Substrate-induced Unlocking of the Inner Gate Determines the Catalytic Efficiency of a Neurotransmitter:Sodium Symporter.*

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Background: The mechanism coupling substrate binding to transport in neurotransmitter: sodium symporters (NSSs) is poorly understood.

Results: Site-directed fluorescence quenching spectroscopy experiments on the NSS homologue LeuT reveal a structural intermediate preceding transition to the inward-open conformation. The substrate-induced, Na+-dependent change required an intact primary substrate-binding site and involved increased water exposure of the cytoplasmic end of transmembrane segment 5. The findings were supported by simulations predicting disruption of an intracellular interaction network leading to a discrete rotation of transmembrane segment 5 and the adjacent intracellular loop 2. The magnitude of the spectroscopic response correlated inversely with the transport rate for different substrates, suggesting that stability of the intermediate represents an unrecognized rate-limiting barrier in the NSS transport mechanism.

Neurotransmitters:sodium symporters (NSSs) mediate reuptake of neurotransmitters from the synaptic cleft and are targets for several therapeutics and psychostimulants. The prokaryotic NSS homologue, LeuT, represents a principal structural model for Na+-coupled transport catalyzed by these proteins. Here, we used site-directed fluorescence quenching spectroscopy to identify in LeuT a substrate-induced conformational rearrangement at the inner gate conceivably leading to formation of a structural intermediate preceding transition to the inward-open conformation. The substrate-induced, Na+-dependent change required an intact primary substrate-binding site and involved increased water exposure of the cytoplasmic end of transmembrane segment 5. The findings were supported by simulations predicting disruption of an intracellular interaction network leading to a discrete rotation of transmembrane segment 5 and the adjacent intracellular loop 2. The magnitude of the spectroscopic response correlated inversely with the transport rate for different substrates, suggesting that stability of the intermediate represents an unrecognized rate-limiting barrier in the NSS transport mechanism.

Conclusion: Stability of the intermediate might represent a rate-limiting barrier in the transport mechanism.

Significance: The data add to our mechanistic understanding of Na+-coupled transport across lipid bilayers.

Specialized integral membrane proteins terminate neurotransmission in the CNS by catalyzing Na+-dependent clearance of neurotransmitters from the synaptic cleft (1, 2). For dopamine, norepinephrine, serotonin, glycine, and γ-aminobutyric acid (GABA), this role is fulfilled by members of the neurotransmitter/sodium symporter (NSS) family (also referred to as the solute carrier 6 gene family) (3). Altered function of these transporters has been implicated in both psychiatric disorders and neurological diseases (4–6). Moreover, NSSs constitute important targets for several pharmaceutical agents, including antidepressants, antiepileptics, and illicit drugs, such as cocaine and amphetamines (7).

Our understanding of how NSSs operate at the molecular level has been substantially improved by the high resolution structural information revealed by crystallization of LeuT, a prokaryotic NSS homologue from *Aquifex aeolicus* (8–10) and more recently of MhsT, a prokaryotic homologue from *Bacillus halodurans* (11), and of the dopamine transporter from *Drosophila* (12). The crystal structures have revealed a structural fold with 11 or 12 packed transmembrane segments of which the first 10 are arranged in a pseudo-symmetric inverted repeat pattern with a primary substrate–binding site (S1) in the center and two adjacent sodium-binding sites (S8, 13) (Fig. 1A). The inverted repeat fold has also been identified in symporters and antiporters.

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2 The abbreviations used are: NSS, neurotransmitter:sodium symporter; TM, transmembrane segment; smFRET, single molecule fluorescence resonance energy transfer; SDFQS, site-directed fluorescence quenching spectroscopy; TMR, tetramethylrhodamine; TMSI, cytoplasmic end of TM5; IL2, intracellular loop 2; DDM, n-dodecyl β-D-maltopyranoside; TCEP, tris(2-carboxyethyl)phosphine; SPA, scintillation proximity assay; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; MD, molecular dynamics; tMD, targeted MD; RMSD, root mean square deviation; sTMD, staircase variation of tMD; TM1i, intracellular portion of TM1; TM1-5maleimide, tetramethylrhodamine 5-maleimide; CMI, clomipramine; NT, N terminus; uMD, unbiased MD.
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antiporters with no sequence homology to NSSs (14–19), suggesting that the fold represents a structural paradigm for ion-coupled transport (20). Importantly, LeuT is widely accepted as a principal model for NSSs (21–25), which is supported by the striking structural similarity among LeuT, MhsT, and Drosophila dopamine transporter despite the evolutionary distance between the three proteins (11, 12).

It is believed that NSSs mediate sodium-driven substrate translocation by a classical alternating access mechanism (26). Consistent with such a model, crystallization efforts on LeuT have outlined three canonical conformational states including an “outward-occluded,” an “outward-open,” and an “inward-open” state (8–10). Nonetheless, despite the importance of these states, the structures represent stabilizable states in crystallography and may not provide a complete picture of the translocation cycle (27). To address this and gain better insight into the structural dynamics of the transport process, biophysical techniques such as double electron-electron resonance and single molecule fluorescence resonance energy transfer (smFRET) have been applied to LeuT (28–31). Of interest, the smFRET data substantiated predictions from computational steered molecular dynamics simulations by supporting the functional role of a second high affinity substrate (S2)-binding site in LeuT situated in a vestibule extracellular to the S1 site (29, 32). To this point, however, our understanding of how substrate binding leads to dissociation of a conserved intracellular interaction network (Fig. 1A) and subsequent isomerization of the transporter to the inward-open state (21) remains incomplete.

Here, we applied site-directed fluorescence quenching spectroscopy (SDFQS) to LeuT as a highly sensitive method to investigate conformational rearrangements linked to substrate binding. Labeling with tetramethylrhodamine (TMR) of a single cysteine inserted at the cytoplasmic end of TM5 (TM5i) enabled detection of a Na+-dependent, substrate-induced increase in aqueous exposure of the fluorphore, consistent with a substrate-induced conformational change at the inner gate. Mutational data suggested the requirement for an intact S1 site, and computational simulations were able to link the enhanced aqueous exposure of the fluorphore to the spatial relation between the discrete outward movement of the N terminus and the cytoplasmic end of TM1 and specific changes in the intracellular loop 2 (IL2)-TM5i region. The data suggest a novel coupling mechanism between the primary substrate-binding site and the inner gate involving the formation of a conformational intermediate that may precede transition to the inward-open conformation.

Experimental Procedures

Construction, Expression, Purification, and Fluorescent Labeling of LeuT—Residues were mutated by the QuikChange method (Agilent Technologies) in pET16b LeuT containing a C-terminal octahistidine tag. The generated LeuT mutants were confirmed by DNA sequencing. LeuT variants were expressed in Escherichia coli C41(DE3) by cultivation in lysogenic broth medium supplemented with 0.1 mg/ml ampicillin until A600 reached −0.6. Expression was induced by addition of 0.1 mM isopropyl β-D-thiogalactopyranoside, and the bacterial culture was cultivated at 20 °C for another 20 h. The membrane fraction was isolated by disruption of the cells (Constant Systems homogenizer, Kennesaw, GA) and solubilized with 1% (w/v) n-dodecyl β-D-maltopyranoside (DDM; Affymetrix, Santa Clara, CA). Detergent-solubilized LeuT was immobilized on Chelating Sepharose Fast Flow resin (GE Healthcare), washed with ice-cold buffer A, and incubated with 200 mM tetramethylrhodamine-5-maleimide (TMR maleimide: Life Technologies) for 16 h at 4 °C. Resin slurry was loaded on a packing column and washed with ice-cold Buffer A (20 mM Tris-HCl (pH 7.50), 200 mM KCl, 20% (v/v) glycerol, 0.1 mM tris-(2-carboxyethyl)phosphine (TCEP), and 0.05% (w/v) DDM) containing 60 mM imidazole until flow-through A541 was reduced to 0. Subsequently LeuT was eluted in ice-cold Buffer A containing 300 mM imidazole. Protein samples were desalted using HiTrap spin columns (GE Healthcare) equilibrated with Buffer A. Protein concentration was determined by measuring the TMR-corrected absorbance at 280 nm (ε = 1.91 cm−2 μmol−1). TMR labeling efficiency was calculated as the TMR:LeuT molar ratio measuring TMR absorbance at 541 nm (ε = 95,000 cm−1 m−1). TMR labeling efficiency was >89% in all TMR-labeled LeuT preparations.

Radioligand Binding—Binding experiments were performed using a scintillation proximity assay (SPA) (33). In a 96-well white wall clear bottom plate, 100 ng or 1 μg of LeuT was used as indicated. [3H]Leucine (0.25 (Y108F) or 20.1 Ci mmol−1) or [3H]tryptophan (0.2 Ci mmol−1) (PerkinElmer Life Sciences) was used at the indicated concentrations together with 0.125 mg ml−1 yttrium silicate-copper His tag SPA beads (PerkinElmer Life Sciences). Binding buffer consisted of 20 mM Tris-HCl (pH 7.50), 200 mM NaCl, 20% (v/v) glycerol, 0.1 mM TCEP, and 0.05% DDM. Nonspecific background was detected in the presence of 5 mM alanine. Samples were incubated for 16 h at 4 °C, and activity was recorded on a MicroBeta™ plate counter (PerkinElmer Life Sciences) with a 1-min counting protocol. No change in sample activity was observed when incubating beyond 16 h. Data were analyzed by non-linear regression analysis and fitted to a single site hyperbolic function or sigmoidal dose-response curve using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Kd values were calculated as follows: 

\[ K_d = \frac{IC_{50}}{L} \times \frac{1}{1 + \frac{L}{K_d}} \]

where L denotes the [3H]leucine concentration and Kd is the dissociation constant for leucine.

Reconstitution and Uptake—Purified LeuT variants were reconstituted at a weight ratio of 1:150 (LeuT:lipid) in E. coli polar lipid extract (Avanti Lipids, Alabaster, AL) liposomes prepared as described (33) except for using a reconstitution buffer (200 mM KCl, 20 mM Tris-HCl (pH 7.50), and 0.1 mM TCEP) supplemented with 0.11% Triton X-100 to destabilize the liposomes. Control liposomes lacking LeuT were prepared in parallel. Detergent was removed by incremental addition of adsorbent Bio-Beads SM-2 (Bio-Rad) to a final concentration of ~250 mg ml−1 followed by incubation overnight at 4 °C. The LeuT-containing proteoliposomes or control liposomes were collected by ultracentrifugation at 323,000 × g for 30 min, resuspended in reconstitution buffer to a lipid concentration of 100 mg ml−1, and flash frozen for storage at −80 °C. Prior to uptake experiments, thawed proteoliposomes were extruded through a 100-nm filter (Avanti Lipids).
Time course accumulation of 1 μM [3H]alanine (84.5 Ci mmol⁻¹; PerkinElmer Life Sciences) was measured in a 96-well 0.22-μm PVDF filter plate (Millipore, Billerica, MA) at 22 °C in assay buffer consisting of 200 mM NaCl, 20 mM Tris-HCl (pH 7.50), and 0.1 mM TCEP. At the indicated time points, samples were rapidly filtered using a MultiScreen™ vacuum manifold apparatus (Millipore) and washed three times with ice-cold 0.1 mM TCEP, and 0.05% DDM unless otherwise stated. For ligand binding experiments, a small aliquot of the ligand (as specified) was incubated with 0.1 ml of scintillation liquid (PerkinElmer Life Sciences) before activity was recorded on a MicroBeta plate counter. Cpm were converted into nmol using known molecular weights of the corresponding [3H]-labeled amino acids. Specific activity retained on the filters was recorded by scintillation counting, and cpm were converted into nmol using known molecular weights of the corresponding [3H]-labeled amino acids. Specific activity retained on the filters was recorded by scintillation counting, and cpm were converted into nmol using known molecular weights of the corresponding [3H]-labeled amino acids.

Fluorescence-based experiments were carried out with 0.5 μg of reconstituted LeuT with 0–8 μM [3H]alanine (60 Ci mmol⁻¹; American Radiolabeled Chemicals, St. Louis, MO), [3H]valine (60 Ci mmol⁻¹; American Radiolabeled Chemicals), [3H]isoleucine (50 Ci mmol⁻¹; American Radiolabeled Chemicals), [3H]methionine (80 Ci mmol⁻¹; American Radiolabeled Chemicals), or [3H]leucine (125 Ci mmol⁻¹; American Radiolabeled Chemicals) in assay buffer containing 50 mM Tris/MES (pH 8.5) and 50 mM NaCl at 22 °C. The bound fraction and nonspecific accumulation were assessed by dissipating the Na⁺ gradient with 25 μg ml⁻¹ gramicidin added 1 min prior to the experiment. The reactions were terminated at t = 1 min by dilution into ice-cold stopping buffer containing 100 mM KP, (pH 6.0) and 100 mM LiCl immediately followed by rapid filtration through 0.22-μm nitrocellulose filters (Millipore). Radioactivity retained on the filters was recorded by scintillation counting, and cpm were converted into nmol using known amounts of the corresponding [3H]-labeled amino acids. Specific uptake was determined as the difference between total activity and 3H-substrate binding (as determined in the presence of gramicidin), and kinetic constants were obtained by data fitting to the Michaelis-Menten function in GraphPad Prism 5.0.

Fluorescence Spectroscopy—Fluorescence-based experiments were carried out with 0.5 μg ml⁻¹ of the TMR-labeled LeuT variants diluted in fluorescence buffer containing 200 mM NaCl or 200 mM KCl (as specified), 20 mM Tris-HCl (pH 7.50), 0.1 mM TCEP, and 0.05% DDM unless otherwise stated. For ligand binding experiments, a small aliquot of the ligand (as specified) was added to the samples followed by incubation on an orbital shaker for 30 min at room temperature. It should be noted that we observed no change in the EC₅₀ value for the quenching response when incubating the samples for up to 60 min. Steady-state fluorescence intensities were recorded on a FluoroMax2 (Horiba Scientific, Edison, NJ) at λₑₓ = 572 nm using an excitation source at λₑₓ = 540 nm and excitation and emission band passes of 5 nm at room temperature. Emission spectra were recorded by varying λₑₓ between 550 and 650 nm by 1-nm increments. Quencher titration was carried out by successive additions of small aliquots containing (i) 1 mM potassium iodide (KI) with 10 mM Na₂S₂O₅ or (ii) 100 mM 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) in the appropriate fluorescence buffers followed by recording of the fluorescence intensity. Fluorescence intensities (F) were corrected for sample dilution, normalized to the initial fluorescence intensity in the sample (F₀), and analyzed by linear regression in GraphPad Prism 5.0. The degree of accessibility was obtained from the Stern-Volmer equation: F/F₀ = 1 + KₛV × [Q] where F₀/F is the normalized fluorescence quenching, [Q] is the quencher concentration, and KₛV is the Stern-Volmer constant.

Fluorescence time course experiments were recorded at λₑₓ = 572 nm (10 data points per s) using an excitation source at λₑₓ = 540 nm and excitation and emission band pass of 5 nm with constant stirring in the cuvette containing 0.5 μg ml⁻¹ TMR-labeled LeuT in buffer consisting of 200 mM NaCl, 20 mM Tris-HCl (pH 7.50), 0.1 mM TCEP, and 0.05% DDM plus 200 mM KI or KCl (control). Data were normalized to the average steady-state fluorescence prior to leucine addition with control conditions (200 mM KCl) subtracted.

Molecular Dynamics Simulations—Based on our established simulation protocols and molecular system, we carried out the MD simulations of LeuT using NAMD (34) as described previously (32). Briefly, all-atom simulations of LeuT immersed in an explicit 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipid bilayer were carried out with the CHARMM22/CMAP and CHARM36/lipid force fields (35, 36). In the isothermal-isobaric (NPT) ensemble, constant temperature (310 K) was maintained with Langevin dynamics, and 1-atom constant pressure was achieved with the hybrid Nosé-Hoover Langevin piston method (37) applied to an anisotropic flexible periodic cell with orthogonal pressure components computed independently. The particle mesh Ewald method was used to evaluate long range electrostatic effects. A time step of 2 fs was used for the unbiased MD simulations, and 1fs was used for the targeted molecular dynamics (tMD) simulations.

In tMD simulations (38), a harmonic potential, V_{tMD} = 0.5k_{tMD} × (RMSD(t) − RMSD_{target}(t))^2, is added to the MD force field to bias a starting conformation toward a target by gradually reducing an instantaneous target RMSD_{target}(t) at simulation time t in response to a user-defined reference value RMSD_{target}(t) (k_{tMD} is a force constant). In this study, we implemented a "staircase" variation of tMD (stMD) (39) in which RMSD_{target}(t) is reduced from RMSD(0) in "staircases", i.e. alternating steps of targeted motion ("move") and constrained equilibration ("pause"); this procedure allowed us to compute averages along transition "pauses" of constant RMSD_{target(T)} values. For each move step, the slope of RMSD_{target}(t) is RMSD(0)/nₛΔt, where nₛ is the number of staircases along the transition and Δtₛ the simulation time of each stMD step so that, at the end of each stMD run with total simulation time T = nₛ(Δtₛ + Δtₚₛ), RMSD_{target}(T) is zero.

In this study, the stMD simulations were performed on the Cα atoms of the N terminus and the cytoplasmic segments of TMs 1, 3, 6, and 8 of LeuT, i.e. residues 1–21, 91–113, 261–267, and 343–368, with Γ = 50 ns, k_{stMD} = 0.25 kcal/mol/Å²/Cα atom, Δtₛ = 0.5 ns, and nₛ = 50. For the execution protocol for the tMD simulations, we devised a stMD wrapper script that runs the tMD module provided in NAMD.

The initial conformation of LeuT-WT for the tMD was a selected frame of the equilibration of the substrate-bound crystal structure of LeuT (Protein Data Bank code 2A65); the targeted conformation is based on an inward-facing conformation resulting from our previous steered MD study (32) with the N
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![Diagram of LeuT](image)

**A** The leucine transporter (LeuT)

**B** Probing small scale conformational changes at the cytosolic face of LeuT. A, two-dimensional diagram of LeuT embedded in the membrane. The cysteine (E192C) introduced for labeling with the sulphhydryl-reactive fluorophore TMR maleimide is indicated in orange (LeuT E192CTMR). Mutations introduced in the primary and secondary substrate-binding sites are show in green (Y108F, F253A, and F253L) and blue (L400S), respectively. The I359Q mutation, shown in red, introduced in the primary substrate site converts LeuT to a tryptophan transporter. The conserved residues involved in the intracellular interaction network (Arg9, Trp9, Tyr369, and Asp590) are outlined in teal. B, structure of LeuT (outward occluded) showing the position of Glu192 with TM1i and TM5i highlighted in green. Glu192 is positioned in close proximity to the conserved intracellular interaction network. C, chemical structure of TMR maleimide that was conjugated to E192C at the cytosolic end of TMS.

Results

Detection of Substrate-induced Conformational Changes by Collisional Quenching—To understand the coupling between substrate binding and conformational rearrangements at the inner gate, we used LeuT as a model system in SDFQS experiments. SDFQS is a highly sensitive method that has been successfully applied to reveal conformational rearrangements in other membrane proteins, such as the β2-adrenergic receptor and the lactose permease of *E. coli* (41–45). Note that LeuT contains no endogenous cysteines, which enables straightforward site-selective incorporation of thiol-reactive fluorophores into targeted cysteine mutants (Fig. 1A).

In the transport cycle, the transition from the inward-occluded to inward-open state is a key step in allowing substrate release to the intracellular side. According to a combined computational and smFRET study (28), the structural rearrangement of such a transition in NSSs involves a tilting of the intracellular portion of TM1 (TM1i) away from the helix bundle in an overall direction that is consistent with the position of TM1i in a crystal structure of LeuT in the inward-open state (Protein Data Bank code 3TT3) (9). Importantly, in the transport mechanism, such a rearrangement of TM1i must coordinate with other nearby structural elements in a controlled manner. One such element is the region that includes the IL2 and TM5i, which has been proposed to have a role in Na+ binding (46). Indeed, *substituted cysteine accessibility method* studies of serotonin transporter (47, 48) have suggested that conformational rearrangements take place in the IL2-TM5i region during translocation. Furthermore, compared with the crystal structure of LeuT in the inward-open state (9), the recent crystal structure of MhsT showed different features of TM5i rearrangements (11), supporting both a critical role for TM5i in the dynamic transition to the...
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To explore the involvement of this dynamic region in substrate-induced conformational rearrangements, we introduced a cysteine at position 192 (E192C) of LeuT. This position was chosen for its location at the junction between TM1i and TM5i so that it is in contact with interior residues and external solvent (Fig. 1, A and B). The construct was expressed in E. coli, purified, and conjugated with the thiol-reactive fluorophore TMR maleimide (Fig. 1, Fig. 2A). TMR maleimide specifically labeled the inserted cysteine as no fluorescence was detected in purified WT LeuT after treatment with 200 μM TMR maleimide (data not shown).

As assessed in a SPA (33), the labeled mutant (LeuT E192C<sup>TMR</sup>) maintained high affinity [3H]leucine binding ($K_D = 108 \pm 14$ nM, mean ± S.E., $n = 4$), although the affinity was moderately decreased compared with WT LeuT ($K_D = 15 \pm 2$ nM, mean ± S.E., $n = 4$) (Fig. 2B and Table 1). We did not observe a difference in the total number of binding sites ($B_{max}$) (Fig. 2B and Table 1), indicating no change in substrate binding stoichiometry. Furthermore, as determined after reconstitution into proteoliposomes and using [3H]alanine as substrate, LeuT E192C<sup>TMR</sup> maintained its ability to transport, although the capacity was partially reduced compared with WT LeuT (Fig. 2C). These results show that despite the mutation and derivatization with TMR the transporter exhibits functional substrate binding and transport.

To evaluate putative conformational changes surrounding the fluorophore, we used a collisional quenching approach. Collisional quenching requires an interaction between the quencher and fluorophore during the excited state of the fluorophore. Upon contact, the fluorophore returns to the ground state without emission of a photon. This makes it possible to assess solvent accessibility of a fluorophore by using a water-soluble quencher (49), such as iodide (I<sup>−</sup>). Indeed, increasing concentrations of KI strongly quenched the fluorescence emitted from free TMR (Fig. 3A). The ability of KI to quench TMR conjugated to the transporter (LeuT E192C<sup>TMR</sup>) was markedly reduced compared with the free fluorophore (Fig. 3B). Plotting $F_0/F$ ($F_0 = $ fluorescence in the absence of KI) against the KI concentration yielded linear Stern-Volmer plots with a slope (the Stern-Volmer constant, $K_{SV}$) of 10.3 ± 0.4 M<sup>−1</sup> (means ± S.E., $n = 4$) for free TMR and a slope ($K_{SV}$) of ~2.2 M<sup>−1</sup> for TMR conjugated to the transporter (Fig. 3C and Table 2). Thus, the fluorophore is less exposed to the solvent when conjugated to the transporter, suggesting that it is partially buried in the protein structure and/or the hydrophobic environment of the detergent micelle.

Incubation with increasing concentrations of leucine enhanced iodide-dependent quenching of LeuT E192C<sup>TMR</sup> as and equilibrium binding constants are shown in Table 1. Data points are means ± S.E. (error bars) from four independent experiments performed in duplicates. C, specific accumulation of 1 μM [3H]alanine in liposomes reconstituted with WT LeuT (circles) or LeuT E192C<sup>TMR</sup> (squares). Data points are duplicate determinations from a representative experiment. D, competition SPA binding of 10 nM [3H]leucine to LeuT E192C<sup>TMR</sup> with leucine in the absence (squares) or presence of 200 mM KI (triangles). Data points are means ± S.E. (error bars) from two independent experiments performed in at least duplicates.
illustrated by a concentration-dependent increase in the slope of the Stern-Volmer plots (Fig. 3D). The enhanced quenching required Na\(^+\) as the response was eliminated in Na\(^+\)-free buffer (200 mM KCl) (Fig. 3E). Removal of Na\(^+\) did not change the basal quenching of LeuT E192C\(^{TMR}\), suggesting that Na\(^+\) binding has no effect per se on fluorophore accessibility (\(K_{SV} = 2.21 \pm 0.05 \text{ M}^{-1}\) in Na\(^+\) versus \(K_{SV} = 2.27 \pm 0.06 \text{ M}^{-1}\) in K\(^+\), means ± S.E., \(n = 5\)). By plotting the \(K_{SV}\) obtained in Na\(^+\) as a function of leucine concentration, we observed a saturable response with an \(E_{50}\) of ~1.9 \(\mu\)M and a maximal \(K_{SV}\) of ~3.4 \(\text{M}^{-1}\) (Fig. 3F and Table 2). Note that the \(E_{50}\) value is larger than the \(K_{SV}\) of leucine obtained in radiotracer binding experiments (~100 nm; Table 1). Importantly, it is unlikely that this higher value is because the binding assay and the quenching assay reflect binding to different pools of transporters; the TMR labeling stoichiometry of purified LeuT E192C was ~89% (see “Experimental Procedures”), and in our binding assay, we did not observe more than one affinity state, and there was no significant decrease in the total number of binding sites (see above). Moreover, the difference is also unlikely caused by addition of the quencher (KI) because the \(^{[3]H}\)leucine binding properties were unchanged even in the presence of 200 mM KI (Fig. 2D).

We also assessed the rate of the leucine-induced iodide quenching response by measuring fluorescence over time with 200 mM KI added to the sample of purified LeuT E192C\(^{TMR}\). As shown in Fig. 4A and consistent with movement of the fluorophore from a lower to a higher KI accessibility, addition of Leu (100 \(\mu\)M) elicited a marked and rapid decrease in the fluorescence signal. Importantly, the response occurred with a rate (\(k_{TMR}\) = 504 h\(^{-1}\)) substantially faster than the rate for the transport (see below).

Next, we tested the effect on LeuT E192C\(^{TMR}\) of the tricyclic antidepressant clomipramine (CMI) that acts as a non-competitive inhibitor of LeuT by binding to the S2 site (50). CMI (100 \(\mu\)M) potentiated the iodide quenching response of LeuT E192C\(^{TMR}\) to leucine (Fig. 4B), but CMI alone did not increase the iodide quenching significantly (Fig. 4B). This suggests that CMI can potentiate the LeuT E192C\(^{TMR}\) quenching response by stabilizing the leucine-induced conformation via its binding to S2 (50). It is unlikely that the effect of CMI relates to spectral interference of the compound as CMI did not affect quenching of free TMR (data not shown).

To further substantiate the specificity of the leucine-induced change in quenching of LeuT E192C\(^{TMR}\), we carried out the same experimental procedure for the adjacent position, 191. For the resulting fluorescently labeled construct (LeuT I191C\(^{TMR}\)), we observed no significant effect of either Na\(^+\) or leucine addition on iodide quenching (Fig. 5, A and B). Position 191 appears to be surrounded by a cluster of hydrophobic residues (Ile\(^{185}\), Leu\(^{183}\), Ile\(^{187}\), and Ile\(^{357}\)) and is in part of the transporter that is not expected to be exposed to a different environment when TM1 is moving away from the helical bundle.

The solvent accessibility of TMR conjugated to E192C was further assessed with the hydrophobic quencher TEMPO, which incorporates into the detergent micelle or lipid bilayer (51) and thereby is capable of quenching fluorescence from fluorophores buried from the aqueous milieu (Fig. 5C) (51). TEMPO strongly quenched the fluorescence from TMR conjugated to E192C, consistent with partial burying of the fluorophore (Fig. 5D). Changing from a Na\(^+\)-free (\(K_{SV} \sim 83 \text{ M}^{-1}\)) to a Na\(^+\)-containing buffer (\(K_{SV} \sim 82 \text{ M}^{-1}\)) did not cause a significant change in accessibility (\(p = 0.8416\)); however, addition of leucine markedly decreased TEMPO accessibility (\(K_{SV} \sim 62 \text{ M}^{-1}\)) (Fig. 5, D and E), suggesting, in full agreement with our findings using iodide as quencher, that TMR conjugated to E192C becomes more solvent-exposed upon leucine binding and thus that leucine induces a conformational change that moves the fluorophore away from a buried water-inaccessible environment.

### Simulations Predict Coordinated Rearrangements between TM1i and TM5i—To predict the character of conformational rearrangements detected in the fluorescence quenching experiments, we used tMD simulations to investigate how the IL2-TM5i region would respond to coordinated rearrangements of the N terminus-TM1i (NT-TM1i) and IL2-TM5i. In the tMD simulations starting from the inward-closed state, the NT-TM1i segment was gradually pulled away from the TM domain in a direction defined in our previous steered MD study (32); this direction is along that identified as well in the inward-open structure of LeuT (Protein Data Bank code 3TT3). The tMD run was followed by extended unbiased MD (uMD) simulations to allow the system to equilibrate. In parallel, we also carried out an uMD simulation of the inward-closed state for comparison.

The distancing of NT-TM1i from the TM domain involves the breaking of three sets of interactions between NT and other TMs: (i) the interactions between NT and TM6 in the cluster of aromatic residues (Trp\(^{5}\)-Tyr\(^{265}\)-Tyr\(^{266}\)), (ii) the cation–π/ionic interactions among NT, TM6, and TM8 (Arg\(^{\beta}\)-Tyr\(^{268}\)-Asp\(^{369}\)), and (iii) an H-bond between NT and TM5i (Thr\(^{19}\)-Glu\(^{192}\)). As a result, the IL2-TM5i region rotated inward to occupy the space left by the displaced N-terminal residues (Fig. 6). The changes are quantified by the angle between the axes of TM1i and TM5i, revealing a substantial increase of this angle relative to the inward-closed state determined from the corresponding uMD simulations (Fig. 6b). Using the same protocol, we carried out tMD -> uMD simulations for LeuT E192C\(^{TMR}\). Importantly, similar rearrangements were observed (Fig. 6, b, c, and d), and these rearrangements were associated with a rotation of TMR.

### Table 1: Radioligand binding constants for LeuT E192C\(^{TMR}\) variants

| LeuT variant | \(K_D\) (nM) | \(B_{\text{max}}\) (% of WT) |
|--------------|--------------|-----------------------------|
| \(^{[3]H}\)Leucine | | |
| LeuT E192C\(^{TMR}\) | 108 ± 14 | 91 ± 4 |
| +L400S | 25 ± 5 | 58 ± 3 |
| +Y108F | >25,000 | ND |
| +F253A | 60 ± 10 | 50 ± 2 |
| +F253L | 94 ± 16 | 32 ± 2 |
| \(^{[3]H}\)Tryptophan | | |
| LeuT E192C\(^{TMR}\) | 50 ± 5 | ND |
| +I559Q | 30 ± 12 | ND |

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buried among the lipid headgroups in the inward-closed state toward the water milieu (Fig. 6, b, c, and d). To quantify the changes of the TMR label, we compared the solvent-accessible surface area of the tricyclic ring of TMR along the simulations and found a significant increase of solvent-accessible surface area of the tricyclic ring of TMR along the simulations (Fig. 6, e). Altogether, the simulations show how the substrate-induced change in fluorescence quenching of LeuT E192CTMR can describe a discrete outward movement of TM1i, supporting the inference from the measurements that this movement in turn leads to a rotation of IL2-TM5i that increases the solvent exposure of TMR.

Conformational Coupling to the Inner Gate Depends on Substrate Binding to S1—To discern whether leucine binding to S1 and/or S2 mediated the observed conformational coupling, we first mutated Tyr108 in TM3 to Phe (LeuT E192CTMR/Y108F).

This mutation is presumed to impair the binding of leucine to both S1 and S2 by disrupting a hydrogen bond to the carboxylate group of S1 (8) and promoting an outward-open conformation (9). As expected (52), LeuT E192CTMR/Y108F displayed a dramatic decrease in [3H]leucine binding affinity (K_D > 25 μM; Table 1). Moreover, we observed marked changes in iodide quenching in our assay. In contrast to our findings for LeuT E192CTMR, an increase in quenching and thus in solvent exposure of TMR could only be detected at higher leucine concentrations yielding an EC_50 of 52 μM (Fig. 7, A and B, and Table 2). We also observed a decrease in the baseline iodide quenching (K_{SV} ~ 1.80 M^{-1} for LeuT E192CTMR/Y108F versus ~2.21 for LeuT E192CTMR) (Fig. 7, A and B, and Table 2).

To block possible substrate binding to S2 (53), we mutated Leu400 in TM10 to Ser (LeuT E192CTMR/L400S). Consistent with previous data for this mutation (32), we found only a modest decrease in [3H]leucine affinity for LeuT E192CTMR/L400S (K_D = 24.9 ± 4.7 nm, mean ± S.E., n = 4) as compared with LeuT E192CTMR but a marked reduction in B_{max} consistent with loss of binding to S2 (53) (Table 1). In the iodide quenching assay, leucine produced a concentration-dependent increase in apparent TMR accessibility in LeuT E192CTMR/L400S of the same magnitude and with a similar EC_50 as that in LeuT E192CTMR (EC_50 ~ 1.3 μM) (Fig. 7, C and D, and Table 2). The baseline quenching and the maximal quenching in response to leucine were also similar although slightly elevated (Fig. 7, C and D, and Table 2).
and D, and Table 2). Together with the blunted response to leucine in LeuT E192CTMR/Y108F, the data suggest that the substrate-mediated conformational change in LeuT E192CTMR requires leucine binding to S1 and not S2.

To further establish the role of S1 binding, we mutated Phe253 to alanine (F253A). Phe253 is part of a hydrophobic pocket accommodating the side chain of leucine bound in S1, and it is critical for trapping S1 leucine by closing access to the extracellular milieu (8, 32). Of interest, the side chain of Phe253 and it is critical for trapping S1 leucine by closing access to the pocket accommodating the side chain of leucine bound in S1, and it is critical for trapping S1 leucine by closing access to the extracellular milieu (8, 32). Of interest, the side chain of Phe253

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To further establish the role of S1 binding, we mutated Phe253 to alanine (F253A). Phe253 is part of a hydrophobic pocket accommodating the side chain of leucine bound in S1, and it is critical for trapping S1 leucine by closing access to the extracellular milieu (8, 32). Of interest, the side chain of Phe253 rotates away in the outward-open conformation, permitting free access to S1 (9). For the resulting mutant, LeuT E192CTMR/F253A, basal TMR accessibility was unchanged (Fig. 7, E and F, and Table 2). The EC50 for the leucine response increased nonetheless 43-fold (Fig. 7, E and F, and Table 2), further substantiating a critical role of S1 leucine binding in the conformational coupling to the cytoplasmic end of TM5. However, the maximum response was unchanged, suggesting that Phe253 is not required per se for the conformational change. When Phe253...
was substituted with leucine (LeuT E192CTMR/F253L), thus introducing a bulkier side chain than that of Ala, the EC_{50} for leucine increased only 11-fold compared with LeuT E192CTMR (Fig. 7, G and H, and Table 2). Of note, the K_{D} values for binding of [3H]leucine to LeuT E192CTMR/F253A/L were similar to that of LeuT E192CTMR, but the B_{max} values were reduced to ~35–55% of LeuT E192CTMR (Table 1) as would be expected from previous data for this mutant (53).

Tryptophan Elicits a Response in the Quenching Assay upon Mutation of Ile^{359} to Gln—Tryptophan (Fig. 8A) binds to LeuT but is not a transportable substrate because the indole side chain sterically hinders the movement of Phe^{253} and thereby stabilizes the transporter in an outward-open conformation (10). If our quenching assay reports a functional step in the transport cycle then we surmise that tryptophan, as an inhibitory, would not elicit a response. Indeed, tryptophan elicited no change in iodide quenching (Fig. 8B). Interestingly, it has been shown that mutating Ile^{359} to glutamine makes LeuT capable of transporting tryptophan (54). We reasoned, therefore, that introducing a glutamine in position 359 of LeuT E192CTMR (LeuT E192C TMR/I359Q) should give rise to a tryptophan response. The resulting mutant (LeuT E192CTMR/I359Q) bound [3H]tryptophan with similar affinity as LeuT E192CTMR (Table 1), and tryptophan did elicit a clear response in the iodide quenching assay (EC_{50} 6.34 μM (3.19; 12.6), mean (95% confidence interval), n = 4) (Fig. 8C). The baseline iodide quenching of LeuT E192CTMR/I359Q was not significantly altered compared with LeuT E192CTMR (2.18 ± 0.05 M^{-1}, mean ± S.E., n = 5) versus 2.21 ± 0.05 M^{-1}, mean ± S.E., n = 5), suggesting no basal change in the conformation surrounding position 192.

The Quenching Response Is Inversely Correlated with Substrate Efficacy—Alanine (Fig. 8A), which is a better substrate for LeuT than leucine (10, 32), also caused a concentration-dependent increase in iodide quenching of LeuT E192CTMR with an EC_{50} of 25.6 μM (12.1;54.0) (mean (95% confidence interval), n = 4). Surprisingly, however, the maximum quenching was lower than in response to leucine (Fig. 8D). We consequently decided to test methionine, another known LeuT substrate (10), as well as valine and isoleucine (Fig. 8A). Like alanine and leucine, a saturating concentration (1 mM) of these amino acids elicited a Na^{+}-dependent increase in iodide quenching of LeuT E192CTMR, but the magnitudes of the responses differed (Fig. 8E). This is unlikely caused by differences in binding affinities because all tested amino acids displayed K_{D} values distributed between those of leucine and alanine, and thus full occupancy...
of the transporter should be obtained at 1 mM (Table 3). We therefore reasoned that the different quenching responses reflected the intrinsic ability of each amino acid to stabilize the substrate-bound state, which gives rise to the measured quenching efficiencies. This could provide a quantitative explanation for the observation that alanine is transported with a 5-fold higher rate (Fig. 8A) with alanine having the highest and leucine the lowest uptake efficiencies (Table 3). Here, we covalently coupled TMR-maleimide to a cysteine inserted at position 192 of LeuT (LeuT E192CTMR) and performed a series of collisional quenching experiments offering dynamic rearrangement data in support of a direct coupling between substrate binding and a conformational rearrangement at the intracellular end of TM5. Thus, leucine elicited a Na+-dependent increase in the accessibility of TMR to the aqueous quencher iodide while decreasing accessibility to the hydrophobic quencher (TEMPO) (51).

The leucine-induced increase in quenching displayed an EC50 value of ~2 μM, which is higher than the affinity of leucine obtained by radioligand binding (KD ~ 100 nM). The EC50 of alanine in the quenching assay was also higher than the affinity determined by radioligand binding, although the difference was smaller (Kd ~ 7 μM versus EC50 ~ 26 μM). Based on the range of results obtained with other substrates we analyzed as described, it is tempting to suggest that the observed discrepancy might indicate a range of coupling efficiencies for the various sub-

**Discussion**

In this study, we used SDFQS to investigate conformational changes linked to substrate binding in LeuT, a prototypic NSS protein. SDFQS measures environmental changes around a single fluorophore and can therefore be used as an extremely sensitive technique for detection of discrete conformational rearrangements (49). The technique has previously shown its strength in delineating conformational changes associated with activation of G protein-coupled receptors (42, 43). Here, we covalently coupled TMR-maleimide to a cysteine inserted at position 192 of LeuT (LeuT E192CTMR) and performed a series of collisional quenching experiments offering dynamic rearrangement data in support of a direct coupling between substrate binding and a conformational rearrangement at the intracellular end of TM5.

The leucine-induced increase in quenching displayed an EC50 value of ~2 μM, which is higher than the affinity of leucine obtained by radioligand binding (KD ~ 100 nM). The EC50 of alanine in the quenching assay was also higher than the affinity determined by radioligand binding, although the difference was smaller (Kd ~ 7 μM versus EC50 ~ 26 μM). Based on the range of results obtained with other substrates we analyzed as described, it is tempting to suggest that the observed discrepancy might indicate a range of coupling efficiencies for the various sub-

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**FIGURE 7. Conformational coupling to the inner gate depends on substrate binding to S1.** A. Stern-Volmer plots of KI quenching data for the S1 substrate-binding site mutant LeuT E192CTMR/Y108F. Experiments were conducted in 200 mM NaCl in the absence of substrate (black circles) or presence of increasing fixed concentrations of leucine (blue squares, 100 mM; blue triangles, 1 μM; blue inverted triangles, 10 μM; blue diamonds, 100 μM; blue circles, 1 mM). B. KSV values obtained from A plotted as a function of leucine concentration. The dashed line shows data for LeuT E192CTMR taken from Fig. 2F and E and F. Stern-Volmer plots of KI quenching data for the S1 substrate-binding site mutants LeuT E192CTMR/Y108F LeuT E192CTMR/F253A LeuT E192CTMR/L400S LeuT E192CTMR-F253L. Experiments were performed as described in A. G and H, KSV values obtained from E and G plotted as a function of leucine concentration. The dashed line shows data for LeuT E192CTMR taken from Fig. 2F. By revealing a right shift in dose-response curves (EC50 values given in Table 1) for the S1 mutants Y108F, F253A, and F253L but not for L400S, the data support the importance of leucine binding to S1 for the conformational response. Data points are means ± S.E. (error bars) of three to four independent experiments using protein from two separate preparations. For F253L, data points are means ± S.E. (error bars) of two to three independent experiments.
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FIGURE 8. The substrate-induced increase in iodide quenching of LeuT E192C\textsuperscript{TMR} is inversely correlated to the maximum attainable uptake rate of the substrate. A, chemical structures of the neutral amino acids used for the experiments in B–G. \( K_{SV} \) values obtained from KI quenching of LeuT E192C\textsuperscript{TMR} in 200 mM NaCl plotted against the indicated concentrations of tryptophan. No significant quenching response was observed. C, \( K_{SV} \) values obtained from KI quenching of LeuT E192C\textsuperscript{TMR}/I359Q in 200 mM NaCl plotted against the indicated concentrations of tryptophan, showing that introduction of I359Q enabled a Na\textsuperscript{+}-dependent substrate-induced quenching response. \( D, K_{SV} \) values obtained from KI quenching of LeuT E192C\textsuperscript{TMR} in 200 mM NaCl plotted against the indicated concentrations of alanine. Alanine induced a concentration-dependent increase in quenching, but the magnitude of the response was smaller than that of leucine. \( E \), maximum I\textsubscript{KI} quenching response for the indicated amino acids. The experiment was performed using a 1 mM concentration of the amino acids in 200 mM NaCl (filled bars) or 200 mM KCl (empty bars). Data points are means ± S.E. (error bars) from three to five independent experiments. Note that fluorescence buffer containing Tris/MES (pH 7.50) instead of Tris-HCl was used to improve the solubility of the tested amino acids and that similar results were obtained using Tris-HCl buffer except with a smaller range of the individual \( K_{SV} \) values. \( F \), uptake experiments on reconstituted LeuT using \([\text{3H}]\)alanine (red symbols), \([\text{3H}]\)valine (purple), \([\text{3H}]\)isoleucine (green), \([\text{3H}]\)methionine (yellow), and \([\text{3H}]\)leucine (blue). Data points are specific uptake in nmol/min/mg of protein given as means ± S.E. (error bars) from three independent experiments. Kinetic constants are shown in Table 4. \( G \), amino acid. G, the catalytic efficiencies \((k_{cat}/K_m)\) for \([\text{3H}]\)alanine, \([\text{3H}]\)valine, \([\text{3H}]\)isoleucine, \([\text{3H}]\)methionine, and \([\text{3H}]\)leucine are inversely correlated with the maximal quenching responses shown in \( E \).

TABLE 3

\[\text{[3H]}\]Leucine displacement constant for LeuT E192C\textsuperscript{TMR}

| Amino acid \( \text{[3H]} \) | \( K_d \) (nM) |
|---------------------|---------|
| Alanine             | 6.600 (4.300; 10.200) |
| Valine              | 1.250 (920; 1.700) |
| Isoleucine          | 249 (199; 312) |
| Leucine             | 77 (60; 99) |
| Methionine          | 1.910 (1,560; 2,350) |
| Tryptophan          | 36,600 (26,100; 51,500) |

Kinetic constants for LeuT

| Amino acid | \( K_m \) (nM) | \( V_{max} \) (nmol/min/mg LeuT) | \( k_{cat} \) (h\textsuperscript{-1}) | \( k_{cat}/K_m \) (h\textsuperscript{-1} nM\textsuperscript{-1}) |
|------------|----------------|-------------------------------|----------------|----------------|
| [\text{3H}]Alanine | 901 ± 140 | 6.38 ± 0.32 | 22.0 ± 1.10 | 0.024 |
| [\text{3H}]Valine | 730 ± 220 | 4.30 ± 0.38 | 14.8 ± 1.30 | 0.020 |
| [\text{3H}]Isoleucine | 749 ± 140 | 3.43 ± 0.19 | 11.8 ± 0.67 | 0.016 |
| [\text{3H}]Leucine | 392 ± 150 | 0.85 ± 0.08 | 2.92 ± 0.28 | 0.007 |
| [\text{3H}]Methionine | 643 ± 310 | 2.20 ± 0.24 | 7.59 ± 0.83 | 0.012 |

In our computational simulations, we observed that, in response to a discrete outward movement of TM1, the increase in aqueous solvent accessibility of TMR attached to position 192 is consistent with the experimentally observed enhanced accessibility of the TMR to iodide upon addition of substrate. Importantly, neither the simulations nor the measured substrate-induced change is likely to reflect the structural context of a full transition to the inward-open configuration. A full transition to the inward-open state is improbable in light of recent smFRET and spin labeling experiments indicating that under similar experimental conditions Na\textsuperscript{+} promotes a more inward-closed configuration, whereas leucine in high Na\textsuperscript{+} was found to have no or little effect on the distribution between the confor-
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mational states detected in these studies (28–30). Conceivably, in the state visited under these conditions, the leucine-induced change observed in the present study is not altering the net distance between the probes sufficiently to be detected. This underlines the unique sensitivity of the current approach and suggests that our data might reflect a distinct Na\(^+\)- and leucine-dependent rearrangement in the IL2-TM5i region that, although not readily detectable by smFRET and spin labeling experiments, describes the formation of a heretofore unrecognized substrate-stabilized structural intermediate that may precede transition to the inward-open conformation.

The possible existence of a substrate-stabilized structural intermediate is interesting to consider in relation to the structure of the NSS member MhsT (11). This structure was solved in a substrate-bound, inward-occluded state and thus in a state that should occur subsequent to the outward-occluded state but precede the formation of the inward-open state. In the solved structure (Protein Data Bank code 4US4), MhsT has both Na1 and Na2 bound; however, a water access pathway reaching the Na2 site has formed. The formation of this path is caused by a striking structural rearrangement of the intracellular part of TM5 involving an unwinding of the helix (11). It is difficult to predict whether an unwinding of TM5 can be generalized to LeuT and other NSS proteins; however, the results in MhsT strongly support a substrate- and Na\(^+\)-dependent structural rearrangement at the intracellular end of TM5 as a key step in the transport cycle that precedes transition to the inward facing state by providing a water access pathway to the Na2 site (11).

By mutating residues impairing leucine binding to S1, we observed major changes in the quenching response. Substituting Tyr\(^{108}\) with phenylalanine (LeuT E192C\(^{TMR}\)/Y108F) caused a rightward shift in the leucine dose-response curve and decreased basal accessibility to TMR attached to E192C. The shift in the dose-response curve substantiated the requirement for S1 binding, whereas the decrease in basal accessibility might reflect the shift of Y108F to an outward-open state (9), a shift that concomitantly would decrease E192C\(^{TMR}\) accessibility. Mutation of Phe\(^{253}\) to alanine (LeuT E192C\(^{TMR}\)/F253A) also caused a right shift of the leucine dose-response curve, further supporting the requirement for S1 binding. Interestingly, substitution of Phe\(^{253}\) with leucine (LeuT E192C\(^{TMR}\)/F253L) instead of alanine caused a more moderate right shift in the dose-response curve, most likely because the side chain is bulkier. It is tempting to speculate that the presence of Phe\(^{253}\) increases the coupling efficiency for each leucine binding event by prolonging the substrate residence time in S1; such a function of the highly conserved residue has recently been proposed for GABA transporter-1 (57).

Mutation of Ile\(^{359}\) to glutamine (I359Q) transforms tryptophan from an inhibitor of LeuT into a transported substrate (54). Hence, it was important that tryptophan did not affect iodide quenching in LeuT E192C\(^{TMR}\) but did increase quenching in LeuT E192C\(^{TMR}\)/I359Q. This provides strong support for the hypothesis that the substrate-induced change reports on a conformational change of functional relevance to the transport process. Of further interest, we compared the effects of a series of substrates, including alanine, valine, isoleucine, leucine, and methionine, on the quenching efficiencies and found that their maximum $K_{SV}$ responses showed an inverse linear correlation with the efficacy of the amino acids as substrates. An intriguing explanation for this correlation is that a larger propensity of the substrate to stabilize a structural intermediate imposes a rate-limiting effect on the catalytic process in the transporter. Interestingly, the ability of the tricyclic antidepressant CMI to potentiate the effect of leucine in the quenching assay may provide additional support for this explanation. Thus, by binding to the S2 site, CMI may increase the conformational coupling by preventing dissociation of leucine from S1; however, as an allosteric inhibitor, it may not allow further progression of the transport cycle. For transport to occur, both the binding of a substrate molecule (but not an inhibitor) in the S2 site (29, 32) and the presence of a Na\(^+\) gradient might be required. Of note, it has been hypothesized before that the lower transport rate of leucine in LeuT is caused by its high affinity for the occluded intermediate, increasing the energy barrier for transitioning to the inward facing state (10).

Summarized, our findings provide, to our knowledge, the first direct evidence for a coupling mechanism between the primary substrate-binding site (S1) and the inner gate in NSSs, suggesting formation of a conformational intermediate that may precede transition to the inward-open state. Such coupling has been proposed for LeuT on the basis of computational simulations in the frame of Shannon information theory with the magnitude of this coupling emerging as a determinant factor in transport (58). Consistent with this theoretical analysis, we show here evidence for this coupling and that the conserved residue Phe\(^{253}\) is critical for the concentration dependence of the effect trapping the substrate in S1. Furthermore, the striking inverse correlation between catalytic efficiencies and the degree of the conformational response provides an attractive structural explanation for why a substrate might be poorly transported despite its binding with high affinity to the transporter. It underscores the inference that, although the structural intermediate could be a key step in transport cycle, an increase in the stability of the intermediate becomes a limiting factor in the maximum attainable transport rate.

Author Contributions—C. B. B., M. B. K., and J. K. generated LeuT constructs. C. B. prepared, purified, and labeled LeuT variants and performed fluorescence quenching experiments with help from J. K., J. S. M., and M. B. K. G. helped with preparations and purifications of LeuT. C. B. B. performed radioligand binding experiments. C. B. B. and M. Q. performed functional reconstitution and radioligand uptake experiments. L. S., Z. L., and S. S. performed molecular dynamics simulations and analysis. C. B. B., M. B. K., L. S., J. K., M. Q., J. A. J., C. J. L., H. W., and U. G. were all involved in designing the experiments and interpretation of the data. C. B. B., C. J. L., and U. G. wrote the manuscript, and all authors commented on the manuscript.

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