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The Mechanism of a Neurotransmitter:Sodium Symporter—Inward Release of Na^+ and Substrate Is Triggered by Substrate in a Second Binding Site

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SUMMARY

Eukaryotic neurotransmitter:sodium symporters (NSSs), targets for antidepressants and psychostimulants, terminate neurotransmission by sodium-driven reuptake. The crystal structure of $LeuT_{Aa}$, a prokaryotic NSS homolog, revealed an occluded state in which one leucine and two Na^+ ions are bound, but provided limited clues to the molecular mechanism of transport. Using steered molecular dynamics simulations, we explored the substrate translocation pathway of LeuT. We identified a second substrate binding site located in the extracellular vestibule comprised of residues shown recently to participate in binding tricyclic antidepressants. Binding and flux experiments showed that the two binding sites can be occupied simultaneously. The substrate in the secondary site allosterically triggers intracellular release of Na^+ and substrate from the primary site, thereby functioning as a ''symport effector.'' Because tricyclic antidepressants bind differently to this secondary site, they do not promote substrate release from the primary site and thus act as symport uncouplers and inhibit transport.

INTRODUCTION

Neurotransmitter:sodium symporters (NSSs) play an essential role in the nervous system by terminating synaptic transmission and recycling neurotransmitters for reuse (Rudnick, 2002). These proteins are secondary active transporters that utilize the Na^+ gradient across the plasma membrane to catalyze the uptake of a variety of neurotransmitters from the extracellular milieu against their concentration gradient in a cotransport (symport) mechanism (Torres et al., 2003). NSS substrates include biogenic amines, such as dopamine, norepinephrine, and serotonin, as well as amino acids (γ-aminobutyric acid, glycine, and proline) and osmolytes (betaine and creatine) (Sonders et al., 2005). The transporters for the biogenic amines dopamine, norepinephrine, and serotonin (DAT, NET, and SERT, respectively) are of particular interest because they are targeted by numerous drugs, including the widely abused psychostimulants cocaine and amphetamine (Amara and Sonders, 1998), as well as antidepressants (Iversen, 2006).

Genes encoding more than 200 putative NSS homologs have been computationally identified in prokaryotic genomes (Beuming et al., 2006), and several of these, including TnaT (Androutsellis-Theotokis et al., 2003), LeuT (Yamashita et al., 2005), Tyt1 (Quick et al., 2006), and MhsT (Quick and Javitch, 2007) were shown to be Na^+-dependent amino acid transporters. The crystal structure of LeuT at 1.65 Å resolution revealed an occluded binding pocket with L-leucine (Leu) and two Na^+ ions, Na1 and Na2, complexed within an extensive network of backbone and side-chain interactions, which for Na1 also includes the carboxylate of the bound substrate Leu (Yamashita et al., 2005).

A stoichiometry of 2 Na^+:1 substrate molecules per transport cycle has been inferred for most NSS (Gu et al., 1994), and this has been supported by direct flux experiments in GAT-1 (Krause and Schwarz, 2005). It is likely that coordinated conformational changes couple the movement of Na^+ down its electrochemical gradient to the uphill movement of substrate (Jardetzky, 1966). We have shown (Quick et al., 2006) that Na^+ produces conformational changes consistent with an ''outward-facing'' conformation and that its absence promotes increased accessibility of cytoplasmic residues consistent with an ''inward-facing'' conformation of the transporter. Nonetheless, how these changes might drive transport remained a mystery.

The dynamic properties of protein-ligand interaction complexes have been shown to be well described with the current methods of molecular dynamics (MD) simulations (Karplus and Kuriyan, 2005; Kong and Karplus, 2007). However, characterization of the complex conformational rearrangements associated with physiologically relevant allosteric mechanisms, such as transport by NSS, requires other types of simulation approaches capable of describing dynamics involving barrier crossings. Therefore, we have used steered MD (SMD) simulations (Israelewitz et al., 2001) to study the dynamics of ligand movement in LeuT. SMD had been used to simulate unbinding or unfolding events in several biomolecular systems, e.g., LacY (Jensen et al., 2007). We used this approach to simulate ligand motion in LeuT by pulling the substrate along a simulated pathway. The energy barrier crossings are accelerated by this SMD procedure through the application of external forces. Thus, nanoseconds trajectories of SMD will reasonably simulate molecular behavior during the natural transport process (Jensen et al., 2007), such as transport by NSS, in which turnover occurs over hundreds of milliseconds to seconds.

Combining the computational studies with experimental analysis of dissociation kinetics, transport, and binding, we were able to probe the molecular mechanism of LeuT-mediated substrate translocation. Our findings identified a secondary substrate binding site at the extracellular vestibule of LeuT and revealed the nature of its involvement in the transport mechanism. Very recently, tricyclic antidepressants (TCAs) have been shown to bind to a similar site in LeuT where they were considered to trap the substrate Leu in the occluded binding site (Singh et al., 2007; Zhou et al., 2007). We show here that two substrate molecules can bind simultaneously to the primary and secondary sites and that binding of substrate in the secondary site is the trigger for inward transport of Na^+ and substrate from the primary binding site along the permeation pathway. We propose an allosteric mechanism of Na^+-coupled symport in which binding of substrate to the secondary site is essential for coupling the energy from the electrochemical ion (Na^+) gradient to the transport of solutes by the NSS.

RESULTS

Identification of a Secondary Substrate Binding Site from Steered Molecular Dynamics Simulations

For the SMD simulations, we constructed and equilibrated a molecular system consisting of Leu-bound LeuT immersed in a solvated lipid bilayer, based on the original LeuT crystal structure (Yamashita et al., 2005). At the end of a 24 ns unconstrained equilibration, the LeuT model was very similar to the LeuT crystal structure (rmsd of 1.4 Å), and the transmembrane segments (TMs) 1, 3, 6, and 8 were even closer to that structure (rmsd 0.7 Å). Pulling the substrate from its occluded binding site (termed the primary binding site), known from the LeuT crystal, toward the extracellular milieu with a force applied to the center of mass of the Leu, we identified energy barriers along the simulated path with specific interactions along the pathway. These are mainly ionic interactions and hydrogen bonds between the carboxyl/amine groups of Leu and LeuT residues (for details and representative results from SMD runs, see Figure S1 available online). With the reorganization of the protein environment in the path around the pulled substrate, several of the interactions between the amine group of the substrate and LeuT are severed. This allows the Leu to be repositioned between the aromatic rings of Tyr108$^{3.50}$ and Phe253$^{6.53}$ in a cleft that emerges between TMs 1, 3, and 6 before exiting the primary binding site (for indexing system, see Goldberg et al., 2003; Beuming et al., 2006). The most remarkable details of the observed structural response to the relocation in this initial movement are (1) the consistent interaction of the carboxylate of Leu with Tyr108 and (2) the change in both backbone angles and the rotamer of Phe253, which suggests a gating role for this residue in enabling the Leu side chain to exit the binding site. Results from simulations exploring the role of Na^+ (see below) further support this role.

Further relocation of the substrate with SMD led to a local equilibrium site where the pulling force drops dramatically, thus identifying a new favorable position. Here, Leu is at ~+10 Å along the z axis (relative to the primary Leu binding site defined to be at ~0 Å) and is partially exposed to extracellular bulk water. This site at the extracellular vestibule was termed "the secondary binding site" (Figures 1A–1C) and consists of two components: a hydrophobic pocket composed of Leu29$^{1.50}$, Tyr107$^{3.49}$, Ile111$^{3.53}$, Trp114$^{3.56}$, Ala319^{EL4}, Phe320^{EL4}, Phe324^{EL4}, and Leu400$^{10.44}$—which accommodates the Leu side chain—and an ionic cleft composed of the Asp404$^{10.48}$ and Arg30$^{1.51}$ that establish direct ionic interactions with the amine and carboxyl groups of Leu, respectively. Recent direct structural studies show that TCAs bind to a very similar site in the extracellular vestibule of LeuT, with Leu and two Na^+ remaining bound below the TCA in positions nearly identical to the original crystal structure (Singh et al., 2007; Zhou et al., 2007) in which the secondary site is empty and a water molecule is poised to mediate the interaction between Asp404 and Arg30 (Yamashita et al., 2005).

The Impact of Na^+ on the Structure of the Binding Site

Because substrate binding is Na^+ dependent (see Figure S2A), we used MD simulations to explore the structural role of the two Na^+ bound near the Leu by comparing the holocrystal structure to results from simulations of constructs in which the substrate was removed (−Leu) in the presence (+Na) or absence (−Na) of the two Na^+ ions (Figures 1D–1F). A number of local structural changes are observed to occur in the primary binding site in the absence of both Na^+ and Leu (−Na/−Leu). These produce a "filling in" of the binding cavity (see Figure 1 and the Supplemental Results). An important role in the physiological mechanism of the transporter is attributable to these changes, as filling and shielding of the cavity in the absence of Na^+ is likely to be a feature of an inward-open conformation in which the primary binding site is difficult to access from the extracellular environment. In contrast, we found for the "+Na/−Leu" state that water molecules penetrate the cavity and break the Tyr108 to Ser355$^{8.60}$ hydrogen bond in −Na/−Leu by binding to each of them separately. In this manner, the presence of Na^+ opens access to the primary site for the incoming substrate, consistent with the reported conformational dependence of access to position 3.53 (one helical turn above Tyr108) in the biogenic amine transporters (Chen and Rudnick, 2000; Loland et al., 2004).

Our 30 ns long MD simulations cannot reveal all the intervening conformational changes associated with binding of Na^+ and Leu, but the end points make the trends quite clear: (1) in the absence of Na^+, the binding site is shielded from the extracellular milieu, and no water molecules are found within the cavity (Figures 1D