What Is an Antidepressant Binding Site Doing in a Bacterial Transporter?

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ABSTRACT LeuT is a bacterial amino acid transporter belonging to a large family of membrane proteins, including the neurotransmitter transporters that are targets for antidepressant drugs. The high-resolution structure of LeuT has provided an important model for understanding structure and function in this family. Two recent papers found that LeuT can bind tricyclic antidepressants, raising the possibility that it may also serve as a model for the pharmacological properties of neurotransmitter transporters.

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Do microorganisms suffer from depression? Do we care? Perhaps we should be paying more attention to the search for antidepressants that affect bacteria. Two recent papers address the issue of antidepressant binding to the bacterial transporter LeuT (1, 2). This amino acid transporter from *Aquifex aeolicus* is homologous to human transporters for the monoamines serotonin (5-HT), norepinephrine (NE), and dopamine (DA), which are targets for the most effective antidepressant drugs. These membrane proteins (SERT, NET, and DAT, respectively) belong to the NSS, or SLC6, family of transporters along with other neurotransmitter transporters for glycine and γ-aminobutyric acid (GABA) and many amino acid transporters in both the animal and prokaryotic kingdoms. LeuT is special because it is the first member of the family to yield the secrets of its structure.

Two years ago, Yamashita *et al.* (3) published a groundbreaking paper showing the high-resolution structure of this membrane protein together with a molecule of the substrate, leucine, bound at a position close to the center of the membrane. One of the features of this structure is an ion pair that acts as a molecular barrier between the bound leucine molecule and the aqueous pathway connecting the binding site with the extracellular medium. The two new papers each show the crystal structure of LeuT with one molecule of a tricyclic antidepressant (TCA) bound just outside this ion pair (see Figure 1). Although the two papers present similar structures, they disagree sharply over the effect of TCAs on leucine binding and come to quite different conclusions about the relevance of the TCA binding site in LeuT for human neurotransmitter transporters.

The TCAs were discovered by accident. Originally tested as an antipsychotic medication, imipramine (IMI) was found to be a poor substitute for chlorpromazine, on which its structure was based, but to have a beneficial effect on patients suffering from depression (4). Intensive synthetic efforts led to the development of many analogues based on the tricyclic structure of IMI, including clomipramine (CMI) and desipramine (DMI), two of the compounds shown to be bound in the LeuT structure. Because of the many unpleasant and outright toxic side effects of the tricyclics, synthetic efforts continued to develop more selective inhibitors for the monoamine transporters, on the basis of the observation that IMI acted by inhibiting SERT. This effort led to the modern antidepressant drugs, including Prozac (fluoxetine), Zoloft (sertraline), Paxil (paroxetine), and Celexa (citalopram). This class of drugs, sometimes referred to as selective serotonin reuptake inhibitors (SSRIs), is notable for reduced side effects and improved patient tolerance, but they have not totally replaced the tricyclic drugs in therapy (5).

The medical and economic importance of the monoamine transporters is further highlighted by the fact that they are targets for psychostimulants, including drugs used to treat attention deficit hyperactivity disorder, such as Ritalin (methylphenidate) and
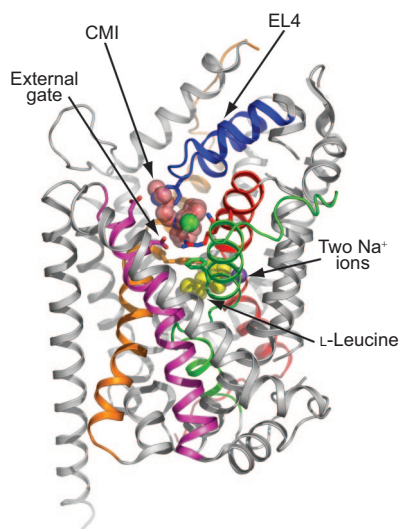
Figure 1. A structural model of the inhibited form of LeuT with leucine and CMI bound (courtesy of Satinder K. Singh, Oregon Health Science University). The structure, viewed from within the membrane plane, shows the bound leucine molecule (yellow), Na⁺ ions (purple spheres), and CMI (pink). Transmembrane helices (TMs) that contribute to the external gate structure are shown in the following colors: TM1, red; TM3, orange; TM6, green; and TM10, magenta. The fourth extracellular loop (EL4) is shown in blue.

Adderall (a mixture of amphetamine isomers). Some of these stimulants are drugs of abuse, such as cocaine, which inhibits SERT, NET, and DAT; amphetamine and its derivatives, which act by a complex mechanism to release the monoamines from their storage organelles and allow them to exit the cell (6, 7); and the recreational drug Ecstasy (3,4-methylenedioxymethamphetamine).

Given this background and the central role of the transporters in neurotransmission, there was intense interest in the structure of LeuT when it was published. In many respects, the structure agreed with and explained biochemical data on transporters in the NSS family. However, in other respects, clear differences existed in structure and

function. For example, the human transporters contain larger hydrophilic elements, including the NH_2- and COOH-terminal segments and the loops connecting transmembrane α-helices. LeuT is an amino acid transporter, and the carboxyl group of the bound leucine molecule in the crystal structure helps to coordinate one of the two sodium ions believed to be transported together with the substrate (Figure 2). In contrast, 5-HT, NE, and DA have no carboxyl group with which to coordinate sodium, but a nearby aspartate residue in the first transmembrane domain is thought to replace the missing carboxyl group. This aspartate is found only in monoamine transporters and is always a glycine in the rest of the family. Although this explanation satisfies the requirement for sodium binding, attempts to convert a monoamine transporter to an amino acid transporter, or *vice versa*, by replacing or introducing the aspartate residue have not been successful.

Another important difference between LeuT and the neurotransmitter transporters is that most of the latter require chloride for transport, but none of the prokaryotic members of the family studied to date have this property. This puzzle has recently been resolved through a combination of efforts, including electrostatics computation and mutagenesis. Two laboratories independently and almost simultaneously pinpointed an acidic residue in bacterial NSS transporters (Glu290 in LeuT) that was missing in the chloride-requiring members of the family (Figure 2). Restoring this carboxylic residue to the neurotransmitter transporters SERT or the GABA transporters GAT-1 and GAT-4 (where it is a serine residue in each case) removed the requirement for chloride, and placing a serine at that position in the bacterial transporters TnaT or Tyt1, which normally do not require chloride, made these transporters chloride-dependent (8, 9).

In light of these differences between prokaryotic NSS transporters and their human relatives, it would seem unrealistic to expect

that they share sensitivity to inhibition by the same drugs. After all, even transporters as similar as NET and DAT, which are 75% identical at the amino acid level, have dramatically different sensitivities to TCAs. And yet, these two recent papers both show that TCAs like IMI, DMI, and CMI each bind to and inhibit LeuT (1, 2). Indeed, both papers report finding TCAs bound in the same site within the LeuT structure. However, the functional data accompanying the structures diverge widely between the two papers and also are at odds with antidepressant binding studies using the human neurotransmitter transporters.

The two papers agree well on the location of bound TCA in the LeuT structure. Singh *et al.* (1) show structures with CMI, IMI, and DMI at resolutions of 1.85, 1.70, and 1.9 Å, respectively, and Zhou *et al.* (2) show a 2.9 Å structure with DMI bound. In all of these structures, a molecule of leucine and two sodium ions were seen in the same
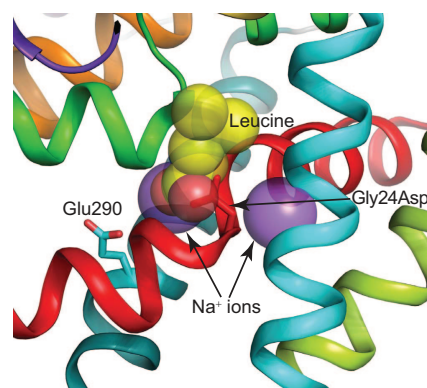
Figure 2. A view of the substrate binding pocket of LeuT. One of the bound Na⁺ ions normally interacts with the carboxyl group of leucine, but in SERT, NET, and DAT, an aspartate residue (shown here replacing Gly24) is positioned to take the place of the carboxyl group missing from these transporters' substrates. Glu290 is predicted to carry a full negative charge, which is stabilized by the nearby Na⁺ ion. In many Cl⁻-dependent transporters in the NSS family, this glutamate is replaced by a serine residue that is proposed to coordinate chloride.

binding site where they were found in the original structure (*3*), and this demonstrates that both substrate and inhibitor can be bound at the same time. In the new structures, the TCA was found just outside the leucine site, separated from that site by a small number of highly conserved amino acid side chains that create an "external gate" between leucine and an aqueous pathway leading to the extracellular medium. This gate consists of Tyr108 and Phe253 and the ion pair between Arg30 and Asp404. In the TCA-free structure, two water molecules were found between these charged side chains. However, in all of the new structures, this ion pair shows a direct salt bridge between Arg30 and Asp404. The difference appears to represent movement of the guanidinium side chain of Arg30 into closer proximity to the Asp404 carboxylate, probably because the bound TCA strengthens the ionic interaction by decreasing the local dielectric constant. Both papers also report that the positively charged amino group in the tail of the TCAs interacts with Asp401 and that the bound TCA was sandwiched between the external gate and the tip of the fourth extracellular loop (between transmembrane domains 7 and 8), which adopts a slightly altered conformation when TCA is bound.

Singh *et al.* observed noncompetitive inhibition of amino acid transport by CMI, an expected consequence of the fact that TCA and substrate can bind together in the same structure. The hallmark of this phenomenon is the inability of high substrate concentrations to overcome the inhibition, and in this case, it makes perfect sense because CMI remained bound even when leucine occupied the substrate site. Similarly, in binding experiments, [³H]leucine was displaced by other amino acids, which are expected to bind to the substrate site, but not by DMI, IMI, or CMI, which all bind to the external site.

These observations directly conflict with those of Zhou *et al.*, which show inhibition of [³H]leucine binding by DMI and nortriptyline, another TCA. How could the two laboratories find such similar structures and yet observe such different binding behavior? The answer may lie in the ability of TCAs to bind even when the substrate site is empty. In this way, DMI could have bound to LeuT first, effectively sealing off the substrate site and preventing leucine binding. It may be relevant that Singh *et al.* initiated their binding studies by adding leucine and TCA simultaneously to LeuT, but Zhou *et al.* preincubated LeuT with TCA before adding [³H]leucine. Under the latter conditions, inhibition of binding might represent an effect on the kinetics of binding rather than a change in equilibrium affinity. Although neither paper directly shows TCA inhibition of binding kinetics, Singh *et al.* show that the reverse reaction, leucine dissociation, was dramatically inhibited by CMI.

How seriously should we take these results in thinking about binding of TCAs and SSRIs to human neurotransmitter transporters? Here again, the two papers differ quite sharply. Zhou *et al.* made mutations in human SERT and DAT in which residues corresponding to those in the LeuT DMI binding site were mutated to match the equivalent positions in NET, which has the highest DMI affinity of the three monoamine transporters. They found the DMI sensitivity modestly increased in these mutants and concluded that DMI binds to the same site in hDAT and hSERT as it does in LeuT and that it inhibits transport the same way. Singh *et al.*, in contrast, suggest TCA binding to a site deeper in the protein, citing recent data from NET and SERT mutants with dramatic changes in TCA affinity.

Functional studies in the human transporters would seem to favor a different mode of inhibition from the noncompetitive transport inhibition described by Singh *et al.* IMI inhibited SERT competitively, and [³H]IMI binding was competitively displaced by 5-HT (*10*). Similar data for NET demonstrated competitive inhibition of transport by DMI (*11*) and competitive displacement of DMI by NE (*12*). Until these differences are resolved, it is prudent to question whether TCA binding to the human transporters really is similar to the phenomenon described in these papers with LeuT. And yet, the promise of using a tractable system like LeuT expression in *Escherichia coli* for development of antidepressant drugs compels us to consider possible explanations for the apparent mechanistic differences between bacterial and human NSS transporters.

One possible explanation lies in a phenomenon observed with several SERT ligands. It has been known for some time that high concentrations of some ligands, particularly 5-HT and citalopram, decreased the dissociation rate of IMI from SERT (*13*), whereas other ligands increased dissociation rate (*14*). The concentrations required to affect dissociation were much higher than those necessary to inhibit transport, an indication of the existence of a second, low-affinity binding site distinct from the inhibitory site. Could the TCA binding site found in LeuT represent the low-affinity site that affected IMI dissociation? If so, it would be surprising to find TCAs binding to this site in the human transporters, because TCAs were repeatedly shown not to affect dissociation in this assay (*13, 14*).

Another consideration is the uncertainty about the location of the substrate site in SERT, NET, and DAT. The lack of a carboxyl group on 5-HT, NE, and DA means that the close interaction between bound sodium and substrate in LeuT (*3*) does not exist in these human transporters. If the amines are not tethered by a carboxyl group, they might bind in such a way as to contact the external gate residues and impinge on a TCA binding site similar to the one found in LeuT. We cannot, therefore, rule out the possibility that in contrast to LeuT, where substrate and TCA can bind simultaneously, the same two binding sites may be mutually exclusive in SERT, NET, and DAT and that this leads to competitive interactions.

The new structures obviously raise many questions about the location of inhibitor binding sites in neurotransmitter transporters. However, they also open up possibilities for a deeper understanding of the way that these important therapeutic agents modulate transporter function. Perhaps the most exciting prospect is that the existence of this new binding site will spur the development of more effective agents with which to treat depression and other psychiatric disorders.

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