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# Serotonin Transporters – Structure and Function

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## Introduction to SERT Mechanism

Serotonin transporter (SERT) selectively transports 5-hydroxytryptamine (5-HT) into nerve cells together with $Na^+$ and $Cl^-$ and, in the same reaction, transports a $K^+$ ion out of the cell. SERT is a member of the SLC6 gene family designated the neurotransmitter sodium symporter (NSS) family 2.A.22 by Saier (1999). Also in this family are many other transporters responsible for reuptake of small neurotransmitters, including glycine, γ-aminobutyric acid (GABA), dopamine (DA), norepinephrine (NE) and 5-HT across the plasma membrane of neurons and glia. The neurotransmitter transporters are plasma membrane proteins that take up extracellular neurotransmitters after release and thereby terminate the transmitters' action at extracellular receptor sites. They represent the first step in the process of transmitter recycling. These plasma membrane neurotransmitter transporters use transmembrane ion gradients of $Na^+$, $Cl^-$ and $K^+$ and an internal negative membrane potential for transport of their substrate neurotransmitters (Rudnick, 2002; Rudnick & Clark, 1993).

SERT is of interest also as a drug target. A variety of compounds that are used to treat clinical depression, including fluoxetine (Prozac), sertraline (Zoloft), paroxetine (Paxil) and citalopram (Celexa), are inhibitors of SERT. In addition to drugs that specifically target SERT, this transporter is also affected by cocaine and amphetamines, psychostimulant drugs that are widely abused. Cocaine acts as a simple inhibitor of SERT and also of the closely related NSS transporters for NE and DA (NET and DAT, respectively) (Gu, Wall & Rudnick, 1994).

**Key words:** Serotonin transporter — SERT — 5-HT — Structure — Function — SLC6 gene family

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## Stoichiometry and Mechanism of Transport

To understand the mechanism of a transporter like SERT in detail requires knowledge of four key properties of the protein: 1) The nature of the binding site, which determines how the transporter can selectively transport one substrate and not another. In cases like SERT, where ions are cotransported with substrate, the relative positioning of substrate and ion binding sites may be critical for coupling. 2) The pathways through which substrate and ions pass in their movement from one side of the membrane to the binding site and then from the binding site to the other side of the membrane. These need to be tightly coupled to each other so that they are not both open simultaneously, which would lead to uncoupled flux through the transporter. 3) Conformational changes that close access from one side of the membrane and open access to the other, which are required for translocation to occur. 4) Control of conformational changes so that they occur only when the appropriate ligands are bound at the binding sites. Otherwise, the transporter would catalyze uncoupled flux of any solute that occupied its binding site.

A generalized mechanism for transport is shown in Fig. 1. It is based on the well-known alternate access model (Jardetzky, 1966; Tanford, 1983). In this model, transporters are believed to function by alternately exposing a substrate binding site to the cytoplasmic and extracellular faces of the plasma membrane. The model allows solutes to be transported from one side of the membrane to the other, but more importantly, it provides a mechanism for a transmembrane concentration difference of one solute to be utilized as a driving force to generate a concentration difference for another solute. The two main mechanisms for this process, named symport and antiport by Mitchell (1963), couple the movement of two solutes moving in the same direction across the membrane or in opposite directions, respectively. In symport, two (or more) solutes bind

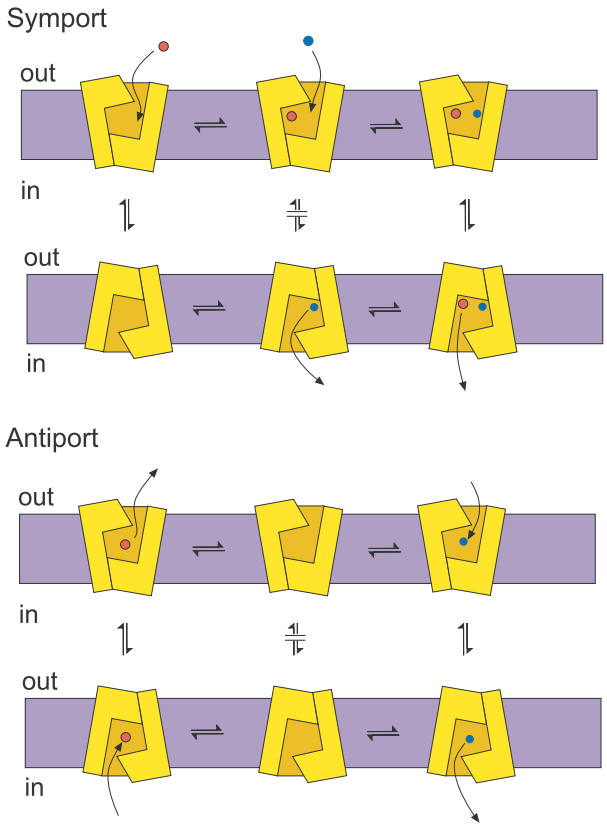


**Fig. 1.** Alternating access mechanisms for symport and antiport. For symport (*upper scheme*), two solutes bind from the ouside to form a ternary complex (*upper right*) with the transporter. A conformational change closes external access and opens a path to the inside (*right*). Following dissociation of the solutes, the empty transporter can undergo a second conformational change to regenerate the outside-facing form. Note that interconversion of the transporter with a partially filled binding site is incompatible with efficient symport of the two solutes. The same mechanism can accomodate antiport (*lower scheme*), if the rules are changed. For antiport, the empty transporter can not change its orientation between inside- and outside-facing forms. For maximum efficiency only the form with solute bound can interconvert.

to the transporter in one conformation, and are later released to the opposite side of the membrane from another conformation (Fig. 1, *Symport*). In antiport, binding of one solute facilitates conversion of the transporter to the form facing the opposite side of the membrane. After dissociation of the first solute, binding of a second solute allows the reverse conformational change leading to dissociation of the second solute on the side of the membrane where the first solute originated (Fig. 1, *Antiport*).

For coupled transport processes such as symport and antiport to work efficiently, the interconversion of transporter forms must obey certain rules (Jencks, 1980). The rule for symport prevents this interconversion from occurring when only one of the symported solutes is bound. Otherwise, each solute could cross the membrane independently, and coupling would be abolished. Conversely, the rule for antiport stipulates that the interconversion must take place only when a solute is bound and not when the binding site is empty. This rule ensures strict coupling between solute fluxes with no uncoupled flux. In more complex transport systems, more than two solutes would be coupled by symport, in which case all solutes would need to bind before interconversion. Symport and antiport can occur as part of the same mechanism if more than one solute molecules move across the membrane together in exchange for one or more solute molecules moving in the opposite direction. In these cases, both symport and antiport rules apply.

SERT catalyzes a complex reaction incorporating both symport and antiport. In 5-HT transport, SERT binds $Na^+$, $Cl^-$ and 5-$HT^+$ in a 1:1:1 stoichiometry and only then undergoes a conformational change that occludes the binding site from the extracellular medium and exposes it to the cytoplasm (Nelson & Rudnick, 1979). Dissociation of $Na^+$, $Cl^-$ and 5-$HT^+$ allows the transporter to return to its original conformation, but only after binding a cytoplasmic $K^+$ ion and releasing it to the extracellular medium (Fig. 2). The overall stoichiometry of this process is a 1:1:1:1 electroneutral exchange of $K^+$ with $Na^+$, $Cl^-$ and 5-$HT^+$ (Rudnick & Nelson, 1978; Talvenheimo et al., 1983; Rudnick, 1998).

The studies that provided the original evidence for this mechanism utilized platelet plasma membrane vesicles (Rudnick, 1977). These studies supported a 1:1:1:1 stoichiometry and also indicated that $K^+$ moved in a step distinct from the one in which 5-HT was transported (Nelson & Rudnick, 1979). SERT is also capable of conducting ionic current that is induced by 5-HT (Mager et al., 1994; Lin, Lester & Mager, 1996; Cao, Mager & Lester, 1997; Cao et al., 1998; ). Although this would, on the surface, appear to argue against a coupled electroneutral stoichiometry, it has become clear that SERT catalyzes an uncoupled flux in addition to the coupled transport process. An alternative mechanism has been put forward in which 5-HT and $Na^+$ movement are coupled within a channel (Adams & DeFelice, 2002; Petersen & DeFelice, 1999), but this mechanism does not explain how $K^+$ countertransport could be coupled to 5-HT uptake in an electroneutral process. It was subsequently demonstrated that interaction with syntaxin 1a blocks the uncoupled SERT current, revealing a coupled electroneutral process with the same stoichiometry that was originally proposed (Quick, 2003).

Following the rules for symport and antiport constrains the conformational changes of SERT. The rules require that for electroneutral 5-HT transport to be tightly coupled to $Na^+$, $Cl^-$ and $K^+$, the conformational changes must allow these solutes to cross the membrane but must also prevent their uncoupled

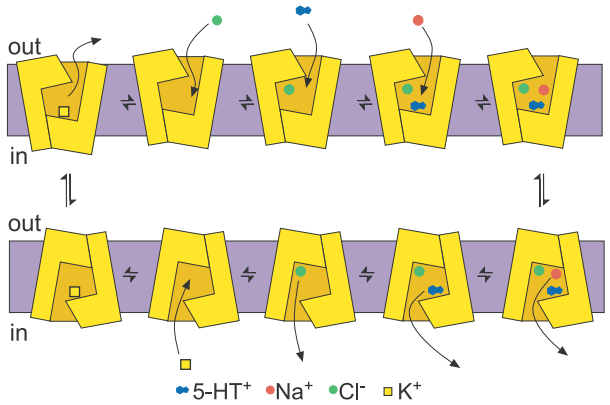


**Fig. 2.** Possible mechanism of serotonin transport. Transport of 5-HT together with $Na^+$ and $Cl^-$ ions requires binding of each solute to the transporter. These binding events are depicted in the three steps on the upper right of the figure. There is no evidence that the binding sequence is strictly ordered. Only after all three solutes are bound, is the transporter able to undergo a series of conformational changes that close off access to the extracellular medium and expose the binding site to the cytoplasm. This conformational change is depicted on the right side of the figure. After dissociation of 5-HT, $Na^+$ and $Cl^-$ on the cytoplasmic side of the plasma membrane, as shown by the three rightmost steps in the lower part of the figure, a cytoplasmic $K^+$ ion is able to bind (lower left). Once $K^+$ has bound, SERT is able to undergo another series of conformational changes that close off access to the cytoplasm and expose the binding site to the extracellular medium. Dissociation of $K^+$ to the medium completes the cycle. Note that for effective coupling of 5-HT influx to influx of $Na^+$ and $Cl^-$ and efflux of $K^+$, transitions between the extracellular-facing and cytoplasmic-facing forms of SERT should occur only when the binding site is occupied with 5-HT, $Na^+$ and $Cl^-$ or with $K^+$.

movement. Thus, conformational changes should occur when 5-HT, $Na^+$ and $Cl^-$ are bound or when $K^+$ is bound, but not when the binding site is only partly occupied (*see* Fig. 2).

## The Structure

The conformational changes required for transport depend on a structure that, until recently, was a topic only for speculation because of the lack of a suitable model. This situation changed as a result of the discovery that the genomes of many prokaryotes (bacteria and archaea) contained genes coding for proteins quite homologous to neurotransmitter transporters. In 2003, the first evidence became available showing that these proteins were actually transporters (Androutsellis & Theotokis et al., 2003). It showed that the TnaT protein of *S. thermophilu*m was a $Na^+$-dependent tryptophan transporter with properties similar to those of other NSS transporters. In 2005, the laboratory of Eric Gouaux provided a high-resolution structure from another bacterial homologue, LeuT from *Aquifex aeolicus* (Yamashita et al., 2005). Although this structure will certainly differ in detail from the structure of the mammalian proteins, it provides a framework for designing further experiments toward a variety of goals, among which are to test the relevance of the structure, to define the particular differences between the bacterial and mammalian transporters, and to understand the molecular motions within the structure that lead to transport.

**Table 1.** Residues in contact with leucine and sodium in LeuT and the corresponding residues in SERT and GlyT1

| LeuT | | | SERT | GlyT1 |
|---|---|---|---|---|
| Leu-$NH_3^+$ | Ala-22 | carbonyl | Ala-96 | |
| | Phe-253 | carbonyl | Phe-335 | |
| | Thr-254 | carbonyl | Ser-336 | |
| | Ser-256 | hydroxyl | Gly-338 | |
| Leu-$COO^-$ | $Na^+$ | | Asp-98 | |
| | Leu-25 | amide | Leu-99 | |
| | Gly-26 | amide | Gly-100 | |
| | Tyr-108 | phenol | Tyr-176 | |
| Leu side chain | Val-104 | | Ile-172 | Ile-178 |
| | Tyr-108 | | Tyr-176 | Tyr-182 |
| | Phe-259 | | Phe-335 | Tyr-356 |
| | Ser-256 | | Gly-338 | Ala-359 (S) |
| | Phe-259 | | Phe-341 | Trp-362 |
| | Ser-355 | | Ser-438 | Thr-458 |
| | Ile-359 | | Gly-442 | Leu-462 (T) |
| Na1 | Ala-22 | carbonyl | Ala-96 | |
| | Thr-254 | carbonyl | Ser-338 | |
| | Asn-27 | amide O | Asn-101 | |
| | Asn-286 | amide O | Asn-368 | |
| | Thr-254 | hydroxyl | Ser-336 | |
| | substrate | carboxyl | Asp-98 | |
| Na2 | Gly-20 | carbonyl | Gly-94 | |
| | Val-23 | carbonyl | Val-97 | |
| | Ala-351 | carbonyl | Leu-434 | |
| | Thr-354 | hydroxyl | Asp-437 | |
| | Ser-355 | hydroxyl | Ser-438 | |

### The Binding Site

One of the most interesting aspects of the LeuT structure is the relationship between the bound leucine and sodium ions. Because of the high resolution of the structure, the Gouaux group was able to identify two $Na^+$ ions bound together with leucine at the active site, thus providing a structural basis for coupling of $Na^+$ and solute fluxes. The carboxyl group of leucine was found to directly coordinate one of these sodium ions (designated Na1). In addition to the substrate carboxyl, Na1 is coordinated by the carbonyl oxygens of Ala-22 and Thr-254, the amide oxygens of Asn-27 and Asn-286, and the hydroxyl of Thr-254 (Table 1). It is informative to compare these residues with the corresponding residues of SERT. Most of these residues are identical, with the conservative substitution of serines for the two threonines. However, the substrate of SERT, 5-HT, has no carboxyl group to coordinate with Na1. Instead,

SERT, NET and DAT all contain an aspartate in the position corresponding to Gly-24, and the β-carboxyl group of this aspartate is in position to coordinate with Na1. Mutation of this aspartate to glutamate or cysteine was strongly inhibitory for SERT function (Barker et al., 1999; Henry et al., 2003). Like LeuT, all of the amino acid transporters in the family contain a glycine at the position corresponding to Gly-24 of LeuT.

The direct interaction between bound $Na^+$ and leucine provides a mechanism for coupling their binding, translocation, or both. Because of this interaction, substrate may not bind in the absence of $Na^+$, emphasizing the close coupling of the two solutes. However, for SERT and DAT, studies have repeatedly indicated binding of substrates and inhibitors in the absence of added $Na^+$ (Humphreys, Wall & Rudnick, 1994). This difference suggests that the ability of amino acid substrates to coordinate directly with bound $Na^+$ may provide an important component to the strength as well as of the specificity of interaction between transporter and substrate.

A second bound $Na^+$ ion in the LeuT structure (Na2) is coordinated by five residues in the LeuT structure. Three of these residues are identical in SERT. One of the two nonidentical residues coordinates with Na2 through its carbonyl oxygen and the other is a Thr-354 in LeuT and an Asp-437 in SERT. Because Asp-437 could participate in coordinating a $Na^+$ ion in SERT similar to Thr-354 in LeuT, it is possible that SERT also binds two $Na^+$ ions. Indeed, previous results comparing the $Na^+$ dependence of 5-HT transport and imipramine binding by SERT suggested that two $Na^+$ ions were involved in the latter process (Talvenheimo et al., 1983).

However, this poses questions about how Na1 and Na2 are related to $Na^+$ ions cotransported with substrate. Some transporters in the family, such as GAT-1, and GlyT1b are known to transport 2 $Na^+$ ions with each substrate molecule (Keynan & Kanner, 1988; Roux & Supplisson, 2000). SERT $Na^+$ stoichiometry has been determined by two methods and found to be one $Na^+$ per 5-HT (Talvenheimo et al., 1983; Quick, 2003). The $Na^+$ binding stoichiometry of LeuT is 2, but the $Na^+$ stoichiometry for transport is unknown. Many interesting mechanistic questions remain to be answered regarding ion coupling in SERT. Is binding stoichiometry always the same as transport stoichiometry or is it possible that only one of the two $Na^+$ ions in the structure is transported? Similarly, does the single sodium symported with 5-HT by SERT represent a single bound $Na^+$ ion or is an additional, non-transported $Na^+$ bound? Does either of the $Na^+$ sites predicted in SERT by the LeuT structure represent the site used for $K^+$ antiport? And finally, how is $Cl^-$ cotransport coupled to 5-HT in SERT?

Residues coordinating with the side chain of leucine are partly conserved in SERT (Table 1). In principle, the binding pocket created by the side chains of Val-104, Tyr-108, Phe-253, Ser-256, Phe-259, Ser-355 and Ile-359, which were found interacting with the side chain of leucine bound to LeuT (Yamashita et al., 2005), would be the primary determinant of substrate specificity. However, more than half of these residues are identical in SERT (Table 1) and only 3 differ. Is it possible that these three residues are sufficient to determine the difference between a leucine and a hydroxytryptophan side chain? This question may point out the limitations of a single static structure. When we have additional structures from the NSS family with other substrates bound, it might be possible to identify the residues responsible for substrate specificity with more confidence.

How well did the predictions from studies of chemical modification agree with the binding site residues found in the LeuT structure? Evidence from many laboratories (Chen, Sachpatzidis & Rudnick, 1997b; Barker et al., 1998; Barker et al., 1999; Chen & Rudnick, 2000; Adkins, Barker & Blakely, 2001; Henry et al., 2003; Melamed & Kanner, 2004; Zhou, Bennett & Kanner, 2004) had suggested that the binding site for the substrate and ions is formed, at least in part, by transmembrane (TM) domains 1 and 3. TM2, although it is adjacent to both TM1 and TM3 in the primary sequence, does not contribute directly to the binding site in LeuT, and results from SERT are in agreement with this observation. Although mutations in this region of SERT have effects on expression and $K_M$ for substrate, most of the positions in TM2 were neither accessible nor affected by substrate binding (Sato et al., 2004). However, mutations in TM2 of DAT have strong effects on the affinity of cocaine (Chen, Han & Gu, 2005; Sen et al., 2005) and may represent part of an inhibitor binding site or it may contribute to the position of TMs 1 and 6.

## Overall Architecture

An unusual aspect to the LeuT structure is that it contains an internal structural repeat consisting of two groups of five transmembrane domains in opposite topological orientations. We previously had no indication that TM domains other than TM1 and TM3 contributed to the permeation pathway, although analysis of channel proteins strongly suggested that it must consist of more than just these two (Spencer & Rees, 2002). From the LeuT structure, it is apparent that TM6 and TM8, the two corresponding TMs from the second 5-TM repeat, also contribute to the binding site. It will be important to examine residues in TM6 and TM8 to evaluate their contribution to the SERT binding site for 5-HT, $Na^+$ and drugs such as cocaine, amphetamines and antidepressants.

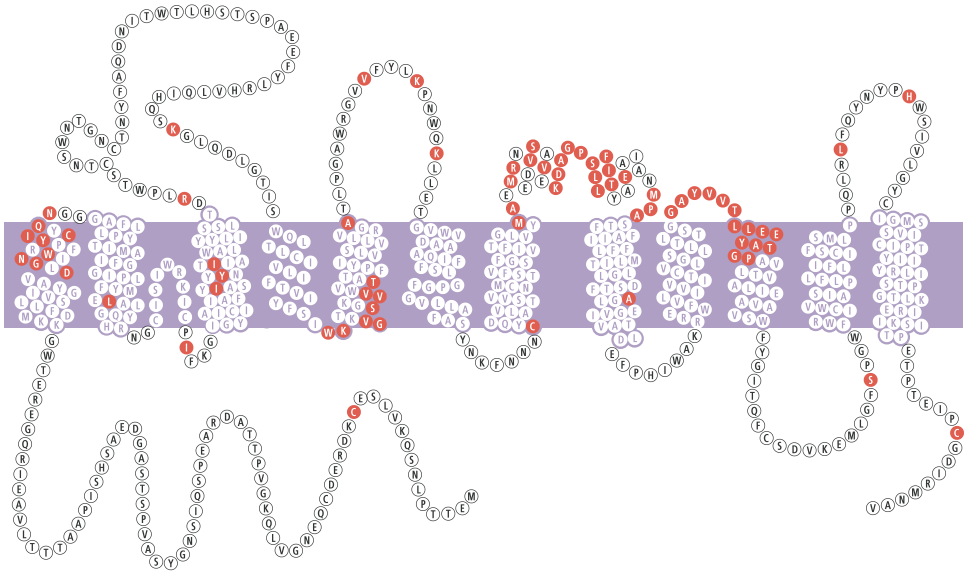

**Fig. 3.** Topology diagram of SERT. The sequence of SERT was aligned with that of $LeuT_{Aa}$ and presented as a topology diagram, using the beginnings and ends of each helix from the LeuT structure. Shaded residues are those where mutation to cysteine or lysine was found to introduce reactivity toward hydrophilic reagents. Not all positions were tested, and positions where reactivity was not detected are not marked.

The inverted structural repeats that comprise the first 10 TMs provide an explanation for a previously puzzling observation. The primary sequence of most NSS family members from animals predicts 12 TMs connected by hydrophilic loops. However, most prokaryotic sequences predicted only 11 TMs. The fact that the central core of LeuT, including the substrate and $Na^+$ binding sites, is formed by two copies of a 5-TM repeat provides an explanation for the functionality of a 10-TM transporter core and suggests that TM12 is not always required for transport function. This was recently confirmed by the demonstration that one of these truncated prokaryotic sequences encodes a functional tyrosine transporter (Quick et al., 2006).

The inverted repeat is becoming a common theme with transport proteins. It has been observed also with aquaporins and CrcB- and DUF606-family membrane proteins (Rapp et al., 2006) (although including the SMR proteins in this category may be premature). In LeuT, it is particularly intriguing that pairs of TMs with opposite topology fulfill similar roles. For example, TMs 1 and 6 both contain non-helical regions near the center of the protein, where contacts are made with $Na^+$ and leucine by both these TMs (Yamashita et al., 2005). Similarly, TMs 3 and 8 both are angled away from the membrane normal and both contribute residues to the substrate binding site. The function of this family of transporters is symmetrical – movement from the extracellular medium to the binding site mirrors movement from the cytoplasm to the same binding site. Moreover, transport by these proteins is reversible, with directionality being determined by the orientation of the $Na^+$ gradient (Rudnick & Wall, 1992). It is likely that transporter functions other than binding, such as permeation and conformational change, may also depend on the symmetrical contribution of pairs of TMs from the two parts of the internal structural repeat. A possible example of this is presented below (*see* "The Permeation Pathway").

## Topology

In the mammalian transporters, glycosylation sites in the second extracellular loop (EL2, between TM3 and TM4) indicated that EL2 is extracellular (Tate & Blakely, 1994). For SERT, many residues predicted by the initial topological predictions to lie in hydrophilic loops were demonstrated to be accessible from the appropriate side of the membrane (Chen, Liu-Chen & Rudnick, 1998; Androutsellis-Theotokis & Rudnick, 2002), indicating a 12-TM structure with NH2- and COOH-termini in the cytoplasm. These studies extensively utilized cysteine scanning mutagenesis of internal and external loops and transmembrane domains. Figure 3 shows a summary of some of the results for SERT superimposed on a topology diagram generated from the crystal structure of LeuT and using an alignment of the NSS family (Beuming et al., 2006). The highlighted positions, where various studies demonstrated chemical reactivity with hydrophilic reagents, indicate that many of these residues are in regions predicted by the structure to be exposed to solvent (Chen, Liu-Chen & Rudnick, 1997a; Chen et al., 1997b; Chen et al., 1998; Chen & Rudnick, 2000; Androutsellis-Theotokis, Ghassemi & Rudnick, 2001; Ni et al., 2001; Androutsellis-Theotokis & Rudnick, 2002; Henry et al., 2003; Mitchell et al., 2004; Sato et al., 2004).

In addition to confirming aspects of the topology, the LeuT structure revealed some incorrect assignments as well. Cysteines were modified at many positions that the LeuT structure predicts to be inaccessible from either face of the membrane. For example, at least one residue, Leu-137, thought to be

part of the IL1 loop between TM2 and TM3, is actually part of TM2. L137C reacted with MTSEA and the reaction was inhibited by 5-HT and cocaine (Androutsellis-Theotokis & Rudnick, 2002) so it was assigned to the IL1 loop. Similarly, Ala-441 was previously thought to be part of IL4 between TM8 and TM9. The LeuT structure revealed that this position is in TM8 only one helical turn away from binding site residues. When expressed in intact cells, A441C did not react with MTSEA, but in membrane preparations MTSEA inactivated A441C binding activity and this reaction was inhibited by 5-HT and cocaine.

The observation that residues predicted by the structure to be buried were nonetheless accessible in SERT suggests that these residues become exposed in other conformations of the protein. Thus, the form of LeuT that crystallized is likely to represent only one conformation of a transporter that must undergo conformational changes to allow substrates to bind and dissociate from both sides of the membrane.

## Conformational Changes

While it might be expected that substrate would protect a cysteine that replaces a residue in close proximity to the substrate binding site, the same behavior would not be expected of positions far from the binding site. However, many of the positions indicated by Figure 3 that react with MTS reagents are not close to the binding site for substrate and ions as defined by comparison with the LeuT structure, and yet they are protected by the presence of 5-HT or cocaine. Because of their distance from the substrate binding site, it is unlikely that these residues are directly occluded by 5-HT or ion binding, but the changes in reactivity are likely to reflect conformational changes in response to occupation of the binding site. Furthermore, many of the changes that were observed required not just 5-HT, but $Na^+$ and $Cl^-$ as well, suggesting that the change in accessibility represented entry of SERT into the transport cycle.

Among the residues proposed to be close to the binding site are Ile-172 and Tyr-176 in TM3. Part of the evidence for this conclusion was the observation that 5-HT could protect a cysteine at those positions from modification by MTS reagents (Chen et al., 1997b). Further studies demonstrated that 5-HT protected even in the absence of $Na^+$, confirming that 5-HT binding was not $Na^+$-dependent (Chen & Rudnick, 2000). Thus, it was remarkable to discover that in many SERT cysteine mutants, the effect of 5-HT on reactivity of a cysteine residue required $Na^+$ (Androutsellis-Theotokis et al., 2001) or both $Na^+$ and $Cl^-$ (Mitchell et al., 2004; Sato et al., 2004).

In the proposed mechanism for SERT-mediated 5-HT transport, at least two important conformational changes are required, as deduced from a variety of approaches (Rudnick, 2002). The first of these occurs when 5-HT, $Na^+$ and $Cl^-$ are bound to the extracellular form of the protein (upper part of Fig. 2). This conformational change occludes the bound substrates from the extracellular medium and exposes them to the cytoplasm. After the bound solutes dissociate to the cytoplasm and a cytoplasmic $K^+$ ion binds to SERT, the second major conformational change converts SERT from the cytoplasmic to the extracellular form (the lower part of Fig. 2), releasing $K^+$ to the medium. The requirement for specific ligand binding is crucial for the stoichiometric coupling of 5-HT, $Na^+$, $Cl^-$ and $K^+$ (Rudnick, 1998). There may also be additional, intermediate conformational changes that lead to occluded forms of SERT, similar to the form of LeuT in the crystal structure, in which the binding sites are exposed to neither side.

Of the many SERT cysteine replacement mutants that have been studied, there are different types of behavior that have been observed. We would like to be able to assign the various changes in cysteine accessibility with different states of the transporter, so as to understand which parts of the protein participate in the conformational changes accompanying binding and transport reactions. It is possible to assign some changes with binding and others with the transport steps. For example, cysteines at some positions (such as I172C and Y176C) were protected by 5-HT or inhibitor binding, did not depend on $Na^+$, and were protected both at 25° and at 4°C (Chen & Rudnick, 2000). This can be interpreted as simple steric occlusion of the reactive residue, which is a property of the outward facing transporter and does not require conformational changes.

A second type of behavior is typified by positions (such as Cys-357) where both 5-HT and inhibitors protected against cysteine modification, but the protection required $Na^+$ and was observed at 25° but not 4°C (Androutsellis-Theotokis et al., 2001). This behavior suggests an allosteric effect due to a conformational change that occurs when both 5-HT and $Na^+$ are bound to SERT. It probably does not represent the translocation step, because the protection was seen with both 5-HT and the non-transported inhibitor cocaine. Apparently, binding induced a conformational change that precedes the translocation event.

Because 5-HT transport requires $Na^+$ and $Cl^-$, it is significant that a third set of residues (such as S404C) was protected only by 5-HT and not cocaine (Mitchell et al., 2004). When tested, the protection required both $Na^+$ and $Cl^-$. This behavior probably represents the conformational change that actually translocates 5-HT across the membrane. The reasoning is as follows: 1) The effect is allosteric, suggesting a conformational change, since some residues

were protected (Mitchell et al., 2004) while others were potentiated (they reacted faster with MTS reagents when 5-HT, $Na^+$ and $Cl^-$ were present) (Sato et al., 2004). 2) Only substrates but not non-transported inhibitors such as cocaine promote the change in reactivity (Mitchell et al., 2004; Sato et al., 2004). 3) Other SERT substrates, such as MDMA, could replace 5-HT (Sato et al., 2004). These data suggest that the presence of 5-HT, $Na^+$ and $Cl^-$ transforms SERT from a predominantly extracellular-facing conformation to one that is predominantly cytoplasmic-facing, having transported 5-HT and released it on the cytoplasmic side of the membrane.

An important goal of research in this area is to understand how the conformational changes within SERT lead to alternate accessibility of the binding site from the two sides of the membrane. To accomplish this goal, it will be necessary to use biochemical approaches with the functional protein in its native environment. The ability to manipulate the state of SERT and to determine the effects on accessibility of cysteine residues placed at specific positions will allow the testing of possible transport mechanisms. The structure of LeuT, even though it does not provide much information about dynamic changes in the transporter structure, provides a useful framework for these studies.

## The Permeation Pathway

Yamashita et al. (2005) described the structure of LeuT as having the shape of a "shot glass" in which the cavity in the glass is an opening in the structure that leads from the periplasmic (extracellular) side of the protein almost to the bound leucine. The walls of the shot glass are formed by the extracellular portions of TMs 1, 3, 6, 8, and 10. Leucine is occluded in the LeuT structure, and there is no pathway for it to dissociate either to the periplasmic or cytoplasmic sides of the membrane (Yamashita et al., 2005). However, only a few residues (Tyr-108, Phe-253, and a salt bridge betweenArg-30 and Asp-404) block the exit of leucine to the periplasmic side. On the other side, there is almost 20Å of packed protein structure separating leucine from the cytoplasmic face of the protein. Taken at face value, the pathways from the periplasmic and cytoplasmic faces to the binding site would appear to be quite different.

It is tempting to speculate that this crystal structure is closely related to the extracellular form of LeuT, in which the binding site is open to the extracellular medium. Small movements of a few side chains would allow leucine dissociation. If so, we can use the symmetry inherent in the inverted structural repeat in the first 10 TMs of the LeuT structure to speculate further that the transporter form that releases bound solutes to the cytoplasm has the shape of an inverted shot glass. The walls of the cytoplasmic pathway should be composed of the regions related, by the symmetry of the repeat, to those that line the extracellular pathway. For example, the extracellular half of TM1 is related to the cytoplasmic half of TM6, TM3 to TM8, etc. Using this approach, we could speculate that the cytoplasmic pathway is lined by the cytoplasmic portions of TMs 6, 8, 1, 3 and 5.

Indeed, there is new evidence from SERT and a bacterial NSS transporter that such a structure is formed during transport. Zhang et al. (2005) were able to react cysteines in the cytoplasmic half of TM5 of SERT that correspond to buried residues in the LeuT structure. Using a series of cysteine substitution mutants designed to assess the extent of IL2, positions from Trp-271 up to Tyr-289 were found to be accessible to reagents on the cytoplasmic side. In the LeuT structure, Phe-203, which corresponds to SERT Tyr-289, is in TM5 more than halfway across the membrane from IL2 (Yamashita et al., 2005).

Within the region of SERT TM5 that was accessible to cytoplasmic reagents, some of the cysteine mutants reacted at rates up to 100-fold greater than others. These positions were in a repeating pattern consistent with an α-helix, one face of which was highly reactive (Zhang & Rudnick, 2005). This is expected from the helical nature of TM5 in LeuT, but also suggested that one face of the cytoplasmic half of TM5 was more accessible to cytoplasmic reagents, at least under some conditions. In membrane preparations from cells expressing these mutants, the reactivity of these TM5 residues was modulated by ligands in a manner consistent with the opening and closing of the cytoplasmic permeation pathway (Zhang & Rudnick, 2006). Cocaine, an inhibitor that binds from the extracellular medium, decreased the accessibility of the reactive TM5 residues, presumably by trapping SERT in the extracellular conformation. However, 5-HT, the normal substrate, increased accessibility. Significantly, the 5-HT effect required both $Na^+$ and $Cl^-$, suggesting that binding of these three solutes allowed SERT to undergo the conformational change that opens up the internal permeation pathway and allows 5-HT, $Na^+$ and $Cl^-$ to dissociate on the cytoplasmic side of the membrane (Zhang & Rudnick, 2006). This requirement for $Na^+$ and $Cl^-$ is consistent with the symport rules described under "Stoichiometry and Mechanism".

In the bacterial tyrosine transporter Tyt1, there are two endogenous cysteine residues (at positions 18 and 238) in the cytoplasmic portions of TM1 and TM6 (Quick et al., 2006). When either one or both of these cysteines was present, Tyt1 was sensitive to inactivation by NEM, but in a mutant with both cysteines replaced, Tyt1 was resistant. The inactivation of wild type or the single cys mutants was decreased by extracellular $Na^+$ and tyrosine alone had no effect. However, in the presence of $Na^+$, tyrosine

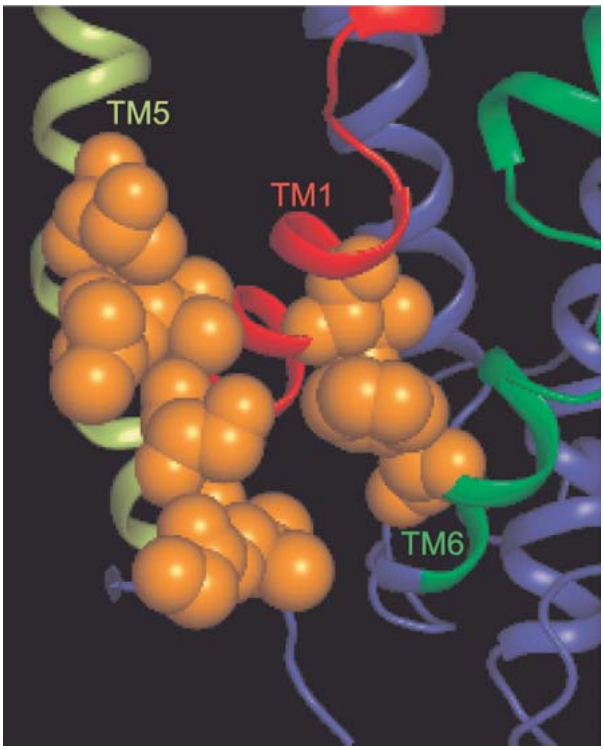


**Fig. 4.** Residues in the cytoplasmic permeation pathway of SERT. Potential permeation pathway residues in TMs 1, 5 and 6 are shown in a SERT homology model based on the structure of LeuT. Residues whose reactivity was altered in a manner consistent with opening and closing of the cytoplasmic permeation pathway in SERT (TM5) and Tyt1 (TM1 and 6) are shown in space-filling representation.

increased the inactivation by NEM. Quick et al. (2006) interpreted these results to mean that Na binding favored the extracellular form of Tyt1, in which the two cysteine residues were unreactive because the internal permeation pathway was closed. When tyrosine was added, it converted Tyt1 to the cytoplasmic form, in which the permeation pathway was open and the cysteine residues were exposed. Figure 4 shows a model of the cytoplasmic part of TMs 1, 5 and 6 of SERT with the reactive face of TM5 and the residues corresponding to Tyt1 shown as space-filling spheres. These positions are close to each other in space and could all contribute to the same permeation pathway.

On the extracellular face of LeuT, TM10 appears to form part of the inner wall of the shot glass. It is related to TM5 in that each one is the last TM in the two repeated 5-TM structural units that make up the core of the LeuT structure. Keller et al. (2004) examined the extracellular half of TM10 by cysteine scanning mutagenesis. They found that residues from Gly-484 through Pro-499 were accessible to extracellular reagents. In the LeuT structure, access from the extracellular medium to the binding site is blocked in part by a salt bridge between Arg-30 and Asp-404 (Yamashita et al., 2005). The corresponding residues in SERT are Arg-104 and Glu-493. However, in SERT, access in the EL5-TM10 region continued past Glu-493 up to Pro-499. According to the LeuT model, this position is almost in the middle of TM10, within 7Å of the bound substrate. The binding site residues for leucine and $Na^+$, formed by TMs 1, 3, 6, and 8, are located in the LeuT structure between the intracellular end of the TM10 accessible region and the extracellular end of TM5 accessible region. Thus, the accessibility of residues in TM5 and TM10 is consistent with these helices forming part of the permeation pathways that allow substrates to enter and exit their binding sites from the cytoplasm and extracellular medium, respectively.

Because the crystal structure may represent a form of LeuT close to the extracellular form, it is quite possible that in the cytoplasmic form, the external pathway exists in a more condensed conformation (the base of the inverted shot glass). Additional evidence exists that the extracellular permeation pathway is more condensed in the cytoplasmic form than is suggested by the LeuT structure. Residues in the tip of EL4 (between TMs 7 and 8) Val-33 and Gln-34 form parts of the periplasmic pathway in LeuT. In SERT, the corresponding residues Tyr-107 and Ile-108 are largely protected by 5-HT in the presence of NaCl (Henry et al., 2003; Mitchell et al., 2004), conditions likely to favor the cytoplasmic-facing form. Tyr-107 is particularly interesting, because the Y107C mutant was protected by 5-HT and rendered more reactive by cocaine (Henry et al., 2003). This is exactly the reverse of the TM5 cysteine mutants, but it is expected if TM1 in the extracellular pathway is occluded when the cytoplasmic pathway is open, and accessible when it is closed.

## Future Directions

Despite the major advance in our understanding provided by the structure of LeuT, many aspects of SERT structure remain unresolved. The difference in ion coupling between SERT and LeuT must be reflected in differences in the structure of the substrate binding site. In addition, there are three regions of SERT where the structure of LeuT provides little or no information. The first of these is EL2, which is much longer in SERT than in LeuT. EL2 is likely to be important for functional expression of SERT because replacing part or all of it with corresponding sequence from NET led to a protein inactive for transport (Stephan et al., 1997; Smicun et al., 1999). The additional sequence not present in LeuT includes glycosylation sites (Tate & Blakely, 1994) and a highly conserved pair of cysteine residues likely to form a disulfide (Chen et al., 1997a). Mutations in

these cysteines, or modification of one when the other was mutated, led to severe loss in activity (Chen et al., 1997a). Thus, it is likely that the parts of EL2 that are unique to animal members of the NSS family are important for function in ways that are not addressed by the LeuT structure.

Most neurotransmitter transporters, like SERT, contain much longer NH2- and COOH-terminal regions than does LeuT. The N-terminal region of SERT has been implicated in the regulation of ion conductance by syntaxin 1a (Quick, 2003). It is likely that these domains are important for regulation through interactions with other intracellular pathways. The NH2- and COOH-terminal regions are also likely targets for agents that control the subcellular localization of SERT, as has been demonstrated for the related norepinephrine and GABA transporters (Perego et al., 1997; Muth, Ahn & Caplan, 1998; Gu et al., 2001; Farhan et al., 2004). The structure of these domains and their potential interaction with the intracellular face of the central region of SERT are still unknown, and will doubtless be the subject of future study.

The images of LeuT, because they show a static structure, cannot tell us how transporters in this family move substrate and ions from the cell exterior to the binding site and then to the cytoplasm. These movements require conformational changes involving the transmembrane domains and possibly also the hydrophilic loops that connect them. Moreover, these movements are triggered by the binding of appropriate substrates and ions to the transporter (in the case of SERT, $Na^+$, $Cl^-$ and 5-HT for the forward reaction and $K^+$ for the return). Understanding the mechanism by which these binding events allow and control the conformational changes, and the nature of the conformational changes themselves, are important goals for future research in this area.

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