Insights into transport mechanism from LeuT engineered to transport tryptophan

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LeuT is a bacterial homologue of the neurotransmitter:sodium symporter (NSS) family and, being the only NSS member to have been structurally characterized by X-ray crystallography, is a model protein for studying transporter structure and mechanism. Transport activity in LeuT was hypothesized to require structural transitions between open-to-out and occluded conformations dependent upon protein:ligand binding complementarity. Here, using crystallographic and functional analysis, we show that binding site modification produces changes in both structure and activity that are consistent with complementarity-dependent structural transitions to the occluded state. The mutation I359Q converts the activity of tryptophan from inhibitor to transportable substrate. This mutation changes the local environment of the binding site, inducing the bound tryptophan to adopt a different conformer than in the wild-type complex. Instead of trapping the transporter open, tryptophan binding now allows the formation of an occluded state. Thus, transport activity is correlated to the ability of the ligand to promote the structural transition to the occluded state, a step in the transport cycle that is dependent on protein:ligand complementarity in the central binding site.

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Introduction

Rapid transport of polar solute molecules across biological membranes is accomplished by a diverse array of transporter proteins specializing in different physiological functions. At chemical synapses, neurotransmitter:sodium symporters (NSSs) catalyse the reuptake of biogenic amine and amino-

acid neurotransmitters from the synaptic cleft into surrounding cells (Iversen, 1971; Amara and Kuhar, 1993; Rudnick and Clark, 1993). The central role of NSS members in neural physiology and disease is reflected in the large number of therapeutic and illicit drugs that target these transporters (Zahniser and Doolen, 2001; Andersen *et al*, 2009). Because of this physiological and therapeutic relevance, the mechanism of these transporters has been under study for decades. Recently, crystallographic data from LeuT, a bacterial NSS homologue, have provided a structural model for the NSS family showing how conserved functional elements are organized and how substrates and inhibitors interact with the transporter (Yamashita *et al*, 2005; Singh *et al*, 2007, 2008; Zhou *et al*, 2007, 2009).

LeuT functions as a sodium-dependent aliphatic and aromatic amino-acid symporter, with uptake activity demonstrated for amino acids ranging from glycine to L-tyrosine. Crystal structures of LeuT–ligand complexes with amino-acid substrates, including glycine, alanine, leucine, methionine, and the isosteric tyrosine analogue 4-fluoro-phenylalanine (4-F-Phe), all adopt an occluded conformation with a single substrate molecule bound in the central S1 binding site (Yamashita *et al*, 2005; Singh *et al*, 2007, 2008). Tryptophan, by contrast, binds LeuT but cannot be transported and is a competitive inhibitor. Furthermore, unlike the substrate-bound complexes, the LeuT–tryptophan complex adopts an open-to-out conformation with one tryptophan molecule bound at the central S1 binding site (Trp601), and a second tryptophan molecule (Trp602) bound at the base of the extracellular vestibule (Singh *et al*, 2008). These results together suggested a structural model for transport in which transport-competent amino acids initially bind to an open-to-out state such as that observed in the tryptophan-bound structure, and if there is appropriate steric and electrostatic complementarity, the LeuT–substrate complex then isomerizes to the occluded state. We suggested that the inhibitory activity of tryptophan was due to the binding of Trp601 in the S1 site which, because of the bulky size of tryptophan relative to the binding site, sterically inhibits the structural isomerization to the occluded state thereby trapping the transporter in an open-to-out conformation (Singh *et al*, 2008; Krishnamurthy *et al*, 2009). This hypothesis proposes that a link between protein structure and transport activity is based, at least in part, on the steric and/or electrostatic complementarity of ligand binding within the S1 binding site.

In this work, we seek to further understand the influence of protein:ligand binding complementarity on the structure and function of LeuT. Here, we focus our efforts to study LeuT with specific mutations in the S1 binding site designed to alter the transport specificity of LeuT to ‘convert’ tryptophan from an inhibitor into a substrate. We conjectured, based on our prior observations, that such an alteration of activity should also manifest itself structurally whereby a

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mutant transporter that exhibits tryptophan transport activity should also adopt an occluded conformation upon binding tryptophan. By using radioligand binding and transport assays, we demonstrate that a single mutation in the binding site, I359 to Gln, suggested by a comparative analysis to the related bacterial tryptophan transporter TnaT (Androutsellis-Theotokis *et al*, 2003), is sufficient to shift the substrate specificity profile of LeuT such that it efficiently transports tryptophan. Using crystallography, we observe that the change in transport specificity is accompanied by structural changes both in the global conformation of LeuT and in the local conformation of the bound tryptophan molecule, which together allow the LeuT–tryptophan complex to adopt the occluded state. Finally, we observe that the second tryptophan, Trp602, present in the open-to-out state is absent in the tryptophan-bound occluded state.

Results and discussion

TnaT model indicates specificity-altering substitutions

To gain insight into the nature of transport versus inhibition, we began by comparing the sequences of LeuT with TnaT, a related bacterial NSS homologue that transports tryptophan (Androutsellis-Theotokis *et al*, 2003). The sequence alignment shows that while the overall conservation is about 29%, regions that are known or predicted to be involved in substrate binding are more highly conserved, such as in transmembrane helix 1 (TM1) and TM6 (Yamashita *et al*, 2005; Beuming *et al*, 2006). Because we are interested in the determinants of substrate specificity, we examined the residues of TnaT that align with the central, S1 binding site residues of LeuT. We found that most of the binding site residues with side chains that contact leucine in the LeuT–leucine complex have shared identity between LeuT and TnaT. Two exceptions, however, are F259 and I359. In TnaT, these residues are substituted by valine and glutamine, respectively (Figure 1A).

In LeuT, the F259 and I359 side chains participate in van der Waals interactions with the sidechain of leucine (Figure 1B). A homology model of TnaT suggests that the F259V and I359Q substitutions could significantly alter the

environment of the LeuT binding pocket (Figure 1B). Substitution of the I359 *sec*-butyl group, a branched non-polar sidechain, with the glutamine propylamide, an unbranched polar sidechain, will change both the steric and electrostatic environment of the binding pocket. Substitution of the F259 phenyl group with the smaller isopropyl group of valine removes an aromatic ring from the binding site and also changes the shape of the binding pocket. We surmised that, in TnaT, these substitutions support tryptophan transport activity by allowing tryptophan to bind in an occluded conformation. Thus, given the overall similarity of LeuT and TnaT, these binding site substitutions introduced into LeuT should also allow tryptophan to bind in an occluded conformation, leading to a gain of tryptophan transport activity. We sought to test this hypothesis by constructing LeuT mutants that contain the TnaT binding site substitutions F259V and I359Q to see if we can, in fact, observe a correlation between a gain of tryptophan transport function and a change in the conformational state of the LeuT–tryptophan complex.

I359Q substitution confers Trp transport activity to LeuT

LeuT variants, constructed in the K288A background (LeuT^K), incorporating both substitutions together (LeuT^K + F259V + I359Q) or as single mutations (LeuT^K + F259V and LeuT^K + I359Q) were purified and reconstituted into liposomes for transport analysis. Here, we used the LeuT^K mutant as the background because it exhibits an apparent increase in substrate flux in comparison to the wild-type transporter under our typical assay conditions (Piscitelli *et al*, 2010). We also note that K288 is not a conserved residue, and, in LeuT crystal structures, K288 is far from the substrate and ion binding sites, with the side chain projecting away from the transporter and not interacting with other residues.

The ability of each variant to transport [³H]tyrosine and [³H]tryptophan was first measured in a time-course assay. Tyrosine was chosen as an assay ligand because it is the largest amino acid that wild-type LeuT can transport and its hydroxyphenyl moiety interacts directly with the side chains of both F259 and I359 (Singh *et al*, 2008). Therefore, though it is more weakly transported by LeuT than other substrates, we reasoned that it would present a more sensitive functional readout of substitutions in the binding pocket that alter interactions with substrate. As expected from previous work with wild-type LeuT (Singh *et al*, 2008), at pH 7, LeuT^K does not transport tryptophan but does demonstrate measurable tyrosine transport (Figure 2A; Supplementary Figure S1; Supplementary Table S1).

The single-mutant LeuT^K + F259V, like wild-type LeuT, does not transport tryptophan (Figure 2A; Supplementary Figure S1A; Supplementary Table S1), suggesting that this substitution alone is insufficient to extend the substrate specificity of LeuT. The F259V variant, however, also displayed reduced tyrosine transport (Figure 2A; Supplementary Figure S1B; Supplementary Table S1), suggesting that the F259V substitution induces changes in the transporter that are suboptimal in the context of LeuT.

The single I359Q substitution, by contrast, demonstrated an appreciable gain of tryptophan transport activity while maintaining tyrosine transport activity (Figure 2A; Supplementary Figure S1; Supplementary Table S1). When I359Q is incorporated together with F259V, the double variant

Figure 1 (A) Sequence alignment of LeuT and TnaT, showing the regions of TM6 and TM8 that contribute to the binding site in LeuT. Yellow highlights residues, which contribute sidechain interactions to the substrate in LeuT crystal structures. Shown in red lettering are the residues F259 and I359 in LeuT that are substituted by V and Q, respectively, in TnaT. (B) Overlay of the LeuT–leucine crystal structure (yellow; PDB ID 2A65), and a homology model of TnaT (green). Leucine is shown in spheres, and the van der Waals radii of the sidechains that contact are rendered as a dotted surface.

Figure 2 (A) Initial rate of [³H]Tyr or [³H]Trp uptake by proteoliposomes incorporating LeuT^K and binding site variants. Error bars indicate s.e.m., $n = 2-5$. (B) Saturation binding of [³H]Trp to LeuT^K and variants measured by scintillation proximity assay. LeuT^K: circles with solid line; F259V: triangles with dashed line; I359Q: inverted triangles with dotted line; F259V + I359Q: squares with dash-dot line. Error bars indicate s.e.m., $n = 2$. (C) Steady-state kinetic measurement of [³H]Trp uptake by LeuT^K + I359Q fitted with Michaelis–Menten rectangular hyperbola. Error bars represent s.e.m., $n = 3$.

also shows both measurable tryptophan and tyrosine transport activity (Figure 2A; Supplementary Figure S1; Supplementary Table S1), although the rate of tryptophan transport is markedly reduced compared with the single I359Q variant. This is consistent with the observation from the tyrosine transport data that indicated the F259V mutation negatively impacts LeuT transport activity.

F259V does not inhibit binding of Trp to LeuT

To determine if the F259V substitution decreases tryptophan transport activity in the double mutant by inhibiting binding, we measured the affinity for [³H]tryptophan binding to LeuT and the three variants. We found that all of the mutants bind tryptophan with similar affinity, each with a K_d of $\sim 20 \mu\text{M}$ (Figure 2B; Supplementary Table S1).

Steady-state kinetics of Trp transport by LeuT^K + I359Q

The activity of LeuT^K + I359Q was further characterized by measuring the steady-state kinetics of [³H]tryptophan transport. The data were well fit by a single-site Michaelis–Menten model ($R^2 = 0.991$) with kinetic parameters of $K_M = 78.9 \pm 8.8 \mu\text{M}$ and $V_{\max} = 7504 \pm 340 \text{ pmol/min/mg}$ (Figure 2C; Supplementary Table S1). The corresponding turnover rate k_{cat} is $\sim 0.6 \text{ min}^{-1}$, which is within the range of turnover rates previously reported for transport activity by wild-type LeuT at pH 7 (Singh *et al.*, 2007, 2008). Altogether, these uptake and binding data demonstrate that the single substitution, I359Q, derived from TnaT, is sufficient to alter the substrate specificity profile of LeuT, converting the activity of tryptophan from a competitive inhibitor to a transportable substrate.

Trp transport active mutants bind Trp in the occluded state

In order to understand the structural basis that underlies the gain of tryptophan transport activity by the I359Q substitution, we co-crystallized each of the three LeuT^K binding site variants with tryptophan and determined the crystal structures by X-ray diffraction (see Supplementary Table S2 for data collection and refinement statistics). The structure of LeuT^K + F259V, the mutant that does not exhibit tryptophan transport activity, in complex with tryptophan is nearly identical to the wild-type LeuT–Trp structure described previously (Singh *et al.*, 2008) (Figure 3A and C; Supplementary Figure S2), with an overall C_α r.m.s.d. of $0.25 \text{ \AA}$ (Supplementary Table S3). LeuT^K + F259V–Trp adopts an open-to-out conformation marked by a solvent accessible pathway through the extracellular vestibule into the central substrate binding site (Figure 3A) with a second tryptophan molecule (Trp602) bound in the extracellular vestibule between the extracellular gate residues R30 and D404 (Supplementary Figure S2B). We previously proposed that this open-to-out conformation signifies a competitively inhibited state where the transporter is locked open and prevented from progressing through the transport cycle due to steric hindrances from the tryptophan bound in the central site (Singh *et al.*, 2008; Krishnamurthy *et al.*, 2009).

The structures of LeuT^K + I359Q and LeuT^K + F259V + I359Q bound with tryptophan, both of which demonstrated a gain of tryptophan transport activity, in contrast to wild-type and the F259V mutant, both adopt an occluded-state conformation in complex with tryptophan (Figure 3B) that is strikingly similar to the conformation previously observed for LeuT bound with transportable substrates (Yamashita *et al.*, 2005; Singh *et al.*, 2008) (Figure 3D; Supplementary Table S3).

Figure 3 (A, B) Cross-sections of the crystal structures of (A) $\text{LeuT}^{\text{K}+\text{F259V}}\text{-Trp}$ and (B) $\text{LeuT}^{\text{K}+\text{I359Q}}\text{-Trp}$ showing solvent accessible surface area (blue). Bound tryptophan molecules are shown as yellow sticks, sodium ions are depicted as magenta spheres. (C) Overlay of the $\text{LeuT}^{\text{K}+\text{F259V}}\text{-Trp}$ (green) and the wild-type LeuT-Trp (PDB ID 3F3A, yellow) crystal structures showing details of tryptophan bound in the central binding site. (D) Overlay of $\text{LeuT}^{\text{K}+\text{I359Q}}\text{-Trp}$ (teal), $\text{LeuT}^{\text{K}+\text{F259V}+\text{I359Q}}\text{-Trp}$ (orange), and wild-type LeuT-Leu (PDB ID 2A65, grey) crystal structures, showing details of the substrate binding site. (E, F) Overlay of the $\text{LeuT}^{\text{K}+\text{I359Q}}\text{-Trp}$ (teal) and wild-type LeuT-Trp (yellow) crystal structures. (E) A zoomed-out view of the transporters, parallel to the membrane plane. (F) A close-up view of the central binding sites showing the details of how tryptophan is differently accommodated in the wild-type and I359Q mutant LeuT structures.

Figures 3E and F illustrate the differences observed between the tryptophan-bound states of wild-type LeuT and the I359Q mutant that suggest the structural basis for the functional changes in tryptophan activity we observed in our transport assays. In the wild-type transporter, the indole ring of tryptophan is nestled into a cleft between the *sec*-butyl sidechain of I359 and the hydroxyphenyl ring of Y108, which participates in an edge-face aromatic interaction with the bound trypto-

phan (Figure 3C and F). The conformational isomer of tryptophan bound in the open-to-out state is therefore stabilized in a more elongated conformer that spans the open binding site, preventing the ‘collapse’ to form the occluded state.

From the structure of $\text{LeuT}^{\text{K}+\text{I359Q}}\text{-Trp}$ it is evident that the I359Q mutation introduces both steric and electrostatic changes in the binding site that influence the conformation of

the indole ring of the bound tryptophan to rotate downward towards the intracellular side of the transporter (Figure 3D). In the I359Q mutant, the glutamine side chain is extended towards Y108 where the amide head group interacts with the aromatic face of the Y108 side chain (Figure 3D and F). The indole moiety of tryptophan is therefore precluded from binding with Y108, thus the ligand adopts a more compact conformation where the indole ring is rotated by $\sim 30^\circ$ about the χ^2 torsion angle. This rotation situates the indole ring such that the aromatic face interacts with the N ϵ of the glutamine amide group as well as forming putative C–H interactions with the C γ methylene. The cytoplasmically oriented indole ring now interacts directly with N21, resulting in an $\sim 90^\circ$ rotation of the asparagine amide sidechain (Figure 3F), engaging it with the aromatic face of the conserved Y265 residue (not shown). Taken together, the indole ring of the bound tryptophan molecule changes orientation such that in the I359Q structure, it is oriented ‘vertically’ in the binding pocket whereas in wild-type LeuT, the ring is oriented ‘horizontally’, acting like a ‘brace’ between TMs 6 and 8. Thus, the I359Q substitution alters the LeuT binding site interaction network, enabling tryptophan to bind with complementarity that allows, for example, TM6a to move closer to TM8 and for the transporter to isomerize to the occluded state. These observations provide new insight into the interplay between the conformation of the ligand and the transporter.

The Trp602 site is absent in the Trp-bound occluded state

The LeuT^{K+I359Q} and LeuT^{K+F259V+I359Q} complexes with tryptophan show that, in the occluded state, a single tryptophan molecule binds in the central, S1 binding site. Furthermore, there is no evidence for a second tryptophan bound within the extracellular vestibule, as is observed in the open-to-out structures. Similar to the wild-type occluded state structures, in the engineered tryptophan transporter structures, extracellular gate residues R30 and D404 are engaged in a water-mediated ionic bridge (Supplementary Figures S3 and S4), ablating the binding of Trp602. This illustrates that the Trp602 site is strictly dependent upon the conformation of the transporter and that in the open-to-out facing structures its presence is not simply an artefact of co-crystallization with tryptophan. Nevertheless, the crystallization experiments described here utilize the detergent β -OG, a molecule that binds within the extracellular vestibule and may influence the conformation of the transporter (Quick *et al.*, 2009). Additional experiments, using different detergents, are required to conclusively determine the consequences of β -OG on transporter conformation.

A structural description of the transport cycle

Our crystal structures of LeuT bound to tryptophan in two correlated structural and functional states provide additional evidence of structural transitions that accompany the transport cycle of LeuT. As we have discussed in previous work, the crystallographic data from LeuT suggest that the transport cycle includes structural isomerization events that alternately open and close extracellular and intracellular ‘gates’ to sequentially open and occlude the central S1 binding to allow substrate and ions to bind and unbind across the membrane (Krishnamurthy *et al.*, 2009). This notion of an

alternately accessible binding site due to coordinated extracellular and intracellular gate transitions has also been proposed for other structurally related yet functionally distinct ‘LeuT-fold’ members based on recent crystallographic data (Abramson and Wright, 2009; Forrest *et al.*, 2010; Shimamura *et al.*, 2010; Weyand *et al.*, 2010; Perez *et al.*, 2011).

In Figure 4A, an open-to-out state of an apo-LeuT molecule is depicted, based on the conformation of wild-type LeuT bound with tryptophan in which the conserved extracellular gate residues are open. The existence of this state for the apo-transporter is supported by EPR measurements (Claxton *et al.*, 2010) and is analogous to the crystal structure of the open-to-out apo state of the sodium-hydantoin transporter Mhp1 (Weyand *et al.*, 2008). In this state, LeuT forms an electrostatically polarized pathway between conserved residues R30 and D404, which may serve to recruit and orient ligands into the extracellular vestibule. This polarized pathway might transiently bind substrates as they permeate through the transporter into the central substrate binding site (Figure 4B). The idea of a transient site is suggested by the open-to-out crystal structures of LeuT in which a tryptophan molecule is observed bound in this location, and on multiple different molecular dynamics simulations (Celik *et al.*, 2008; Shi *et al.*, 2008). We suggest that the occupancy of this site is dependent on the conformation of the transporter and that it is a weak, low affinity binding site. After passing through the extracellular gate, substrate binds into the central site (Figure 4C) whereupon, given the correct protein:ligand complementarity, the extracellular gate closes forming a stabilized occluded state. The transition from open-to-out to occluded appears to involve the inward movement of TM1b and TM6a towards TM3 and TM10, as observed in the LeuT crystal structures of the open-to-out and occluded states (Singh *et al.*, 2008), which is also supported by EPR measurements of spin label pairs located on the extracellular surface of LeuT (Claxton *et al.*, 2010). Furthermore, the crystal structures of other LeuT-fold transporters bound with ligand also show the transporter adopting a similar occluded-state conformation (Faham *et al.*, 2008; Weyand *et al.*, 2008; Ressler *et al.*, 2009), suggesting that ligand binding in other transporters is similarly accompanied by a structural transition from an open state to an occluded state.

From the occluded state, LeuT can then isomerize to open the intracellular gate composed of residues that are conserved across the NSS family (Kniazeff *et al.*, 2008) (Figure 4D), allowing substrate and/or ions to dissociate (Figure 4E). While no crystal structure of LeuT has captured an inward-facing conformation, structures of the structurally related LeuT-fold transporters Mhp1 and vSGLT have been crystallized in an inward-open conformation. These structures indicate that movement of a single helix largely accounts for the opening of the intracellular gate: TM5 for Mhp1 (Shimamura *et al.*, 2010) and TM1 for the bacterial glucose transporter vSGLT (Watanabe *et al.*, 2010). For LeuT, this structural transition appears to involve outward movements of TM1a based on a series of single-molecule FRET studies (Zhao *et al.*, 2010, 2011) and possibly TM6b from models based upon the two-fold pseudosymmetry of TMs 1–5 and 6–10 (Yamashita *et al.*, 2005; Forrest *et al.*, 2008), substituted cysteine accessibility assays (Forrest *et al.*, 2008; Kniazeff *et al.*, 2008) and molecular modelling (Forrest *et al.*, 2008; Shaikh and Tajkhorshid, 2010).

Figure 4 Mechanism of substrate permeation and conformational changes of the LeuT transport cycle. Core helices of LeuT are shown with TMs 3 and 10 truncated for clarity. TMs 1 and 6 are coloured yellow. Select residues that are observed to interact with substrate during permeation and/or are known conserved functional elements are labelled and shown as sticks. Tryptophan, coloured cyan, is the substrate, with Na^+ indicated by purple spheres. Red arrows indicate the direction of TM helix movement required to progress to the subsequent state. (A) Na^+ -bound open-to-out state, modelled on the open-to-out conformation of the wild-type LeuT-tryptophan structure (PDB ID 3F3A). (B) Na^+ -bound open-to-out state with substrate bound between extracellular gate residues R30 and D404, proposed to be a transient state (indicated by double-dagger symbol) along the transport cycle. (C) Na^+ and tryptophan bound occluded intermediate state, based on the LeuT^K+I^{359Q}-Trp structure. (D, E) Putative inward-facing conformation to illustrate opening of the intracellular gate preceding inward Na^+ and substrate release. (D) Na^+ and tryptophan bound inward-facing state. (E) Inward-facing state with Na^+ and substrate dissociated (apo state).

Materials and methods

Homology modelling of TnaT

Pairwise alignment of the TnaT sequence from *Symbiobacterium thermophilum* (GenBank accession BAA24689.2) with LeuT was performed using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). A three-dimensional model of TnaT was constructed using the Swiss-Model (Arnold *et al.*, 2006) web interface from the ClustalW-generated sequence alignment and the coordinates of the occluded-state LeuT structure (PDB ID 2A65). Similar results were achieved using Modeller 9v5 (Eswar *et al.*, 2006) both with and without explicit modelling of the bound sodium ions. Tryptophan and sodium ions were modelled manually into the resulting homology model of TnaT guided by the locations of the ions and the α -amino and -carboxylate position of bound leucine in the template LeuT structure. The model of TnaT bound with sodium and tryptophan was subjected to a round of geometrical energy minimization to alleviate clashes using Refmac5 (Collaborative Computational Project, 1994; Murshudov *et al.*, 1997; Vagin *et al.*, 2004).

Expression and purification

LeuT was expressed and purified as previously described (Yamashita *et al.*, 2005; Singh *et al.*, 2008). Specifically, membranes were solubilized with 40 mM *n*-dodecyl- β -D-maltoside (C_{12}M) and the protein was purified and manipulated in 1 mM C_{12}M , except for crystallization experiments, as described subsequently. Mutations were introduced by polymerase chain reaction using the Quik-Change method (Stratagene, Inc.). All binding and transport assays were conducted with LeuT constructs containing the K288A background mutation (LeuT^K) (Piscitelli *et al.*, 2010). To displace endogenously bound high-affinity ligands, protein was solubilized from membranes in the presence of 100 mM L-alanine followed by purification either without added ligand (for binding and transport assays) or with 50 mM L-tryptophan (for crystallization of LeuT-Trp complexes).

Preparation of LeuT proteoliposomes

LeuT was reconstituted into proteoliposomes at a protein:lipid ratio of 1:100 (w:w) as previously described (Singh *et al.*, 2007). Reconstitution was carried out in the presence of 20 mM HEPES-Tris pH 7, 100 mM KCl. Proteoliposomes were concentrated by centrifugation at 300 000 g for 30 min and resuspended in reconstitution buffer to a protein concentration of 0.5 mg/ml, determined by the modified amido black assay method (Kaplan and Pedersen, 1985). Proteoliposomes were flash frozen in liquid N_2 and stored at -80°C . Prior to each assay, proteoliposome stock suspension was thawed then diluted 25-fold into internal buffer (20 mM HEPES-Tris pH 7, 500 mM KCl), subjected to two freeze/thaw cycles in liquid nitrogen and room temperature water and reconstituted by centrifugation at 300 000 g for 30 min. The pelleted proteoliposomes were resuspended back to the original volume using internal buffer. The resuspended liposomes were then extruded using an Avestin Mini-extruder coupled to two 1 ml Hamilton syringes with a 400-nm polycarbonate filter, passing the suspension through the filter 15–21 times.

Transport assays

For time-course assays, reactions were initiated by adding proteoliposomes into external buffer (20 mM HEPES-Tris pH 7, 500 mM NaCl) at 27°C with 1.2 μM [^3H]tryptophan (20 Ci/mmol) or 1 μM [^3H]tyrosine (40 Ci/mmol). Final LeuT concentration was 8 $\mu\text{g}/\text{ml}$. At time points, 100 μl of the reaction mix was removed and quenched in a glass test-tube containing 1.8 ml of ice-cold internal buffer.

For the steady-state kinetic measurements, proteoliposomes were diluted into external buffer at 27°C with 0.3–400 μM [^3H]tryptophan (0.2 Ci/mmol). Final LeuT concentration was 16 $\mu\text{g}/\text{ml}$. Reactions were incubated for 2 min, then 100 μl from each was quenched in a glass test-tube containing 1.8 ml of ice-cold internal buffer. For both time-course and steady-state assays, non-specific uptake was assessed by control reactions in the absence of sodium. After each time or concentration series was collected, quenched reactions were filtered through GSWP02500 nitrocellulose filters, pre-wetted with

ice-cold internal buffer, followed by three 2 ml washes with ice-cold internal buffer. Filters were placed in glass scintillation vials, 6 ml of Ultima Gold scintillation fluid was added and filters were allowed to dissolve for ~5 h before measuring tSIE-corrected d.p.m. values using the Packard TriCarb LSC. Data were analysed using GraphPad Prism version 4.0. Steady-state measurements were fitted with a Michaelis-Menten equation or subjected to an Eadie-Hofstee transformation and analysed by linear regression.

Binding assays

For saturation binding analysis, 70 nM LeuT with C-terminal His₆ tag was incubated with 2 mg/ml Cu²⁺-YSi SPA beads (GE Healthcare) in binding buffer (20 mM HEPES-Tris pH 7, 100 mM NaCl, 1 mM C₁₂M) in the presence of 0.3–600 μM [³H]tryptophan (0.2 Ci/mmol) in a white-walled clear bottom 96-well Isoplate (Perkin-Elmer). The reactions were mixed on an orbital microplate shaker at room temperature. Prior to reading, each plate was allowed to rest for at least 1 hour to allow the beads to settle. Readings were taken at 2, 20, 40 and 60 h, though no significant change was observed after 20 h incubation. Radioactivity from each well was read using a Wallac Microbeta plate reader specifying sample type as SPA and measuring detector-normalized counts per minute. Non-specific radioligand binding was assessed by parallel binding measurements with 5 mM unlabelled leucine added. Specific binding was determined for each experiment by the difference between the mean of duplicate measurements of total and non-specific binding. Data were analysed using GraphPad Prism version 4.0.

Crystallization, data collection, model refinement

LeuT constructs with the binding site mutations F259V, I359Q, and F259V + I359Q were purified by metal ion affinity chromatography in 1 mM C₁₂M and then fractionated by size-exclusion chromatography in the presence of 40 mM β-octyl glucoside and 50 mM L-tryptophan as previously described (Singh *et al*, 2008). The proteins were concentrated to 3–4 mg/ml and dialysed overnight against crystallization buffer (20 mM HEPES pH 7, 200 mM NaCl, 40 mM β-octyl glucoside, 50 mM L-tryptophan) using a 25 000-kDa MWCO membrane. Hanging-drop crystallization screens were set up in 24-well plates with 100 mM HEPES pH 7 or pH 7.5, 200 or 400 mM NaCl and 16–26% PEG 550-MME at protein:reservoir ratio of 1:1, 2:1, and 1:2 (v:v). Plates were incubated at 20 °C and in the dark to minimize photo-oxidation of tryptophan (Asquith and Rivett, 1971; Kerwin and Remmele, 2007). Crystals of a rod-like morphology grew to 20–100 μm in the smallest dimension within 7–10 days and were harvested and cryoprotected as described previously (Yamashita *et al*, 2005). Diffraction screening was

carried out using synchrotron radiation at the Advanced Light Source, beamlines 8.2.1 and 5.0.2 (Lawrence Berkeley National Lab, Berkeley, CA) and at the Advanced Photon Source, beamline NE-CAT 24-ID E (Argonne National Lab, Chicago, IL). The best-diffracting crystals typically grew at 20–24% PEG 550-MME at both 200 and 400 mM NaCl.

Data sets from cryogenically cooled crystals were collected with λ = 1.000 Å, Δφ = 0.5–1.0° per frame, with a minimum of 180° total crystal rotation. Data were indexed, integrated, and scaled using HKL2000 (Otwinowski and Minor, 1997). Molecular replacement was carried out with data from each crystallization experiment using MolRep (Vagin and Teplov, 1997) with either the occluded state structure of LeuT (PDB ID 2A65) or the open-to-out structure (PDB ID 3F3A) with all ligands removed as the search model. The highest scoring solution was then subjected to an initial round of rigid-body refinement followed by simulated annealing and iterative rounds of positional and B-factor refinement with manual model rebuilding using Phenix (Adams *et al*, 2002) and Coot (Emsley and Cowtan, 2004) with geometrical validation using Molprobity (Davis *et al*, 2007). Complete data collection and refinement statistics are reported in Supplementary Table S2.

Accession codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 3QS4, 3QS5, and 3QS6.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: CLP and EG designed the research and prepared the manuscript; CLP performed the research and analysed the data.

Conflict of interest

The authors declare that they have no conflict of interest.

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