Conformational dynamics of a neurotransmitter:sodium symporter in a lipid bilayer

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Edited by Christopher Miller, Howard Hughes Medical Institute, Brandeis University, Waltham, MA, and approved January 26, 2017 (received for review August 10, 2016)

Neurotransmitter:sodium symporters (NSSs) are integral membrane proteins responsible for the sodium-dependent reuptake of small-molecule neurotransmitters from the synaptic cleft. The symporters for the biogenic amines serotonin (SERT), dopamine (DAT), and norepinephrine (NET) are targets of multiple psychoactive agents, and their dysfunction has been implicated in numerous neuropsychiatric ailments. LeuT, a thermostable eubacterial NSS homolog, has been exploited as a model protein for NSS members to canvass the conformational mechanism of transport with a combination of X-ray crystallography, cysteine accessibility, and solution spectroscopy. Despite yielding remarkable insights, these studies have primarily been conducted with protein in the detergent-solubilized state rather than embedded in a membrane mimic. In addition, solution spectroscopy has required site-specific labeling of nonnative cysteines, a labor-intensive process occasionally resulting in diminished transport and/or binding activity. Here, we overcome these limitations by reconstituting unlabeled LeuT in phospholipid bilayer nanodiscs, subjecting them to hydrogen–deuterium exchange coupled with mass spectrometry (HDX-MS), and facilitating interpretation of the data with molecular dynamics simulations. The data point to changes of accessibility and dynamics of structural elements previously implicated in the transport mechanism, in particular transmembrane helices (TM)s 1a and 7 as well as extracellular loops (ELs) 2 and 4. The results therefore illuminate the value of this strategy for interrogating the conformational mechanism of the more clinically significant mammalian membrane proteins including SERT and DAT, neither of which tolerates complete removal of endogenous cysteines, and whose activity is heavily influenced by neighboring lipids.

Significance

Most studies of neurotransmitter:sodium symporter (NSS) function and dynamics have been carried out in detergent even though the activity of these integral membrane proteins is heavily modulated by surrounding lipids. Here, we reconstituted the prokaryotic homolog LeuT into nanodiscs and subjected the preparation to hydrogen–deuterium exchange mass spectrometry to reveal a global view of the hallmarks of the transporter in two disparate conformations. The data were interpreted with the aid of molecular dynamics simulations, allowing unprecedented atomic-level insights into the dynamics of an unmodified, unlabeled NSS in a native-like lipid bilayer environment.

Author contributions: S.A. and S.K.S. conceived the project and designed the HDX-MS and accompanying biochemical experiments; S.A. and D.J.D. performed the HDX-MS experiments; S.A., D.J.D., and P.L.W. analyzed the HDX-MS experiments; A.N. and L.R.F. designed, carried out, and analyzed molecular dynamics simulations; and S.A., L.R.F., and S.K.S. wrote the manuscript with input from all authors.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1613293114/-/DCSupplemental.
distance or accessibility changes that must then be extrapolated to the entire protein, almost all of these studies have been conducted in detergent micelles, which can be poor substitutes for the membrane (20). For example, the affinity of LeuT for the substrate Ala, and the $D_{\text{max}}$ for both Ala and Leu are at least 1.5-fold greater in a lipid bilayer than they are in the detergent $n$-dodecyl-$\beta$-D-maltoside (DDM) (21), and TM1a adopts a different orientation in 1-palmitoleoyl-2-oleoyl--sn-glycero-3-phosphocholine (POPC) liposomes than it does in DDM (22). Lipids, especially cholesterol and sphingolipids, seem to play an even more significant role for eukaryotic NSS members, with specific lipids markedly altering transporter conformational dynamics to the point of modulating how a drug, substrate, and/or ions interact with the protein (23–26). Moreover, the mere presence of detergent may have unforeseen consequences (27). In particular, $n$-octyl-$\beta$-D-glucoside ($\beta$-OG) binds in the extracellular vestibule of LeuT (28, 29), presumably inhibiting activity (29), and the mild, widely used detergent DDM appears to influence substrate-binding properties depending on its concentration (2 versus 6 mM) (30). Hence, it is critical that such investigations be carried out in a more physiologically relevant membranous medium.

The second issue with published LeuT studies is that conformational dynamics have been monitored exclusively via cysteine accessibility, smFRET, EPR, and DEER. Although such methods are powerful, they require covalent modification of dozens of strategically placed cysteines with methanethiosulfonate reagents (SCAM) (31), fluorophores (smFRET) (32), or spin labels (EPR and DEER) (33, 34) on a fully active, cysless protein target. Constructing such a variant is an arduous process that has thus far been unattainable for mammalian NSS members. Even for an endogenously cysless protein like LeuT, introduction of nonnative cysteines demands that the functional and structural fidelity of each mutant be scrutinized before use.

Here, we overcome these experimental obstacles by gauging global conformational dynamics via hydrogen–deuterium exchange coupled with mass spectrometry (HDX-MS) on LeuT reconstituted into phospholipid bilayer nanodiscs and interpreting the results with the help of MD simulations. To test the validity of our strategy, we focused on two conditions under which LeuT should adopt the most divergent conformational ensembles. Significantly, these conformations include those stabilized by psychoactive antagonists in LeuT’s mammalian counterparts (35, 36). HDX is an isotopic labeling technique in which amide hydrogens in the peptide backbone become “exchanged” for deuterium upon incubation in $D_2O$-containing buffer. The solvent-exposed regions readily exchange at the earliest time of incubation, allowing HDX to probe the structure of a protein. Furthermore, local fluctuations result in transient exposure of otherwise unexchangeable amide hydrogens. Over time, these transient exposure events result
in increasing deuterium incorporation at any given site, a direct measurement of conformational dynamics. When HDX is coupled with proteolytic digestion and MS, the extent of deuterium incorporation can be localized to specific peptides (37). Furthermore, supplementing the analysis with MD simulations of hydrated protein structures embedded in a lipid bilayer and under related conditions can pinpoint individual residues and dynamic behaviors responsible for specific observations, more directly than would be possible by comparison with static X-ray crystallographic structures. Here, for the NSS family member LeuT, we demonstrate major differences in TM1a, TM7, EL2, and EL4, when comparing conditions that favor outward- or inward-facing conformations. Importantly, the deuteration rates predicted from MD simulations of inward and outward conformations mirror the trends observed in HDX-MS data, furnishing singular insights into the conformational flexibility of unmodified LeuT and, by extension, its eukaryotic counterparts, in a more native environment.

Results

Substrate Binding to LeuT-WT and LeuT-Y268A in Nanodiscs. To investigate the HDX signature of the outward- and inward-facing conformations, we used two LeuT variants: LeuT-WT and -Y268A. As mentioned above, the equilibrium of LeuT is biased toward an outward-open conformation in the presence of Na\(^+\) alone, that is, in the absence of substrate. By contrast, the Y268A mutant biases the equilibrium toward the inward-facing conformation (15). These two constructs were each introduced into nanodiscs. Although it is not possible to evaluate sodium-dependent substrate ([\(\text{H}\)Leu or \([\text{H}]\text{Ala}\)] transport in such a nonvesicular preparation, sodium-dependent \([\text{H}]\)-substrate binding can be assessed to ensure that the protein is correctly folded and inserted into the bilayer. We therefore examined the ability of both LeuT-WT and -Y268A to bind \([\text{H}]\text{Leu}\), using a previously published scintillant proximity assay (SPA)-based protocol (21), differing only in that LeuT was now reconstituted into biotinylated nanodiscs. These data confirmed that, in the presence of 50 mM sodium, both variants can indeed bind \([\text{H}]\text{Leu}\) with similar \(B_{\text{max}}\) values but with vastly different dissociation constants \(K_d\) (46 versus 916 nM for LeuT-WT and -Y268A, respectively) (Fig. S2). The ~20-fold lower affinity of the Y268A mutant for Leu most likely reflects its conformational bias toward inward-open conformations.

Peptide Mapping and Coverage of LeuT Peptides via MS. Having established that nanodisc-reconstituted LeuT was correctly folded, we next tested whether peptides obtained upon proteolysis of LeuT-WT could be identified by MS. Data collected from multiple peptide identification runs were used to generate a coverage map for WT could be identified by MS. Data collected from multiple peptide identification runs were used to generate a coverage map for 67 peptides, with an average redundancy of 2.04 (Fig. S3).

Overall Conformational Signatures of LeuT in Lipid Nanodiscs as Revealed by HDX-MS. Comparison of HDX-MS data for LeuT-WT and LeuT-Y268A in the presence of saturating sodium allowed us to identify elements of the protein whose accessibility or conformational ensembles differ between these outward- and inward-favoring conditions. In particular, peptides encompassing the N-terminal peptide, TM1a, EL2, the cytoplasmic end of TM7, and surrounding the extracellular vestibule (TM1b, EL4, and TM6a), all showed significant differences in deuteration uptake. Fig. 2 shows deuteration kinetics for a selection of these peptides. The difference in deuterium exchange rates between outward- and inward-favoring conditions \(\Delta \%D\) mapped from the N to the C terminus clearly highlights the distinct accessibility or conformational dynamics of the two LeuT variants (Fig. 3). Specifically, the extracellular entrance (TM1b, EL2, EL3, and EL4b) was more heavily deuterated in outward-favoring LeuT-WT, whereas the N-terminal peptide, TM1a, the intracellular end of TM7, the small extracellular loop following TM7, and EL4a, all exhibited greater deuteration in inward-favoring LeuT-Y268A (Figs. 2 and 3).

Slow Conformational Dynamics in TM7 and EL2. Amide-H/D exchange events are typically classified as falling within one of two distinct kinetic regimes, EX1 or EX2. EX2 delineates the kinetics of protein dynamics that are faster than the chemical rate of hydrogen to deuterium exchange for peptide(s) residing within a region of the protein. Although the amplitudes of the motions contributing to EX2 exchange may span a wide range, these motions are generally rapid and uncorrelated. Less common is exchange within the EX1 kinetic regime, in which protein movements...
are significantly slower compared with the chemical rate of exchange (41, 42). EX1 kinetics has traditionally been associated with cooperative motions that simultaneously expose multiple adjacent amide hydrogens for exchange, which might occur, for example, by local unfolding. In HDX-MS measurements, EX1 kinetics is generally characterized by a signature bimodal isotopic distribution in the mass/charge ratio, where longer deuterium incubation periods result in the exchange-protected (low mass) and exchange-prone (high mass) envelopes gradually shrinking and accumulating, respectively. This change in isotopic distribution reflects the increasing proportion of protein that has sampled an exchange-prone state of such a slow, cooperative conformational change (41, 42). Such kinetic behavior was observed for peptides at the intracellular end of TM7, that is, the fragment containing amino acids 278–285, hinting that these peptides or their packing with neighboring protein segments underwent conformational changes with slow kinetics (Fig. 4 A and C). Interestingly, this EX1 behavior was observed only in the inward-favoring condition (Y268A). These observations of cooperative “unfolding” (or repacking) of the region involving TM7 are concordant with previously reported DEER data, indicating that movement of TM7 occurs during opening and closing of the intracellular gate (15).

Similar EX1-type deuterium uptake kinetics was also observed for residues 142–148 (EL2; Fig. 4B), which are involved in closure of the extracellular side, when EL4 packs more closely against them (Fig. 4A). Together, these observations demonstrate that differences in conformational ensembles could be detected using HDX-MS and provide clues about the dynamics of areas of the transporter that undergo concerted conformational transitions.

**Agreement Between Experimental and in Silico-Predicted Deuterium Uptake.** The aforementioned EX1-type kinetics of EL2 and TM7 is likely to reflect local unfolding on a relatively slow timescale. By contrast, differences in the EX2-type HDX results for different conditions may be more reflective of faster-scale fluctuations affecting the solvent accessibility of the peptide backbone under those conditions. We therefore hypothesized that interpretation of these data could be aided by comparison of ensembles of structures generated with MD simulations (43, 44) starting with X-ray crystallographic structures of different conformations of LeuT. The outward- and inward-open conformational ensembles of LeuT were mimicked in silico using MD simulations of the following, respectively: the substrate-free, sodium-bound, outward-open structure [Protein Data Bank (PDB) ID code 3TT1] after reversing the Y108F mutation (12); and the substrate-free, inward-open, structure (PDB ID code 3TT3) after reversing the modifications at the Na2 site (T354A and S355A) and TM7 (K288A) (10), but maintaining the intracellular gate mutant Y268A. In each case, the protein was embedded in a hydrated lipid bilayer. Analysis of multiple ~450-ns-long trajectories revealed that the root-mean-squared deviation (rmsd) from the X-ray structure of the Ca atoms in the transmembrane helices is only 1.0 ± 0.1 Å for the outward-facing system and 1.7 ± 0.1 Å for the inward-facing system, each averaged over the last 100 ns of three independent trajectories, indicating the protein structure was stable.

To compare the in silico and in vitro data as directly as possible, we computed the expected extent of deuterium incorporation for each of the peptides based on the so-called protection factor, which is an empirical parameter reflecting the degree to which each amide proton forms hydrogen bonds and is surrounded by other protein groups (44) (Materials and Methods). However, this analysis approach was developed for water-soluble proteins, and therefore it was unclear whether it could reasonably be applied to simulations of membrane proteins, or whether its application to membrane proteins would require timescales longer than ~100 ns (44) to account for differences in dynamics of the protein and its environment. In
particular, the residue protection factor is computed as an ensemble average, contingent on the environment as well as thermal fluctuations, and thus also on trajectory length. Therefore, for each simulation, we calculated the protection factor as a function of extending the length of the trajectory up to ~500 ns. The protection factor for each of the independent trajectories stabilized after ~200-ns simulation (Fig. S4A), indicating that this timescale was both required and sufficient to obtain an ensemble of LeuT conformations whose hydrogen bonding and packing were adequately sampled. The small variation between the three trajectories underscores the additional conformational sampling achieved by the use of multiple independent trajectories for direct comparison with experimental data.

As expected, the residues with the smallest protection factors were the most dynamic and exposed to aqueous solution (Fig. S4B). Importantly, for the peptides detected by MS, the predicted deuterium uptake based on these protection factors for the two simulated states of LeuT followed the same trend as the observed uptake in both outward-favoring (WT) and inward-favoring (Y268A) conditions (Table S1, Fig. 5, and Fig. S5). For example, when we extrapolated the computed protection factors into values of expected deuterium exchange after 10 min of exposure, the predicted values correlated with the experimental data for 21 peptides in the outward-favoring condition with Pearson’s correlation coefficient of 0.84 (Table S1). For reference, when using only the X-ray crystal structure (3TTI; inward-open) to predict deuterium uptake, the corresponding correlation coefficient is only 0.47, emphasizing the requirement for an ensemble average of conformations upon which to compute the protection factors (44).

The overall agreement between the predicted and measured deuterium uptake levels provided support for the assumption that >200-ns-timescale simulations recapitulate many important features of the solvent accessibility of the ensembles being measured experimentally (Figs. S4 and S5), and engender confidence that the computation of the predicted deuterium fraction is a reasonable strategy for membrane proteins. For example, the simulation data correctly predicted overall higher deuteration of the N-terminal segment (peptides 1–12, 1–14, and 1–16), and EL4b (residues 325–331) as well as lower deuteration in TM1b (residues 29–34), and EL4a (peptide 306–313), when comparing outward- to inward-facing systems (Fig. 5). Thus, MD simulations may be useful for providing molecular detail to the measured deuteration and to differences between the two conformations.

**Conformational Dynamics of TM1a, the N-terminal Peptide, and TM7.** A striking feature of the inward-open crystal structure of LeuT is the seemingly unrestrained repositioning of TM1a into the predicted hydrophobic core of the membrane bilayer, relative to the outward-facing structures (6) (Fig. 6A). Indeed, the extent of TM1a movement has been a source of contention in the field given that the crystal conditions contained mostly detergent and only a minute quantity of lipid [1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE)], and that biophysical and computational studies have indicated a more restrained tilt of the helix (10, 15, 22, 45). Our HDX-MS data demonstrate that TM1a (amino acids 17–28) as well as the attached N-terminal peptide (amino acids 1–16) were less protected against deuterium exchange under the inward-favoring conditions and thus apparently free to sample a larger conformational space with greater solvent accessibility than was available under the outward-favoring conditions (Figs. 2, 3, and 5, and Fig. S6). MD simulations also captured this difference in that the predicted deuteration was significantly higher for the N-terminal peptide in the inward-open system (Fig. 5 and Fig. S4). Surprisingly, however, there was no significant increase in the predicted deuteration for residues in TM1a, in contrast to the increase predicted for the N-terminal peptide. Nevertheless, TM1a helix was observed to explore a wider range of tilt angles in simulations of the inward-open state (Fig. 6B) relative to those of the outward-open state, indicating greater flexibility. The average value of this tilt angle was smaller than that obtained for the crystal structure (Fig. 6B), consistent with free-energy calculations (10, 22), possibly reflecting the difference between lipid and detergent environments. Importantly, directly quantifying aqueous accessibility of individual amino acids identified several residues in TM1a for which either the backbone or side chain exposed a larger surface area to water during the simulations of the inward-open structure, consistent with an increased propensity for deuteration (Fig. 6B and Fig. S6 B and C). Of particular note is residue L14, whose increased solvent accessibility is consistent with the higher measured deuteration of residues in peptides containing residues 12–14 and 12–16 in the inward-favoring conditions (Fig. 3 and Fig. S6B), as well as M18 and N21 (Fig. S6 A and C), whose increased solvent accessibility matched the higher measured deuteration of peptides covering residues 17–28 (Figs. 2 and 3, and Fig. S6B). In this way, the simulations also help narrow the segment responsible for measured changes from ≥6-residue-long peptides down to individual amino acids.

In TM7, residues in its ostensibly buried segment (S278 and A282) also became more accessible to water in simulations of the inward-facing structure, partly due to separation of TM1a and TM6 away from TM7 (Fig. S6). This finding is concordant with increased HDX measured in peptides 278–282, 278–285, and 278–289 in inward-favoring LeuT-Y268A compared with outward-favoring LeuT-WT (Fig. 3).

The above analysis of the solvent-accessible surface area indicates that the simulation of the inward-facing Y268A mutant did capture the exposure of TM1a and TM7 implied by the experimental measurements for Y268A, suggesting reasonable agreement of deuterium uptake trends over 21 LeuT peptides between in vitro (green and orange) and in silico (blue) HDX experiments. Left and right panels depict data for deuterium uptake for peptides in the outward- and inward-favoring states of the transporter, respectively, compared with simulation data starting with the outward- and inward-open states, respectively.

**Fig. 5.** Agreement of deuterium uptake trends over 21 LeuT peptides between in vitro (green and orange) and in silico (blue) HDX experiments. Left and right panels depict data for deuterium uptake for peptides in the outward- and inward-favoring states of the transporter, respectively, compared with simulation data starting with the outward- and inward-open states, respectively.

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overlap between in silico and in vitro ensembles. What then explains the underestimation in the predicted deuteration exchange at these segments (Fig. 5)? We posit that this discrepancy may reflect limitations in the approach used to translate MD trajectories into predicted deuteration for helices that buttress narrow aqueous pathways in membrane regions, although it is also possible that the HDX kinetics in this region are slower than the simulation timescale.

**Insights into the Conformational Dynamics of EL4.** The peptides that include EL4a (amino acids 306–311, 306–313, 312–324, and 314–324) all exhibited a significant reduction in deuteration uptake under the inward-favoring versus outward-favoring conditions (Fig. 3), whereas residues in EL4b (amino acids 325–331) exhibited the opposite pattern. The MD trajectories mirrored these findings. Specifically, peptides 306–313 and 312–314 of EL4a were slightly more protected in the simulations of the inward-open structure, whereas peptides in EL4b became less protected (residues 325–331; Fig. 5). These observations uphold expectations based on the relative orientations of EL4a and EL4b seen in the inward-open structure where the V-shaped tip of EL4 dips into the extracellular vestibule, rendering EL4a more exposed to the extracellular milieu, while concomitantly shielding EL4b by virtue of the latter’s proximity to EL2 and TM2 (6). Analysis of the trajectories revealed that this difference in predicted deuteration uptake reflected not only the packing of EL4 against its neighboring elements but also changes in, and increased flexibility of, backbone dihedral angles (Fig. 7 B and D), most strikingly for A319, which resides in the short loop connecting EL4a to EL4b.

Analysis of experimentally determined deuteration uptake kinetics for residues in the V-shaped tip of EL4 (amino acids 312–324; Fig. 6B) under inward-favoring conditions unveiled a bimodal isotopic distribution in the mass/charge ratios (Fig. 7B). However, detailed inspection of the mass spectra (Fig. 7 A and B) revealed that the exchange-protected envelope did not diminish, suggestive that Y268A adopts two noninterconverting conformations in this region. Quantification of isotopic distribution envelopes for this peptide revealed that ~30% of the peptide population remained protected throughout the deuterium exposure (Fig. 7C). Curiously, this behavior was absent for the same peptide under outward-favoring conditions (Fig. 7A). It should be noted that for a few other peptides, this pattern of “static” (or very slow) bimodal behavior was observed in both inward and outward favoring conditions, with notable exceptions being the peptide encompassing EL4 (amino acids 312–324; Fig. 7A) and another in TM7 (amino acids 278–289) for which the protected envelope remained static exclusively in the outward-favoring condition (Fig. S7 and Table S2). It is also worth noting that peptides displaying such static bimodal behavior colocalized, primarily on the extracellular side of the transporter (Fig. S7).

**Discussion**

NSS members are integral membrane proteins that play a crucial role in terminating synaptic transmission and in shaping the duration and magnitude of synaptic signaling. They are the targets of numerous psychoactive agents, and their dysfunction is implicated in multiple neuropsychiatric illnesses (1). Large-scale conformational changes are integral to their function, and these movements are modulated by interaction with substrates, inhibitors, ions, pre-synaptic proteins, and lipids. Because of their extraordinary clinical importance, it is essential to build a solid mechanistic grasp of their conformational dynamics in their native lipidic environment.

The objective of this study was to develop a strategy that could delineate the conformational signatures of two divergent conformations of an unlabeled NSS protein in a membrane-like milieu. To achieve these goals, we incorporated LeuT, a thermostable prokaryotic NSS homolog, into lipid bilayer nanodiscs. To circumvent the need to label specific residues, we monitored the conformational hallmarks of LeuT using HDX-MS, a technique that has previously been used to probe the conformational dynamics of other membrane proteins (40–48). We biased the conformational equilibrium to more outward-favoring orientations by adding saturating Na+ to WT LeuT. To shift the equilibrium toward inward-favoring conformations, we capitalized on the well-characterized Y268A mutant. To avoid complications related to removal of Na+ and to permit direct comparison with the outward-favoring condition, we used assay buffer with identical components, including 100 mM NaCl. Despite the fact that Na+ unambiguously shifts the equilibrium of WT LeuT toward outward-favoring orientations, the same is...
Altered dynamics of EL4 in LeuT under outward- and inward-favoring conditions.

**A** Mass over charge spectra measured without deuteration (UD) and after deuteration for four different time windows, for a peptide in EL4 comprising residues 312–324, indicating a unique population of more highly deuterated peptides in inward-favoring (Right) relative to outward-favoring LeuT (Left). The total isotopic peak (blue) is decomposed into protected (green) and unfolded (red) fractions, when applicable. **B** Position of EL4a and EL4b in the outward-open (orange) and inward-open (green) structures of LeuT. The position of A319 and its neighbor G318 (ball-and-sticks) are shown in detail in the Inset. (C) Fraction of peptide 312–324 deuterated (unfolded) as a function of incubation time for LeuT-WT (orange) and -Y268A (green) LeuT. **D** Backbone \( \psi \) (Upper) and \( \phi \) (Lower) dihedral angles in A319 in the EL4ab loop during MD simulations of the outward-open (3TT1; Left) and inward-open structures (3TT3; Right). For each simulation system, three independent trajectories are shown (black, red, and cyan points) sampled every 100 ps. For reference, the \( \phi, \psi \) values of the X-ray structures are as follows: \(-58.3^\circ, -46.7^\circ\) in 3TT1; and \(-84.0^\circ, 141.2^\circ\) in 3TT3.

**Fig. 7.** Altered dynamics of EL4 in LeuT under outward- and inward-favoring conditions. (A) Mass over charge spectra measured without deuteration (UD) and after deuteration for four different time windows, for a peptide in EL4 comprising residues 312–324, indicating a unique population of more highly deuterated peptides in inward-favoring (Right) relative to outward-favoring LeuT (Left). The total isotopic peak (blue) is decomposed into protected (green) and unfolded (red) fractions, when applicable. (B) Position of EL4a and EL4b in the outward-open (orange) and inward-open (green) structures of LeuT. The position of A319 and its neighbor G318 (ball-and-sticks) are shown in detail in the Inset. (C) Fraction of peptide 312–324 deuterated (unfolded) as a function of incubation time for LeuT-WT (orange) and -Y268A (green) LeuT. (D) Backbone \( \psi \) (Upper) and \( \phi \) (Lower) dihedral angles in A319 in the EL4ab loop during MD simulations of the outward-open (3TT1; Left) and inward-open structures (3TT3; Right). For each simulation system, three independent trajectories are shown (black, red, and cyan points) sampled every 100 ps. For reference, the \( \phi, \psi \) values of the X-ray structures are as follows: \(-58.3^\circ, -46.7^\circ\) in 3TT1; and \(-84.0^\circ, 141.2^\circ\) in 3TT3.

apparently not true for Y268A (15). Specifically, the distance distributions between spin labels on pairs of residues in TM9 and TM1a, or in TM9 and TM5, used to characterize the inward-facing conformation, do not change for Y268A in the presence and absence of 200 mM NaCl (15). On the other hand, smFRET data suggest that Na\(^{+}\) can modulate the inward-outward distribution of Y268A to some extent (13). Thus, Na\(^{+}\) is apparently able to modulate the dynamics of Y268A, and yet cannot completely overcome its bias to inward-favoring conformations. Indeed, the HDX pattern of the Y268A variant reported here, consistent with an inward-facing conformation, is in harmony with the latter finding.

Broadly speaking, transition from the outward- to inward-open conformation in LeuT involves (i) occlusion of the extra-cellular vestibule, obstructing access to the substrate-binding site, and (ii) opening of the cytoplasmic pathway, partly facilitated by the untethering of the N-terminal peptide and TM1a from the scaffold (6, 12, 49). This study provides support for the notion that these global changes, with the same key players, which were previously described predominantly in detergent micelles, also occur in a lipid bilayer. Both HDX-MS and MD data for amino acids along TM1 clearly demonstrate the larger conformational space available to the N-terminal peptide and TM1a in inward-favoring versus outward-favoring conditions, albeit with a smaller average tilt angle than was reported in the corresponding inward-facing crystal structure (6) (Fig. 4). Note that crystallographic capture of the inward-open conformation of LeuT required multiple mutations in addition to Y268A at the cytoplasmic side, as well as a conformation-specific antibody (6). One or more of these modifications may contribute to the distinct nature of TM1a in the MD simulations (10) in addition to the bilayer environment (11).

We note that, to the best of our knowledge, the simulations of the inward-facing conformation of LeuT-Y268A presented here are unique, because all previous studies either examined the inward-facing conformation after restoring the missing Tyr at position 268 (10, 22, 45, 49) or considered the allosteric effect of the Y268A modification in the outward-occluded conformation (11). Although the available evidence shows that the Y268A modification strongly affects the ability for LeuT to stabilize outward-facing conformations and to allosterically detect ligand binding in those conformations (11), comparison of our simulation data with that reported for previous studies (10, 22) revealed no obvious difference due to the Y268A modification in the nature of the inward-facing conformations, presumably because position 268 was entirely solvent exposed, and therefore unable to mediate direct interactions. Nevertheless, free-energy calculations could be used in the future to help rule out an effect of Y268A, and of other modifications used in such studies, on specific features of the inward-facing conformations of LeuT.

The overall pattern of measured deuterium uptake was well captured in predictions from MD simulations, as were the increases in dynamics and accessibility of the N-terminal peptide and EL4 identified experimentally. However, differences in the behavior of TM1a and TM7, both of which are accessible to water via a narrow aqueous pathway, and in contact with lipid, were not reflected by changes in predicted deuterium uptake. Although beyond the scope of the current study, it will be of interest to explore whether this discrepancy reflects a limitation of the “protection factor” descriptor for such pathway-lining regions, which are of particular importance in integral membrane proteins, or whether the relative dynamics are simply inherently slower than the simulation timescales. Nevertheless, it is encouraging that the molecular simulations on timescales of \( \sim 200 \) ns show good convergence of the protection factors and that the overall correlation with the experimental data are high, despite the inherent differences between the in vitro and in silico systems.

In other regions of the protein, slow, EX1-type kinetic behavior for peptides belonging to EL2 and TM7 illuminate their structural plasticity. The observed EX1 kinetics data provide unprecedented insights into LeuT conformational dynamics that cannot be gleaned from either solution spectroscopy or static crystal structures. Another interesting feature of the HDX-MS data is the presence of a static bimodal isotopic envelope distribution for a selection of peptides. These observations suggest local conformational heterogeneity that may reflect preferential stabilization of certain conformations by the lipid nanodisc environment (50). Indeed, two recent studies have demonstrated disparate behavior of transporters in lipids versus detergent. First, Sohail et al. (22) reported different distributions for the N-terminal segment of LeuT in detergent micelles compared with POPC liposomes, as measured by lanthanide-based resonance energy transfer and in agreement with MD simulations. Second, Martens et al. (51), in a spectroscopic study of the proton-coupled multidrug antiporter LmrP, demonstrated that an inward-facing conformation was preferentially stabilized when the protein was incorporated into nanodiscs rather than solubilized in detergent. As such, the local heterogeneity reported by

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this static bimodal behavior may reflect conformational changes of LeuT that are stabilized upon incorporation into nanodiscs. However, measurements of HDX patterns of LeuT reconstituted into both lipidic and detergent environments will be needed to directly compare the effect of detergent on its conformational dynamics.

This study provides a probe-independent, global view of the dynamics of a membrane-bound transporter within a lipid bilayer, interpreted in atomic-level detail by integration with molecular simulations. Going forward, such data will be of enormous importance in understanding the relevance both of the snapshots provided by crystal structures as well as of information about local protein environment provided by other biophysical techniques such as smFRET and EPR. This success represents a crucial leap forward on the road to probing the dynamics of the more clinically relevant mammalian NSS members such as SERT and DAT, whose activities are heavily influenced by the specific properties of surrounding lipids and where retention of “wild-type” levels of activity in cyless mutants has not yet been realized. Furthermore, with these tools, we will now be able to delve into the effects of, for example, inhibitor binding as well as the dynamic sequence of disease-associated polymorphisms, especially those located outside of canonical drug binding sites and/or facing the lipid bilayer, without having to mutate and/or otherwise modify the protein.

Materials and Methods

Protein Preparation. MSP. MSP1E13D1 was expressed in Escherichia coli BL21 (DE3) cells and purified as described (21). Following proteolytic cleavage of the polyhistidine tag used for purification, MSP was biotinylated by incubating with a 20- fold molar excess of NHS-Sulfo-S-S-biotin (Pierce) for 30 min at room temperature and then removing unreacted biotin with Zeba spin columns (Thermo Scientific) as per manufacturer’s instructions.

WT LeuT and the Y268A mutant (WT-LeuT and -Y268A). LeuT-Y268A was generated with PCR-based site-directed mutagenesis and verified by DNA sequencing. Both LeuT variants, each with a C-terminal 8×His tag as previously reported (52) with some modifications. Briefly, membranes were washed three times in buffer A [20 mM Tris (pH 8.0), 200 mM KCN] containing 10 mM 1,4,7,10-pentaacyclooctadecane (15-Crown-5) (S) to ensure efficient removal of sodium and thereby any endogenously bound leucine (52). As an added precaution, sodium-free labware was used throughout. Purification was initiated by solubilizing the washed membrane in buffer A containing 40 mM DDM followed by N2+affinity [HiTrap IMAC Sepharose FF (GE Healthcare)] and gel filtration [Superdex 200 10/300 GL (GE Healthcare)] chromatography in buffer A containing 1 mM DDM. The His tag was left intact to facilitate downstream nanodisc purification and radioligand binding experiments via SPA (21, 54). Purified protein was concentrated to 1 mg/mL and stored at −80 °C until use.

Phospholipid Preparation and Nanodisc Assembly. The two purified LeuT variants were separately reconstituted into nanodiscs as described elsewhere (21) with some minor adjustments. Lipid stocks [POPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG)] were prepared in chloroform and mixed in a 3:2 molar ratio to obtain a 40 mM final lipid stock. After the chloroform suspension had been dried under nitrogen gas, the thin lipid film was solubilized in buffer B [20 mM Tris HCl (pH 7.4), 100 mM NaCl, 0.5 mM EDTA] containing 80 mM sodium cholate. LeuT-MSP/lipids were mixed at a molar ratio of 1:20:1,000 to initiate reconstitution of LeuT into the nanodiscs. After incubating on ice for 1 h, SMZ-Biobeads [10% (wt/vol)] were added to the mix, and the tube was rotated at 4 °C overnight. LeuT-containing nanodiscs (LeuT-ND hereafter) were separated from empty nanodiscs by passing through Ni2+-NTA resin, washing with 10 column volumes of buffer B containing 20 mM imidazole, and eluted in the same buffer containing 300 mM imidazole. The nanodiscs were then concentrated and purified further on a gel filtration column (Superdex 200 10/300 GL; GE Healthcare) in buffer B. Purity and concentration of the LeuT-nanodisc preparations were calculated with SDS/PAGE, as detailed previously (21, 38).

Leucine Binding Assay. Saturation binding of [3H]Leu to both the LeuT-nanodisc preparations was assessed by SPA as outlined (21). Briefly, LeuT-containing nanodiscs were first preincubated with Cu2+ chelating YSI SPA beads (PerkinElmer) in buffer C [150 mM Tris-Mes (pH 7.5), 50 mM NaCl, 20% (vol/vol) glycerol] for 2 h at 4 °C to allow capture onto the bead surface. The resulting mixture was then incubated with 10–1,500 nM [3H]Leu (specific activity, 109 Ci/mmol; PerkinElmer) with or without 1 μM Leu-Enk for 1 h at room temperature before being transferred into the individual wells of a 96-well white-wall clear-bottom plate (Isoleat-96; PerkinElmer) and read in a MicroBeta counter (PerkinElmer) in SPA mode until counts plateaued, indicating equilibrium had been reached. The assay was performed three separate times, each time in triplicate. Nonspecific binding was measured in the presence of 5 mM Ala. Counts per minute were converted to picomoles using a calibration curve relating counts per minute to a known concentration of radioactive substrate, accounting for the manufacturer-provided efficiency of the SPA beads (60%). Data were analyzed via nonlinear regression in GraphPad Prism 6.0.

Optimization of Peptide Coverage Map for Downstream HDX-MS Experiments. LeuT-ND samples were concentrated in buffer B using 10-kDa-cutoff Amicon ultrafiltration filters to obtain a final sample concentration of ~20 μM. Peptide identification and corresponding coverage map of LeuT was obtained from undeuterated controls. A typical sample workflow involved diluting 10 μL of LeuT-ND (20 μM) into 90 μL of buffer B at room temperature followed by quenching with 0.8% formic acid, nanodisc solubilization, and pepsin digestion.

To maximize the number of peptides identified and determine the optimal experimental setup to achieve maximal capture of LeuT peptides, the following variables were explored: detergent type (sodium cholate, Tween-20, maltosides (decyl- and dodecyl), and maltose neopentyl glycols (decyl and dodecyl)), detergent concentration; pepsin species (porcine versus Aspergillus), digestion conditions, brand (Thermo Fisher, Sigma, or POROS), and location [inline versus free (immobilized or batch) at different concentrations]; quench solution (0.8% formic acid in LC-MS grade water with or without various concentrations of guanidine HCl or acetonitrile) to facilitate efficient pepsin digestion; removal of MSP peptides with Neutravidin UltraLink beads (Thermo Fisher), varying both amount and duration; and removal of solubilized phospholipids with ZrO2 HybridSPRE beads (Supelco), again varying both amount and duration.

HDX-MS Experiments. Based on the above trials, the optimal sample scheme selected for HDX purposes was as follows: 10 μL of LeuT-ND (20 μM) was diluted with 90 μL of buffer B containing 50% (wt/vol) Neutravidin Ultralink beads (Pierce) and 10 μL of 2.5 mM DDM were sequentially added. Subsequent to vortexing for ~5 s, the sample was spun down through a 0.45-μm Spin-X microcentrifuge tube filter (Costar) for 30 s at which 10 μL of 1 mg/mL porcine pepsin (Sigma) was added to the flowthrough, mixed by vortexing for ~5 s, and incubated on ice for 4 min. To remove solubilized phospholipids, 10 μL of 300 μM ZrO2 HybridSPRE-Phospholipid beads (Supelco) equilibrated in 0.8% formic acid were added and incubated on ice for 1 min. All steps after dilution were performed either on ice or at 4 °C to minimize back-exchange during the actual deuteration experiment (see below). The resulting mixture was spun down through a 0.45-μm Spin-X microcentrifuge tube filter for 30 s and then immediately injected into a Waters HDX nanoAcquity ultra-performance liquid chromatography (UPLC) system (Waters). Peptide fragmentation (UPLC) system (Waters), peptide fragments were trapped on an Acquity UPLC BEH 300 C18 peptide trap and separated on an Acquity UPLC HSS T3 C18 attached to an Acquity UPLC BEH C18 guard column. A 7 min, 5–35% (vol/vol) acetonitrile (0.1% formic acid) gradient was used to elute peptides directly into a Waters Synapt G2 mass spectrometer (Waters). MS3 data were acquired with a 20–30 V ramp trap collision energy for high-energy acquisition of product ions and lock mass (Leu-Enk) for mass accuracy correction. Peptides were identified using the ProteinLynx Global Server 2.5.1 (PLGS) from Waters.

H/D exchange reactions were performed using the workflow outlined above except that 90 μL of buffer B was replaced by deuterated buffer B. All deuteration reactions were performed at 25 °C and quenched at various times (10 s, 1 min, 10 min, and 2 h) for both LeuT-WT as well as -Y268A. All deuteration time points and controls were acquired in triplicate. Deuterium uptake by the identified peptides over time was calculated with Waters’ DynamX 2.0 software. When considering all time points and both constructs, these conditions allowed for HDX analysis of 35 peptides (27% of the LeuT sequence, i.e., about one-half of the peptides detected by MS could be analyzed for deuterium exchange) (Fig. 53). Plots of the difference in deuterium uptake of any individual time point were determined according to Houde et al. (56, 58). Specifically, the 98% confidence interval for the #D was determined to be ±0.33 deuterium atoms incorporated at any single time point, or ±0.66 deuterium atoms incorporated when considering the sum of the #D over all four incubation time points. For peptides displaying
bimodal spectra, the two peaks were deconvoluted using HX-Express2 (60). The deuterium level and relative intensity of each individual envelope were extracted, and the relative deuterium level of the exchange-protected envelope alone was used to compute $\Delta(\%WT-Y268A)$ for the purposes of comparison with predicted deuterium based on MD simulations. The change in relative intensity of the unprotected peak (\%U) as a function of time was used to ascertain whether the two species were interconverting. To account for back-exchange, experiments with fully deuterated controls were performed. Specifically, LeuT in buffer A containing 1 mM DDM was lyophilized and then dissolved in D$_2$O (99.9%; Cambridge Isotopes with 1 mM DDM), with the process being repeated twice. Ten microliters of deuterated LeuT were further incubated in 90 $\mu$L of buffer B containing 6 M guanidine-DCl for 2 h at 25°C. The rest of the UPLC and MS procedure was unchanged. Out of the 35 peptides, 21 displayed a well-defined spectral isotopic envelope for the fully deuterated control. For these peptides, deuterium uptake was normalized as follows: $\%D = 100 \times (m_t - m_u)/(m_t - m_0)$, with $m_t$ the centroid mass at incubation time $t$, $m_u$ the centroid mass of the undeuterated control, and $m_0$ the centroid mass of the fully deuterated control. The 98% confidence intervals were adjusted accordingly and were determined to be ±8%.

**MD Simulations.** Simulations were carried out on LeuT monomers starting with two different structures: outward-open sodium-bound, or inward-open sodium-bound, each in a patch of dimyristoylphosphatidylcholine (DMPC) bilayer, and hydrated with 100 mM salt solution. DMPC was selected based on hydrophobic matching with LeuT monomers (61). The simulations of outward-open LeuT have been described previously (12). Briefly, the starting point for the protein was PDB ID code 3TTL, with Na$^+$ ions occupying both Na1 and Na2 sites; the Y108F mutation introduced into the crystallization construct was reverted to WT Y108. Protonation states were assigned according to methods from the electrostatics calculations (10,62) performed on the corresponding X-ray structures (12). Thus, E112, E287, and E419 were protonated in both systems. E290 was deprotonated in the outward-open state (with sodium bound at Na1 and Na2) and protonated in the inward-open state (where sodium is not bound). The starting point for the inward-open system was based on PDB ID code 3TTL, but because of concerns over the possible loss of a proton conformation with the minimum free-energy conformation of TM1a based on umbrella sampling calculations of LeuT in a lipid bilayer environment, kindly provided by Prof. B. Schi (University of Aarhus, Aarhus, Denmark) (10). This protein structural model contained WT sequence for the Na2 binding site (T354, S355), although we included the intracellular gate mutation (Y268A), for comparison with the obtained WT sequence for the Na$_2$ binding site (T354, S355), although we maintained WT sequence for the Na$_2$ binding site (T354, S355). The 98% confidence intervals were adjusted accordingly and were determined to be ±8%.

During the simulations, the membrane plane was kept at a constant area, while constant temperature (310 K) was maintained through Langevin dynamics, and constant pressure (1 atm) was achieved with the Nosé–Hoover Langevin piston algorithm (63). Nonbonded interactions were switched off smoothly from 10 to 12 Å, and particle mesh Ewald summation was used to compute long-range electrostatic interactions (71, 72). The simulation time step was 2 fs. After introducing the protein into the adapted hydrated lipid patch, the entire system was energy minimized and equilibrated as described (12) using GROMIN (69), with a total of 1,200 steps of energy minimization followed by 8 ns of constrained MD during which constraints were progressively released.

MD trajectories ($n = 3$; timescale, 450–480 ns per trajectory) were examined every 100 ps (unless otherwise stated) with CHARMm c36b2 (73), VMD (74), MDAnalysis (75), as well as in-house scripts based on the Radou et al. (64) approach that compute hydrogen bonds and local contacts among each amide hydrogen during the simulation so as to predict deuterium uptake for each residue (76, 77). Specifically, the fraction of deuterium D, taken up by a backbone nitrogen in a given experimental exposure time $t$, is predicted to depend on the protection factor of that residue, $P$, according to the following:

$$D(t) = 1 - \exp \left( -\frac{k_BT}{P} \cdot t \right),$$

where $k_B$ is the intrinsic exchange rate, deduced from experimental measurements for every amino acid type, and depends on the amino acids adjacent in the sequence (78). $P$ is computed for a simulation of a given length using the following:

$$P = \frac{n_P}{n_P + n_N},$$

where $n_P$ and $n_N$ have been parameterized using experimental HDX data (77). $N_i$ is the number of contacts of the amide nitrogen with any atom in all other residues within 6.5 Å, and $N_i$ is the number of hydrogen bonds it forms, according to Gromacs, version 4.6.7 (79–82). Calculation of the protection factor for the crystal structures used the same procedure.

For calculation of the rmsd and superposition of structures, transmembrane helices were defined as follows: TM1a (12–21), TM1b (25–38), TM2 (42–72), TM3 (88–124), TM4 (166–184), TM5 (192–215), TM6a (241–255), TM6b (260–268), TM7 (276–306), TM8 (337–369), TM9 (375–395), TM10 (399–427), TM11 (446–476), and TM12 (483–510). For computation of the tilt angle, we defined TM1a as the axis connecting the centers of mass of residues 11, 12, and 13, and of residues 16, 17, and 18; all six residues remained helical >70% of the simulation time. Figures of structures and simulation snapshots were made with Pymol (Schrodinger).

**Acknowledgments.** We thank Bruce Davis and Sitaram Meena in the S.K.S. Laboratory for valuable discussion, Gary Rudnick for helpful comments on a revised version of the manuscript, Mahmoud L. Nasr for preliminary HDX-MS experiments, Stephen Silgar for pMSPIE3D1 (Addgene plasmid no. 20066), Zhi Yue and Jana Shen for help in implementing the method of Radou et al. (44), and Birgit Schi for the coordinates of the inward-facing free energy minimum. This work was supported by the Alfred P. Sloan Foundation (S.K.S.); the Brain and Behavior Research Foundation (S.K.S.); a Goodman–Gilman Yale Scholar Award (to S.K.S.); NIH/National Institute of Mental Health Grant ROI MH088305 (to S.K.S.); the Division of Intramural Research of the NIH, National Institute of Neurological Disorders and Stroke (L.R.F.); and the University of Maryland Baltimore, School of Pharmacy Mass Spectrometry Center (SOP1841-IQB2014).


