mutants with nonconservative substitutions at these positions were impaired in their ability to transport under low Na⁺ conditions. These same mutants also had impaired transport ability in high Na⁺ (Table II), suggesting that their principal defect could be a highly reduced affinity for Na⁺. Nonconservative mutations at many of the positions neighboring these residues also impaired activity in low Na⁺, although most of these mutants had close to wild type activities in high Na⁺. Whether these residues play direct or indirect roles in Na⁺ dependence is not yet known. They may interact with Na⁺ directly, perhaps as part of a Na⁺ binding site or of a water-filled pore through which Na⁺ enters the cell. Alternatively, they might have indirect effects on Na⁺ dependence. One possibility is that these residues form interactions with residues in other TMs that are important for keeping the Na⁺ binding site in its correct three-dimensional conformation. Another possibility is that they are involved in the coupling of Na⁺ binding to later steps in the transport cycle. For example, Na⁺ binding might trigger a conformational change in the transporter that is needed to allow serotonin to pass into the cell. Residues in TM7 may be involved in making this conformational change.

Mutations at another of the six critical residues, Tyr-385, had no apparent effect on Na⁺ dependence (Fig. 4). This residue lies on the other side of the helix from the stripe, perhaps near or at the surface of the membrane. As an aromatic residue, Tyr-385 could potentially be involved in binding of the substrate, serotonin. The aromatic rings of tyrosine, tryptophan, and phenylalanine are known to be energetically favored binding sites for cations, such as the amine group of serotonin, through their cloud of π-electrons (26, 27). G-protein-coupled serotonin receptors, as well as the related dopamine and muscarinic acetylcholine receptors, have been hypothesized to possess a ligand binding pocket made up primarily of aromatic residues. The cationic amine group of serotonin is thought to bind to the anionic carboxyl group of an aspartate residue. This interaction is thought to be stabilized by a large surrounding cluster of conserved aromatic residues (28). This hypothesis is in part based on the finding of an aromatic binding pocket in the crystal structure of another amine-binding protein, acetylcholine esterase (29). It is tempting to speculate upon a similar role for the aromatic face of TM7 (Fig. 3B), although all but one of these residues (Phe-380) can accept nonaromatic substitutions. If a large number of residues were involved, then the energetic contribution of each residue to binding might be small enough that single mutations might not be very delete-
Aromatic residues in other regions of SERT and its relatives have also been implicated in both substrate and competitive inhibitor binding. For example, mutation of Tyr-176 in TM3 of SERT to cysteine renders the protein sensitive to hydrophilic methanethiosulfate reagents, and this position can be protected by the presence of serotonin (21). Mutation of the analogous tyrosine, Tyr-140, in the γ-aminobutyric acid transporter-1 (GAT1) leads to a specific defect in the recognition of its substrate, γ-aminobutyric acid (30). In TM11 of the human SERT homologue, the presence of Phe-586 has been shown to confer high affinity binding of the competitive inhibitor imipramine (31, 32). Two other aromatic residues, Trp-69 and Trp-222, also play important roles in the activity of γ-aminobutyric acid transporter-1 (33, 34).

Previous work with DAT has implicated TM7 in substrate binding. Uhl and co-workers (12) hypothesized that TM7 of DAT is involved in substrate binding by analogy with the binding of catecholamines to the β-adrenergic receptor. Studies of mutant β-adrenergic receptors have suggested the participation of an aspartic acid residue in TM1, which is proposed to bind the amine group, and two serines in TM5, which are proposed to form hydrogen bonds with the hydroxyls of the catechol ring (35). Uhl and co-workers (12) used site-directed mutagenesis to show that an aspartate in TM1 and two serines in TM7 are important for activity in DAT. In their study, simultaneous mutation of both serines in TM7 to alanine decreased transport activity to about 25% of wild type. In the present study, we mutated the analogous serines in TM7 of SERT to alanine individually. The S372A mutant had 46 ± 4% of wild type transport activity, and the S375A mutant had 86 ± 6% activity. The loss of either hydroxyl side chain by itself did not strongly affect transport activity, although it is still possible that loss of both at once might be as deleterious for SERT as it was for DAT. The critical aspartate in TM1, which is found in SERT as well, could also potentially be associated with an aromatic binding pocket, as hypothesized for serotonin receptors.

We will be performing more detailed kinetic analyses of the TM7 mutants in order to determine what role, if any, the critical stripe of residues might play in substrate and/or Na⁺ binding. One implication of the hypothesis that they form part of an aromatic binding pocket that surrounds the aspartate in TM1 is that TM7 would need to lie close to TM1 in the three-dimensional structure of the protein. According to recent results from the dopamine transporter, TM3 could be nearly as well (36). We plan to test these ideas using cross-linking agents and other probes of three-dimensional folding. These studies will provide us with important knowledge about the three-dimensional structure of the serotonin transporter. Integration of this knowledge with the functional data obtained from these and further mutations should then allow us to develop a model for how this physiologically important protein performs its function.

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Critical Residues in TM7 of the Serotonin Transporter

28106

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