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Molecular dynamics simulations of Na^+ and leucine transport by LeuT

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ABSTRACT

Molecular dynamics simulations are used to gain insight into the binding of Na^+ and leucine substrate to the bacterial amino acid transporter LeuT, focusing on the crystal structures of LeuT in the outward-open and inward-open states. For both conformations of LeuT, a third Na^+ binding site involving Glu290 in addition to the two sites identified from the crystal structures is observed. Once the negative charge from Glu290 in the inward-open LeuT is removed, the ion bound to the third site is ejected from LeuT rapidly, suggesting that the protonation state of Glu290 regulates Na^+ binding and release. In Cl^--dependent transporters where Glu290 is replaced by a neutral serine, a Cl^- ion would be required to replace the role of Glu290. Thus, the simulations provide insights into understanding Na^+ and substrate transport as well as Cl^--independence of LeuT.

1. Introduction

Neurotransmitters released from the presynaptic terminals in the course of synaptic transmissions must be removed rapidly from the synaptic cleft by transporting them back into cells. This process involves several families of transporters, of which the solute carrier family 6 is one of the largest [1,2]. The leucine transporter (LeuT) from *Aquifex aeolicus* is a bacterial homolog of the Na^+-coupled neurotransmitter transporters (NSS) belonging to this family with broad specificity for small hydrophobic amino acids [3]. LeuT is considered as a prototype for studying NSS, owing to their similarities in many characteristics such as sequence, structure and function [4].

Crystal structures of LeuT in three distinct conformations (outward-open, outward-occluded, inward-open) have been reported [3,5]. These structures are consistent with the alternating access model proposed decades ago [3,5,6]. This model posits that the transporter must carry a binding site for the substrate and be able to switch between at least two different conformations, in which the binding site of the substrate is open to either the intracellular or the extracellular space. The structures of LeuT also suggest that LeuT transports two Na^+ ions with one substrate molecule in one cycle [3], as the crystal structure of LeuT in an outward-occluded conformation contains two Na^+ binding sites (Na1 and Na2) and one substrate binding site (S1) near the center of the protein [3]. The Na1 and Na2 sites are primarily formed by four (Ala22, Asn27, Thr254 and Asn286) and five (Gly20, Val23, Ala351, Thr354 and Ser355) residues, respectively. However, the precise sequence of events leading to the substrate transport remains to be elucidated [7,8].

Molecular dynamics (MD) simulations have been used extensively to understand the mechanism of substrate/ion binding and transport in LeuT [8–17], primarily focusing on the outward-occluded structure [3]. For example, a second substrate binding site (S2) not seen in the crystal structures, formed by residues Arg30 and Asp404, was predicted from MD simulations [8,9] and validated by experiment [8]. In the crystal structure of the outward-facing LeuT, a second tryptophan molecule is indeed seen to bind the S2 site [18]. It is unclear whether the substrate binds to S2 transiently before binding to S1 [19], or the presence of the S2 substrate helps trigger the required conformational changes to the transporter for subsequent substrate and ion release [20]. The outward-open and inward-open structures of LeuT reported more recently are in the apo form and less well-studied [5].

Using the outward-open and inward-open structures of LeuT, we carry out a series of unbiased MD simulations to determine where in the transporter Na^+ and leucine stably bind. A sodium ion in the reservoir is consistently attracted to Glu290 in both the outward-open and inward-open LeuT. Once the negative charge of Glu290 in the inward-open LeuT is removed by side chain protonation, the ion is released from LeuT rapidly. Although the importance of Glu290 in LeuT transport and Cl^- dependence was proposed previously [21], the exact mechanism was not obvious

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because Glu290 is involved in neither Na1 nor Na2. Our simulations provide a mechanistic understanding of how the changes in the protonation state of Glu290 may regulate the ion and substrate transport of LeuT [22].

2. Methods

2.1. Molecular dynamics simulations

The crystal structures of LeuT in the outward-open (PDB ID 3TT1 [5]) and inward-open (PDB ID 3TT3 [5]) states are considered. The crystal structures represent two mutant forms of LeuT, referred to as LeuT^{K}(Y108F) and LeuT^{K}(TSY), respectively [5]. The two mutants are back mutated to the wild type, in which the K288A background mutation is retained as it enhances substrate transport [23]. The conformation of the ten residues in the N-terminus missing in the inward-open structure is modeled using the SWISS-MODEL homology modeling server [24]. LeuT in either the outward-open or the inward-open conformation is placed in the middle of a 1-palmitoyl-2-oleoyl-*sn*-glycerol-phosphocholine bilayer. Approximately 15,800 water molecules, 60 Na^+ and 60 Cl^- are added, corresponding to a salt concentration of 0.2 M. Two additional Cl^- are added to maintain charge neutrality. The resulting system is ~$90 \times 90 \times 90$ Å^3 in size in all cases. Unless otherwise stated, the side chains of all glutamate and aspartate residues are assumed to be in the deprotonated form. Details of our simulation parameters are given in the Supplementary Material.

3. Results

3.1. Na1q in the outward-open LeuT

The crystal structure of LeuT in the outward-open state reveals two Na^+ binding sites, Na1 and Na2, similar to that in the outward-occluded state [3,5]. To verify if our simulations are able to reproduce the underlying physics governing the interactions between ions and LeuT, we perform two independent equilibrium simulations on the outward-open LeuT in the presence of two Na^+ ions at Na1 and Na2 in the starting configuration. The interactions between the two ions and their binding sites remain unchanged after 50 ns in both simulations (Fig. S1), indicating that the two ion binding sites revealed from the crystal structure are indeed stable.

Having shown that the simulations are able to reproduce some basic characteristics of the crystal structures, we carry out unbiased simulations to identify potential additional Na^+ binding site in the outward-open LeuT. Specifically, we perform unbiased simulations in the absence of Na1 and Na2 ions. All Na^+ ions are placed in the bulk and allowed to bind LeuT spontaneously.

In the two unbiased simulations of 200 ns each, two Na^+ ions are attracted to the central cavity of LeuT. In the outward-open LeuT, two acidic residues, Glu112 and Asp404, are located at the extracellular end of LeuT (Fig. 1A). These two residues may be involved in generating a negative electrostatic field for Na^+ ions. One Na^+ ion consistently binds to Glu290 (Fig. 1B), referred to as the Na1q site, at 165 ns and 75 ns of the two simulations, respectively. The other Na^+ ion binds to Ser355, which is involved in the Na2 site (Fig. 1B). This ion is also within 5 Å to the backbone oxygen atoms of Gly20 and Val23, two other residues of the Na2 site. The binding of the ion to Ser355 is stable, as the distance between the ion and Ser355 is predominantly less than 3 Å once bound (Fig. 1C). The simulations predict that the Na1q binding site is present in the outward-open LeuT.

3.2. Effect of protonation on Na^+ binding

The protonation state of acidic residues are not only important for generating electrostatic field for Na^+ ions, but also may be important for the conformational changes of LeuT [25]. Previous pKa calculations predicted that the side chain of Glu290 assumes different protonation states in the outward-open and inward-open LeuT [22]. In the outward-open LeuT the side chain of Glu290 is likely deprotonated and charged, with a predicted pKa of 5.4. However, the side chains of three residues (Glu112, Glu287 and Glu419) in the outward-occluded LeuT have been predicted to be protonated [9,21]. Our calculations employing the PROPKA program [26,27] suggest that Glu112 (pKa 7.6), Glu287 (pKa 7.6) and Glu419 (pKa 12.6) in the outward-open LeuT should also be predominantly protonated at a neutral pH. However, the pKa values predicted from those empirical methods could be overestimated, since accurate prediction of pKa is challenging even with sophisticated methods [28,29]. For example, the desolvation effect, which is considered as a major contributor to the pKa shift in PROPKA [27], is difficult to predict accurately. The protonation states of those glutamate residues thus remain to be re-examined with other methods. Here, we examine how neutralizing Glu112, which is in close proximity to Na1 and Na2 (Fig. 1A), would affect Na^+ binding to the outward-open LeuT.

The starting configuration is generated from that shown in Fig. 1B, but with the side chain of Glu112 protonated. The system is equilibrated twice for 50 ns each. In both simulations the ions bound to Na1q and Na2 remain stable, indicating that the extent to which the protonation state of Glu112 may affect Na^+ binding is limited on the time scale of our simulations. The side chain of Glu112 is hydrated by several water molecules when it is deprotonated (Fig. S2). Once the side chain of this residue is protonated, the hydration number reduces to one, as the addition of a proton causes the side chain to move to an inward position and form two hydrogen bonds with Phe349 (Fig. S2). This inward motion appears to be inconsistent with the crystal structure, in which the hydrogen bonding between Glu112 and Phe349 is not evident [5]. Therefore, it is possible that Glu112 is charged, which would help generate a negative electrostatic field for the initial binding of Na^+ to LeuT. Similarly, we find that the side chain of Glu287 in the deprotonated form is also fully hydrated in the outward-open LeuT (Fig. S2). As the same Na1q site was predicted from simulations with Glu287 in the protonated form [25], it can be concluded that the protonation state of Glu112 and Glu287 has limited effect on Na^+ binding.

3.3. Binding of leucine to the outward-open LeuT

The structure of the outward-open LeuT was crystallized in the absence of a leucine substrate [5]. Our next aim is to examine the substrate binding of the outward-open LeuT using MD simulations. To save the computing resources required for the initial binding of substrate to the transporter, we place a leucine substrate inside LeuT, at a position approximately corresponding to that in the outward-occluded state [3]. The complete unbinding of leucine from LeuT observed in our simulations reveals a secondary substrate binding site at the extracellular end of the transporter, consistent with previous experimental and computational observations [8].

A leucine molecule is placed above the side chains of Phe259 and Val104, with its head group interacting with several residues in close proximity to Ser355. Two Na^+ ions are placed at the Na1 and Na2 sites. Three simulations each on a time scale of 200 ns are performed. In two of the simulations the position of the substrate remains unchanged, but in the third simulation a complete unbinding of the substrate from LeuT is observed. First the substrate