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Ligand binding in the extracellular vestibule of the neurotransmitter transporter homologue LeuT

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Abstract

The human monoamine transporters (MATs) facilitate the reuptake of monoamine neurotransmitters from the synaptic cleft. MATs are linked to a number of neurological diseases and are the targets of both therapeutic and illicit drugs. Until recently, no high-resolution structures of the human MATs existed, and therefore studies of this transporter family have relied on investigations of the homologues bacterial transporters such as the leucine transporter LeuT, which has been crystalized in several conformational states. A two-substrate transport mechanism has been suggested for this transporter family, which entails that high-affinity binding of a second substrate in an extracellular site is necessary for the substrate within the central binding site to be transported. Compelling evidence for this mechanism has been presented, however, a number of equally compelling accounts suggest that the

transporters function through a mechanism involving only a single substrate and a single high-affinity site. To shed light on this apparent contradiction, we have performed extensive molecular dynamics simulations of LeuT in the outward-occluded conformation with either one or two substrates bound to the transporter. We have also calculated the substrate binding affinity in each of the two proposed binding sites through rigorous free energy simulations. Results show that substrate binding is unstable in the extracellular vestibule and the substrate binding affinity within the suggested extracellular site is very low (0.2 M and 3.3 M for the two dominant binding modes) compared to the central substrate binding site (14 nM). This suggests that for LeuT in the outward-occluded conformation only a single high-affinity substrate binding site exists.

Keywords

LeuT, monoamine transporters, molecular dynamics, free energy calculations

Introduction

The human monoamine transporters (MATs) facilitate the reuptake of serotonin, dopamine and norepinephrine neurotransmitters from the synaptic cleft.¹ The transport of substrate against the concentration gradient is coupled to the downhill co-transport of Na⁺ and Cl⁻. MATs are associated with a large number of neurological diseases and are the targets of both therapeutic and illicit drugs.² Mechanistic studies of the SLC6 transporter family, to which the MATs belong, have been based on investigations of structural data from homologues bacterial transporters, since high-resolution structures of the human MATs has not been solved until recently, when the human serotonin transporter (SERT) was finally crystallized.³ In particular, studies based on crystal structures of the

Na⁺-dependent bacterial leucine transporter LeuT from *Aquifex aeolicus* have yielded unprecedented insights into eukaryotic SLC6 transporter function.⁴ Also recently, crystal structures of *drosophila* dopamine transporter have been published,^{5,6} serving as templates for improved homology models. The first structure of LeuT revealed 12 transmembrane helices (TMs) and a surprising internal structural repeat relating TMs 1–5 to 6–10 by a pseudo two-fold rotation in the membrane plane, now known as the LeuT fold (Figure 1).⁷ Furthermore, the structure also included a leucine molecule bound in the center of the transporter and contained two bound Na⁺ ions, in line with LeuT being a Na⁺-dependent transporter. The central substrate binding site is referred to as the S1 site, while the two Na⁺ ion binding sites are known as the Na1 and Na2 sites (Figure 1).

Figure 1. The S2 site in LeuT. To the left, LeuT is shown in blue ribbons (PDB code 2A65⁷). The leucine bound in the S1 site is shown in sticks with the carbon atoms in purple and the two bound Na⁺ ions are shown as yellow spheres. The S2 site is marked as an orange surface. To the right, the residues proposed to be involved in substrate binding in the S2 site are shown.

Substrate transport by LeuT and other SLC6 transporters is thought to follow the alternating access mechanism. Herein the transporter alternates between conformations in which the substrate binding site is accessible either from the extracellular or intracellular milieu (see Figure 2).⁸ Thus, when the transporter is outward-facing, the substrate and ions may bind to their respective binding sites from the extracellular side. The transporter then changes to an inward-facing conformation which allows for release of substrates to the intracellular side. Based on the LeuT structure, it has been suggested that the major conformational change from outward- to inward-facing consists of a movement of a four-helix bundle (TM1-2, TM6-7) with respect to a scaffold (TM3-5, TM8-10).⁹ This is also in line with a comparison of outward- and inward-facing structures of LeuT.¹⁰ An allosteric two-substrate mechanism has been suggested based on a combination of steered molecular dynamics (SMD) simulations and experimental binding and transport studies.¹¹ The mechanism suggests that release of substrate from the S1 site to the intracellular milieu is dependent on having a second substrate bound in the extracellular vestibule (Figure 1 and 2). This second site, commonly referred to as the S2 site, was initially identified based on SMD simulations where leucine was pulled out of the S1 pocket and towards the extracellular side.^{11, 12} The S2 site consists of a hydrophobic pocket comprised of L29, Y107, I111, W114, A319, F320, F324, and L400, which accommodates the leucine side chain and an ionic cleft composed of R30 and D404, which accommodates the charged amino- and carboxyl group of leucine (Figure 1). Shi et al. showed that wild type (wt) LeuT can bind two leucine molecules per transporter, whereas mutations in the S2 site (I111C or L400C) decrease the amount of bound leucine by ~50 %, ¹¹ suggesting that wt LeuT is able to bind leucine in the S1 and S2 sites simultaneously. Furthermore, the transport of alanine, which is actually transported faster by LeuT than leucine, was also seen to be markedly impaired for each of the two S2 mutants.¹¹ Additionally, SMD simulations of a homology model of the human dopamine transporter, suggesting the presence of a similar site in this transporter,¹³ have led to

the suggestion of an allosteric mechanism for MATs, similar to the one proposed for LeuT. Furthermore, single-molecule FRET studies of the L400S S2 mutant of LeuT showed that addition of alanine does not induce an increase in the transition rate between the inward-open and closed state, in contrast to what is observed for wt LeuT.¹⁴ Although several studies indicate the importance of the S2 site, the two-substrate mechanism has been challenged by a number of experimental and computational studies as well as by development of potent inhibitors consisting of molecular fragments placed in the S1 and S2 sites simultaneously and connected through a linker.¹⁵⁻¹⁷ In a study by Piscitelli et al., the LeuT:leucine ratio was measured using three different experimental methods, all of which suggested a single high-affinity binding site rather than two distinct binding sites.¹⁸ Additionally, the same study included uptake experiments, which suggested that transport is well described by a simple, single-substrate mechanism.¹⁸ Based on these results, Piscitelli et al. concluded that the S2 site is most likely a transient site that the substrate passes through on its way to the S1 site.¹⁸ This is also supported by MD simulations of a homology model of the human serotonin transporter for which the transition from the outward-facing to inward-facing conformation was only observed when the substrate serotonin was bound in the S1 site and no substrate was present in the S2 site.¹⁹ Further support for the single-substrate mechanism is the lack of crystal structures of any LeuT-fold transporter with substrate bound in the S2 site, despite multiple structures with inhibitors bound in the extracellular cavity.²⁰⁻²² It may be argued that if binding of substrate in the S2 site leads to immediate release of substrate from the S1 site, the state containing two bound substrates may be transient, and thus not easy to capture by crystallography. It should, however, then be possible to trap a substrate in the S2 pocket in the inward-facing state, which is not the case for the inward-facing crystal structure of LeuT, as it contains no substrate in either of the two sites, although a density that does not fit well with a substrate molecule is observed in the extracellular cavity.¹⁰ In accordance with earlier reports in the literature,^{23, 24} the

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4 relevance of the S2 allosteric mechanism hence remains unclear, and the aim of this study is to shed
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6 light on the role of the S2 site through the use of extensive computational methods. Using two known
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8 substrates, leucine and alanine, as well as the LeuT inhibitor tryptophan, we have applied MD
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10 simulations to probe the stability and conformational effects of ligand binding to the S2 site. A central
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12 part of the discussion of transport mechanism revolves around whether the S2 site is a low-affinity site
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14 that the substrate binds transiently to on its way towards the S1 site¹⁸ or whether it is a high-affinity site
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16 that retains a stable, longer-lasting interaction with a substrate subsequent to substrate binding in the S1
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18 site and thereby serve as a functional site.¹¹ To investigate this, the substrate binding affinities of the S1
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20 and S2 sites have been calculated through utilizing the free energy method thermodynamic integration
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22 (TI), hereby providing one more clue into the functional role of the S2-site.
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Figure 2. Suggested transport mechanisms for LeuT. (a) In the single-substrate mechanism, the binding of substrate (purple) in the S1 site and ions (yellow) in the Na1 and Na2 site causes a conformational change from outward- to inward-facing, which enables the release of substrate and ions to the intracellular side, followed by a return to the outward-facing conformation. (b) In the two-substrate mechanism, binding of a second substrate in the S2 site is necessary for the release of ions and substrate from the S1 site to the intracellular side (8).

Results and discussion

Firstly, the results of equilibrium MD simulations exploring the binding and effect of substrates and inhibitors in the S2 site are presented. This is followed by the results of determining the binding affinity for leucine and tryptophan in the S1 and S2 sites.

The binding of leucine, alanine, and tryptophan in the S2 site of LeuT.

The stability and conformational effect of ligand binding in the S2 site was assessed through MD simulations. Five simulation setups were constructed; two setups with substrate in the S1 site, **Leu-S1** and **Ala-S1**; two setups with substrate in the S1 and in the S2 site, **Leu-S1/S2**, and **Ala-S1/S2**; and a setup with the inhibitor tryptophan bound in both sites, **Trp-S1/S2**. The first four setups apply an outward-occluded conformation of the protein,^{7, 20} while the latter is based on a crystal structure in an outward-open conformation,²⁰ reflecting the conformations observed in crystal structures of LeuT with leucine, alanine and tryptophan bound, respectively. The latter setup is included in the analysis to serve as a control since it is known from x-ray crystallography that tryptophan, which is an inhibitor of LeuT, binds to both sites simultaneously.²⁰ In the setups with leucine or alanine bound, two different approaches were used to place the substrate in the S2 site. In the first approach, the substrate was positioned in the S2 site of the outward occluded crystal structures (PDB code 2A65⁷ for leucine and 3F48²⁰ for alanine) according to the crystal structure position of the tryptophan molecule found in the S2 site by overlaying the protein structures and extracting the coordinates of the common amino acid skeleton (Figure S1). Three simulations repeats were performed (referred to as **Leu-S1/S2-TX** and **Ala-S1/S2-TX**, with X=1, 2, 3 referring to the simulations repeat and **T** to the position of tryptophan in the S2 site as the source). In the other approach, molecular docking was applied to find the six most likely binding mode of substrate in the S2 site (Figure S2), and a single simulation were performed for each

docking pose (referred to as **Leu-S1/S2-PX** and **Ala-S1/S2-PX**, with X=1-6 referring to the docking Pose). The substrate-bound, outward-occluded conformation of LeuT was used as a starting point for all of the simulations with leucine or alanine bound either in the S1 site or in the S1 and the S2 sites (PDB codes 2A65⁷ for leucine and 3F48²⁰ for alanine). Since tryptophan stabilizes the outward-open conformation of LeuT, the **Trp-S1/S2** simulations were performed using this conformation (PDB code 3F3A²⁰). Common for all of the setups is the presence of a bound Na⁺ ion in each of the two Na⁺ ion binding sites, Na1 and Na2. MD simulations of Na⁺ bound, substrate-free LeuT have shown that under these conditions the ion in Na1 site can move to a quasi-stable site termed Na1'.²⁵ In addition, another site, termed Na1'', have been proposed to play a role as transient Na⁺ binding site for Na⁺ ions prior to binding to the Na1 and Na2 sites.²⁶ However, both crystallographic data^{7, 20} and MD simulations²⁷ support that ligand binding in the central S1 binding site is associated with stable binding of Na⁺ in the Na1 and Na2 sites. As all of the setups in our study include a ligand bound in the S1 site, the simulations are hence performed with the Na1 and Na2 sites each occupied by a Na⁺ ion. Due to the presence of two LeuT monomers in each simulation system, each simulation results in two monomer trajectories for analysis. An overview of the simulation setups is given in Table 2 in the Methods section.

Briefly, the different starting positions of the substrate in the S2 site cover a rather large part of the extracellular vestibule; the tryptophan-based ones all have their amino acid backbone as the inhibitor tryptophan in the S2 site with the carboxylate interacting with R30 and the ammonium group with D404 (Figure S1). The side chain methyl (with alanine in S2) and isopropyl (leucine in S2) is pointing towards T409. In contrast, in the study by Shi *et al.*¹¹ the leucine in the S2 site is placed with its side chain near I111 and L400 while the charged groups are interacting with R30 and D404. A similar

position of the ligand is found in docking pose P1 and P5 for leucine and docking pose P2 for alanine, whereas the remaining poses explore other orientations (Figure S2). Overall, the different orientation explored in this study gives a very thorough sampling of the extracellular pocket.

The stability of ligand binding is determined by measuring the z-coordinate of the center of mass of the ligand after aligning the simulation system such that the z-axis coincides with the membrane normal and the center of the central binding site, S1, is placed at $z = 0$. Initially, simulations of up to 100 ns were performed. For the simulations with substrate bound in the S2 site (**Leu-S1/S2**, and **Ala-S1/S2**), the substrate remained bound in the S2 site during the 100 ns in a subgroup of the simulations, and these were extended further up to 300 ns. For all the simulations, the ligand in the S1 site remains bound during the entire simulation (Figure S3). As seen from Figure 3, tryptophan also binds stably in the S2 site, which fits well with the fact that it has been co-crystallized in this site.²⁰ The results for alanine binding to the S2 site are, on the other hand, quite different, since alanine escapes from the binding pocket towards the extracellular milieu very rapidly in 16 of the 18 trajectories, and in the majority of the simulations dissociates already during the equilibration of the system. Rebinding of alanine to the S2 site is never observed and overall the results suggest that binding of alanine to the S2 site is not favorable. On the other hand, leucine remains bound to the S2 site for an extended time in some of the simulations and remains bound throughout 300 ns in a single trajectory (monomer A of **Leu-S1/S2-P5**). The results thus suggest that leucine binding in the S2 site may be possible.

Interestingly, the initial position of leucine in the S2 site in the **Leu-S1/S2-P5** setup is similar to the one suggested by Shi et al.¹¹ when they proposed the two-substrate mechanism for LeuT. In that study, simulations with leucine in the S2 site were also performed, and the substrate was observed to remain in the S2 site during the simulation time of 30 ns. In our simulation of the **Leu-S1/S2-P5** setup, we

assess the stability of this binding pose of leucine in S2 for a much longer time-scale than what was possible in 2008. We find that the leucine bound in the S2 site of monomer A maintains the ionic interactions with R30 and D404 the majority of the time. However, the initial binding mode is not preserved during the full simulation length of 300 ns as a rotation of the ligand occurs at ~185 ns. This results in a binding mode in which the hydrophobic tail points in approximately in the opposite direction as in the starting position. Additionally this simulation reveal that the leucine that was originally bound in the S2 site of monomer B binds in the top of the extracellular vestibule of monomer A and remains there during the last 50 ns of the simulation. It is likely that the binding of a second molecule in the extracellular vestibule contributes to the stable binding of leucine in the S2 site that is observed for this trajectory.

Figure 3. Displacement of ligand in the S2 site along the membrane normal. The time evolution of the z-coordinate of the ligand COM is shown using a running average with a window size of 1 ns. Each trajectory has been aligned such that $z = 0$ corresponds to the center of the S1 site. The data from the simulations in which substrate has been positioned in the S2 site according to the position of tryptophan

are shown in gray tones, while data from the simulations initiated from the docking poses are shown in colors.

Due to the rapid release of alanine from the S2 site in the majority of the simulations it is difficult to investigate the conformational effect on LeuT of having alanine in the S2 site. Therefore, the following analyses only include comparisons between the **Leu-S1**, **Leu-S1/S2** and the **Trp-S1/S2** simulations to evaluate how the conformation of LeuT is affected when the S2 site is empty, when a substrate is bound, and when an inhibitor is bound, respectively. Since leucine is observed to leave the S2 site during some of the **Leu-S1/S2** simulations, the analysis of these trajectories is split into two parts, where the ligand is considered bound when the z-coordinate of the center-of mass (COM) of the ligand is below 13 Å, while in the remaining snapshots the ligand is considered unbound.

The S1 site is gated towards the extracellular side by an aromatic lid consisting of Y108 and F253 as well as a salt bridge between R30 and D404 (Figure 4a). In the Trp-bound LeuT crystal structure, the amino- and carboxyl groups in the tryptophan molecule in the S2 site interact with the residues in the salt bridge, thus preventing the gate from closing. As seen from the distance distribution in Figure 4b, the residues are kept apart during the simulations with tryptophan in the S2 site (green line). From the simulations with leucine in the S2 site, it can be seen that the R30-D404 interaction is mainly formed when the substrate is released from the S2 site (purple dashed line). In accordance with this, the salt bridge interaction between R30-D404 is present most of the time in the simulations initiated without substrate in the S2 site (blue line). The extracellular gate is expected to close prior to the conformational change from outward- to inward-facing. EPR studies have also shown that substrate binding leads to a closed extracellular vestibule²⁸ which, based on our results, is not consistent with substrate binding in the S2 since this is observed to keep R30 and D404 apart in some of our

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simulations. Hence, the results suggest that either the allosteric transport mechanism is not valid or movement of the substrate from the S2 site into another part of the extracellular vestibule is necessary for closure of the extracellular gate and subsequent transport to occur.

Figure 4: Results of equilibrium MD simulations of LeuT with ligands bound in the S1 and S2 sites.

(a) The residues in the extracellular gating network in LeuT, consisting of Y108 and F253, which form an aromatic lid, and a salt bridge interaction between R30 and D404. The leucine substrate in the S1 site is shown in purple sticks. (b) The shortest distance between any of the three nitrogen atoms in the R30 side chain and any of the two oxygen atoms in the D404 side chain in the simulations. (c) SASA of Y108 and F253 side chains in the aromatic lid positioned just above the S1 site.

The crystal structures used for the leucine and alanine-bound setups are both in the outward-occluded conformation while the tryptophan-bound structure is crystalized in an outward-open conformation. Thus, all the simulations are initiated from structures in which the intracellular pathway to the S1 site is completely closed and the extracellular pathway is either partly (leucine, alanine) or fully (tryptophan) open. To examine if any changes in protein conformation occur during the simulations, the solvent accessible surface area (SASA) of the residue side chains in the aromatic lid was calculated (Figure 4a, 4c). For the leucine-bound simulations, the absence of substrate in the S2 site leads to a low accessibility of the residues in the aromatic lid corresponding to occlusion of the extracellular vestibule. However, the presence of substrate in the S2 site gives rise to a less occluded conformation, which suggests that the substrate in the S2 site prevents closure of the extracellular vestibule. The highest accessibility is observed for the simulations with tryptophan bound, which is consistent with the observation that tryptophan locks the transporter in an outward-open conformation.

Substrate binding modes in the S2 site

In order to determine the overall most likely binding modes of each of the two substrates, a clustering of the binding modes observed during the simulations was performed. For leucine, the clustering was based on the **Leu-S1/S2-T1, Leu-S1/S2-T2, Leu-S1/S2-T3, Leu-S1/S2-P1, Leu-S1/S2-P2, Leu-**

S1/S2-P4 and **Leu-S1/S2-P5** simulations, as substrate was observed to stay > 50 ns in the S2 pocket in at least one monomer in all of these simulations. For alanine, the clustering was based on the **Ala-S1/S2-P6** simulation, since alanine remains bound for an extended period of time in the S2 site of both monomer A and B for this setup (red and cyan line in Figure 3, respectively). The cluster analysis showed two dominant binding modes for binding of substrate in the S2 site (Figure 5). As mentioned in the introduction, a binding mode consisting of hydrophobic interactions between the side chain of leucine and residues L29, Y107, I111, W114, A319, F320, F324, and L400 and salt bridge interactions between the amino and carboxyl groups of leucine and the side chains of residues R30 and D404 has previously been proposed.¹¹ This proposed binding mode is re-found in our study as binding mode 1 from the cluster analysis. In particular leucine is seen to interact with residues I111 and L400 in binding mode 1, both of which have been suggested to be important for substrate binding in the S2 site based on binding and uptake assays.^{11, 14} Binding mode 2 is similar to the experimentally observed binding mode for tryptophan in the S2 site.²⁰ To our knowledge, it has not been described before in the context of substrate binding in the S2 site. In this binding mode, the amino and carboxyl groups of the substrate also interact with D404 and R30, respectively. However, the substrate is rotated ~180° with respect to binding mode 1, such that the side chain points in the opposite direction compared to binding mode 1. Thus, the hydrophobic side chain of the substrate is positioned in a pocket lined by G249, F252, F253, T409, V412, V413 and W469. During the simulations, binding mode 1 is only observed for leucine, whereas mode 2 is seen for both leucine and alanine. For leucine, the cluster size for each of the two binding modes is similar, although with a slight preference for binding mode 1.

Figure 5. Two possible substrate binding modes in the S2 site of LeuT. The substrate is shown in sticks colored according to element with the carbon atoms in purple for leucine and in cyan for alanine. The surrounding residues are shown in thinner sticks colored according to element with the carbon atoms in orange. (a) Binding mode 1 shown for leucine. (b) Binding mode 2 shown for leucine and alanine.

The binding affinity for leucine in the S2 site

The above results suggest two possible binding modes for substrate binding to the S2 site. According to the suggested allosteric S2 transport mechanism, the S2 site is a high affinity substrate binding site. Thus, by determining the binding affinity of each of the two suggested binding modes it is possible to assess the relevance of the S2 site during transport as well as which, if any, of the two modes is preferred. To determine the binding affinity of leucine in each of the two binding modes we performed TI calculations using a thermodynamic cycle consisting of decoupling of the interactions between leucine and the environment for leucine in water and in the S2 site. As a control, the binding affinities

of leucine and alanine in the S1 site were also determined through TI calculations, since the results can be compared with the experimentally determined affinities. As an additional control, the binding affinity of tryptophan in the S2 site was calculated. Although the binding affinity of tryptophan in the S2 site has not explicitly been determined experimentally, the crystal structure with tryptophan bound in both the S1 and S2 site²⁰ illustrates that binding of tryptophan in the S2 site is possible, and thus suggests a favorable binding affinity. It has been shown for flexible biomolecules that combining multiple independent simulations at each λ window during TI rather than running a single longer simulation per window enhances sampling and leads to more reliable results.^{29, 30} Hence, this approach, known as independent-trajectory TI (IT-TI), was applied. 24 λ values between 0 and 1 were sampled with nine simulations of 1-3 ns at each value (see methods section for more details). Rotational and translational restraints were applied to the ligand to reduce the conformational sampling workload following an approach similar to the procedure described by Roux and co-workers.^{31, 32} The total binding energy is calculated as

$$\Delta G_{binding} = \Delta G_{dec}^W - \Delta G_{dec}^P + \Delta \Delta G_{trans} + \Delta \Delta G_{rot} \quad (1)$$

The two first terms in Equation 1 corresponds to the free energy change associated with decoupling the ligand in water and in the binding site, while the two last terms account for the free energy change associated with the translational and rotational restraints, respectively. The energies determined by IT-TI are listed in Table 1. The calculated affinities for leucine (14 nM) and alanine (1.9 μ M) in the S1 site matches the order of magnitude for the experimentally determined binding affinities for leucine and alanine binding to LeuT (20-77 nM and 0.5 μ M, respectively)^{18, 20, 33} nicely, which validates the use of IT-TI for estimating binding affinities for this type of system. Noskov has previously published computed affinities for leucine and alanine binding in the S1 site.³⁴ In this study, a free energy

perturbation (FEP) approach rather than IT-TI was applied, which resulted in free energies of binding of -13.7 kcal/mol and -11.4 kcal/mol for leucine and alanine, respectively. The corresponding K_d values are ~0.2 nM and 9 nM, both of which deviate substantially more from the experimentally determined affinities than the results from our IT-TI calculations. The FEP calculations in the study by Noskov³⁴ were done using a simplified version of the simulation systems in which only the environment within 20 Å of the ligand is described in atomic detail, and the remainder of system accounted for by a generalized solvent boundary potential method, whereas in the study at hand the entire simulation system is described in atomic detail. Furthermore, the collective simulation time is also substantially longer in the presented IT-TI calculation. These differences in the two protocols are likely to be the cause of the differences in the resulting binding energy estimates. The calculated result for tryptophan binding in the S2 site (0.1 mM) suggests favorable binding, which is in line with the observed binding of tryptophan to this site in a crystal structure of LeuT obtained with a high concentration of tryptophan in the crystallization experiment.²⁰ The result for tryptophan further supports using IT-TI to estimate binding affinities for the S2 site. The calculated binding affinities of leucine to the S2 site suggest that leucine binds with a very low affinity to the site in both of the two examined binding modes (0.2 M and 3.3 M). Hence, based on these results, the S2 site seems unlikely to play a role as a high-affinity binding site in the transport cycle of LeuT, but can be a possible transient binding site. However, our simulations are initiated from the outward-occluded conformation of LeuT, and we cannot rule out the possibility that substrate binding in the S2 site causes a conformational change of LeuT to occur, which in turn increases the substrate binding affinity in the S2 site. It is also possible that transient binding of substrate in the S2 site is sufficient to propagate a signal through the transporter to the intracellular gating network, ultimately causing an opening of the intracellular pathway to the S1 site and release of the substrate from this site. Such a network of interactions connecting the extracellular vestibule with

the central and intracellular parts of the transporter has been proposed based on MD simulations and mutational studies.²⁷ On the other hand, the substrate would still need to initially bind in the S2 site for a period of time for any substrate-induced changes to occur, and based on the very low affinities that we obtain, this is rather unlikely.

Table 1. Free energy components (kcal/mol) and binding affinity for the binding of leucine and tryptophan to LeuT

Setup	ΔG_{dec}^W	ΔG_{dec}^P	$\Delta\Delta G_{trans}$	$\Delta\Delta G_{rot}$	$\Delta G_{binding}$	K_d^{calc}	K_d^{expa}
Leu, S1	57.3 (± 0.2)	78 (± 3)	4.5 (± 0.1)	4.9 (± 0.2)	-11 (± 3)	14 nM	20-77 nM
Ala, S1	60.0 (± 0.2)	77 (± 2)	4.51 (± 0.04)	4.3 (± 0.3)	-8 (± 2)	1.9 μ M	0.5 μ M
Trp, S2	60.0 (± 0.2)	71.1 (± 0.6)	1.8 (± 1.7)	3.6 (± 0.5)	-6 (± 2)	0.1 mM	-
Leu, S2-1	57.3 (± 0.2)	65.0 (± 0.7)	2.5 (± 0.8)	4.1 (± 0.3)	-1.1 (± 1.1)	0.2 M	-
Leu, S2-2	57.3 (± 0.2)	64.6 (± 1.6)	3.3 (± 0.4)	4.8 (± 0.1)	0.7 (± 1.7)	3.3 M	-

^aData from^{18, 20, 33}

The calculated binding affinities for leucine binding to the S2 site do not support the hypothesis that the S2 site is a high affinity substrate binding site. Rather, the results suggest that the S2 site is a low affinity site in the outwards-occluded conformation of LeuT to which the substrate may bind briefly while passing from the extracellular milieu to the central binding site. This is also supported by the equilibrium simulations where the substrate is seen to leave the S2 site in the majority of the simulations. Furthermore, our results indicate that binding of substrate in the S2 site prevent formation of R30-D404 salt bridge. Since this interaction has been suggested to be important for the closure of the extracellular vestibule, which is a necessity for transport to occur, this also speaks against the allosteric

S2 mechanism. Overall, the presented results are consistent with the experimental results suggesting that LeuT only has a single high-affinity substrate binding site and that this site is the central S1 pocket.¹⁸ Rather than being an allosteric site, our findings point to the S2 site functioning as a low-affinity attraction site that facilitates binding of substrate in the high-affinity S1 site. It has been argued that the experimental conditions such as the type and concentration of detergent can obscure the S2 site in LeuT,³³ which is supported by a number of LeuT crystal structures with detergent bound in the S2 site.³⁵ However, detergent binding in the S2 site is not an issue in our simulations as no such molecules are present in the simulations. We thus find that even in the absence of detergent, the S2 site is not a high-affinity substrate binding site. It has also been suggested that the presence of detergent in S2 may trap the transporter in an inhibited state rather than a functionally relevant conformation.³⁵ A LeuT crystal structure obtained using the detergent *n*-dodecyl seleno- β -D-maltoside has however shown that this detergent does not bind to the S2 site yet still the obtained conformation of the substrate-bound outward-occluded is very similar to what is obtained when using other detergents.³⁶ Moreover, even if the starting structures used in our study have a slightly perturbed conformation due to the presence of detergent in the S2 site during crystallization, the relaxation of the structure that occurs during the MD simulations should allow the protein to adapt to the substrate positioned in the S2 site. Bahar and co-workers have similarly studied the transport mechanism of LeuT by MD simulations.^{26, 37, 38} While they in one study observed stable binding of alanine in the S2 site of LeuT for ~20 ns when alanine was simultaneously bound in the S1 site,³⁷ later studies suggested that substrate in the S2 site does not accelerate substrate release from the S1 site³⁸ and that binding of substrate in the S2 site is not favored,²⁶ which is in line with our results. Similarly, Madura and co-workers have also studied the conformational flexibility of LeuT using a combination of advanced computational methods and find that the inward conformation can be reached without including a second substrate in the S2 site.^{39, 40}

An important question is whether the S2 site may still function as an allosteric site in the human MATs. Based on SMD simulations of a homology model of human dopamine transporter, it has been suggested that this transporter also functions through an allosteric mechanism where dopamine binding in the S2 site triggers an opening of the transporter to the intracellular site and release of dopamine from the S1 site.¹³ For the human norepinephrine transporter, a combination of homology modeling, molecular docking simulations, and site-directed mutagenesis studies⁴¹ has led to the suggestion of an extracellular substrate binding site in this transporter, similar to the S2 site in LeuT. However, the authors suggest that the function of this site is to attract the substrate into the extracellular vestibule and orient it for binding in the S1 site, rather than being part of a two-substrate mechanism. A similar suggestion of the S2 site as a transient site attracting the substrate towards the S1 site has also been made for LeuT based on SMD simulations.¹² For the human serotonin transporter, a conformational change from outward- to inward-facing has been observed in MD simulations without a substrate in the S2 site, while other simulations showed that substrate binding in the S2 site was not stable,¹⁹ overall suggesting that for this transporter, a substrate in the S2 site is not a necessity. For the human dopamine transporter, an outward-to-inward facing transition has recently been seen to occur in simulations where substrate is only present in the S1 site.⁴² Recently, several crystal structures of the human serotonin transporter have been reported.³ One of these structures show binding of the inhibitor *S*-citalopram both in the central S1 binding site and in a site in the extracellular vestibule. Although the crystal structure does reveal that binding of inhibitors in the extracellular vestibule of the serotonin transporter is possible, it does not shed light on whether substrate binding might occur in this site as well. Consequently, it would still be interesting to investigate the binding, effect and affinity of substrate in the S2 site for each of the three human MATs using a similar approach as the one described herein, to assess the relevance of the S2 site for these pharmaceutically important transporters.

Methods

A detailed description of the simulation system preparation and simulation protocols can be found in the supplementary information. Protein crystal structures were prepared using the Schrödinger 2011 suite.^{43,44} Each simulation system consists of a LeuT dimer embedded in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) lipid membrane and surrounded by water and 0.1 M NaCl. Equilibrium MD simulations were performed in NAMD version 2.8⁴⁵ at 310 K and at a pressure of 1 atm. The protein, ligands and ions was described by the CHARMM22 force field parameters with CMAP corrections^{46,47} and the CHARMM36 parameters⁴⁸ were used for the lipids. Simulations with leucine bound in either the S1 site or both the S1 and S2 sites were based on the outward-occluded conformation observed in the X-ray structure of LeuT with leucine bound in the S1 site (PDB code 2A65⁷). Simulations systems with alanine bound to LeuT were also based on the outward-occluded conformation of LeuT (PDB code 3F48²⁰) while the simulations systems with tryptophan bound to LeuT were based on the outward open conformation of LeuT (PDB code 3F3A²⁰). Simulations were performed for LeuT with leucine or alanine present in the S1 site (**Leu-S1**, **Ala-S1**), and LeuT with leucine, alanine or tryptophan present in both the S1 and the S2 site (**Leu-S1/S2-T**, **Ala-S1/S2-T**, **Trp-S1/S2**). For the latter systems, the substrate in the S2 site was placed according to the position of tryptophan in an outward-open crystal structure of LeuT, as marked by the **-T** in the names.²⁰ For each of these five simulation setups, three independent 100 ns simulations were performed. For **Leu-S1/S2-T** and **Ala-S1/S2-T** simulations in which the substrate in the S2 site remained bound after 100 ns the simulations were further extended up to a maximum of 200 ns, at which point substrate release from the S2 site was observed in all of these trajectories. Additionally, a set of distinct binding modes of leucine and alanine in the S2 site were determined through molecular docking calculations. In short, the

results of a rigid docking and an induced fit docking in the S2 site was combined for each of the two substrates and the ‘Clustering of Conformers’ script in the Schrödinger 2011 suite was applied to obtain a diverse subset of binding modes. Based on the clustering results, six possible binding modes for substrate binding in the S2 site were chosen for each of the two substrates and simulation systems with substrate in both the S1 and S2 site were made for each of these binding modes. These are referred to as **Leu-S1/S2-PX** and **Ala-S1/S2-PX**, respectively, with X = 1-6 corresponding to each of the six binding modes. A single simulation was performed for each of the **Leu-S1/S2-PX** and **Ala-S1/S2-PX** setups with a simulation length of 10 ns to 300 ns depending on the substrate stability in the S2 site. In Table 2, an overview of the simulation setups is given.

Table 2. Simulation setups.

Setup	Conformation ^a	PDB	Ligand in S1 site		Ligand in S2 site	
			Type	Placement ^b	Type	Placement ^b
Leu-S1	Occ	2A65	Leucine	X-ray	-	-
Ala-S1	Occ	3F48	Alanine	X-ray	-	-
Trp-S1/S2	Open	3F3A	Tryptophan	X-ray	Tryptophan	X-ray
Leu-S1/S2-T	Occ	2A65	Leucine	X-ray	Leucine	Trp
Ala-S1/S2-T	Occ	3F48	Alanine	X-ray	Alanine	Trp
Leu-S1/S2-P	Occ	2A65	Leucine	X-ray	Leucine	Docking
Ala-S1/S2-P	Occ	3F48	Alanine	X-ray	Alanine	Docking

^a Occ: Outward-occluded. Open: Outward open. ^b X-ray: Placement is based on what is observed in LeuT crystal structures. Trp: Placement is based on the position of tryptophan in the S2 site in the 3F3A PDB structure. Docking: Placements is based on docking of the ligand in the S2 site of LeuT.

Clustering of the binding modes observed for leucine and alanine in the S2 site during the MD simulations was done based on all the **Leu-S1/S2** simulations as well as the **Leu-S1/S2-P1**, **Leu-S1/S2-P2**, **Leu-S1/S2-P4** and **Leu-S1/S2-P5** simulations for leucine, while for alanine the clustering was based solely on the **Ala-S1/S2-P6** simulation (as described in the results section). The snapshots were clustered based on the position of the heavy atoms in the S2 substrate using the clustering algorithm implemented in VMD, which is based on the Quality Cluster algorithm.⁴⁹

The Gibbs free binding energy of the ligands in the S1 and S2 site of LeuT was determined using IT-TI calculations. The simulations were performed in NAMD version 2.9. IT-TI was performed for 21 equally spaced λ windows between 0 and 1 with a spacing of 0.5 as well as additional windows at $\lambda = 0.925, 0.975, 0.99$ (24 windows in total). The simulation time for each independent trajectory was at least 1 ns per λ window and simulations were extended up to 3 ns until the standard deviation of the cumulative average of $dH(\lambda)/d\lambda$ was ≤ 1.0 kcal/mol for all simulation repeats at the given λ windows. The simulation time of each λ window is given in Table S1. Rotational and translational restraints were applied to the ligand similar to the procedure described by Roux and co-workers^{31, 32} to reduce the conformational sampling workload by biasing the ligand to be close to its bound configuration. For the S1 site, the simulations were based on the **Leu-S1** and the **Ala-S1** simulation setups described above. For each substrate (leucine and alanine), three copies of the simulation system were equilibrated independently, and the endpoint of each equilibration was used as the starting point for the TI-IT calculations. For each equilibrated system, three independent TI calculations were performed at each λ

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4 window. A similar procedure was applied to determine the binding affinity of tryptophan in the S2 site,
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6 using three independently equilibrated copies of the **Trp-S1/S2** setup as starting points. For leucine
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8 binding in the S2 site, the affinity of each of the two different binding modes of leucine determined
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10 from clustering of equilibrium MD simulations was assessed. Three snapshots were chosen for each of
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12 the two binding modes, and for each snapshot a simulation system was built and equilibrated, and
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14 subsequently three independent TI calculations were performed at each λ window. In order to
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16 determine the free energy change associated with decoupling leucine, alanine, and tryptophan,
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18 respectively, in water, nine independent TI calculations were also performed for a simulation system
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20 consisting of a water box with dimensions $40 \text{ \AA} \times 40 \text{ \AA} \times 40 \text{ \AA}$ with the ligand in the center of the box
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22 and a NaCl concentration of 0.1 M. The energy associated with applying the translational and rotational
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24 constraints, respectively, to the fully interacting ligand in the binding site was calculated from three
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26 independent TI calculations in which the restraints were gradually added to the ligand. The energy
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28 associated with adding the restraints to the fully decoupled ligand was estimated using analytical
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30 expressions, which are given in the supplementary information. The uncertainty of each of the terms in
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32 Equation 1 has been calculated as the standard deviation of the free-energy change over the
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34 independent TI trajectories.
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44 **Supporting information**

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46 A detailed description of the applied methods, illustrations of the starting position of ligands in the S2
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48 site, plots of ligand displacements during simulations.
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J.G., H.K., Y.M., and B.S. contributed to research design. J.G. conducted the simulations. J.G. and Y.M. participated in data analysis. The manuscript was written through contributions of all the authors.

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