Supporting Information

Radioligand Binding to Nanodisc-reconstituted Membrane Transporters by Scintillation Proximity Assay

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Experimental Procedures

Expression and purification of C-terminally histidine (His) - tagged LeuT

LeuT was expressed in *E. coli* C41 (DE3) cells and purified as described previously[1] with some changes. Specifically, membrane was resuspended in buffer A (20 mM Tris [pH 8.0], 200 mM NaCl, 100 mM alanine, 1 mM n-dodecyl-β-D-maltoside [DDM]), homogenized, and solubilized for 1 hour at 4°C in the presence of 40 mM DDM. After insoluble aggregates were removed by ultracentrifugation (40,000 rpm for 40 min at 4°C in a Beckman Ti-45 rotor), Ni^{2+}-NTA resin (Qiagen) was added to the supernatant and the entire suspension was batch-bound overnight at 4°C. Resin was washed with buffer A containing 40 mM imidazole and LeuT eluted with 300 mM imidazole.

Eluted fractions were collected, concentrated, and applied to a gel filtration column (Superdex 200 10/300 GL [GE Healthcare]) pre-equilibrated with buffer B (20 mM Tris [pH 8.0], 200 mM KCl, 1 mM DDM) containing 20 mM Ala. Peak fractions were pooled, concentrated, and dialyzed at 4°C against three changes of buffer B over 24 hours until the calculated Ala concentration fell below 100 nM. LeuT purity and monodispersity were assessed by SDS-PAGE and tryptophan fluorescence size exclusion chromatography (FSEC)[2],

respectively. The C-terminal His tag was not removed to enable downstream nanodisc purification and SPA radioligand binding experiments (see below).

Expression and purification of MSP

The MSP1E3D1 and MSP1D1 plasmids were obtained from Addgene and expressed in *E. coli* BL21(DE3) cells as outlined before[3, 4] with a few modifications. Specifically, expression was performed in LB medium at 37°C and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside when the OD_{600} reached 0.6. Cells were harvested 4 hours post-induction and stored at -80 ºC until use.

For purification, cells were resuspended in 20 mM sodium phosphate (pH 7.4) containing 1 mM PMSF and lysed by sonication in the presence of 1% Triton X-100. The lysate was cleared by centrifugation at 30,000 x g for 30 min at 4 ºC and then batch-bound with Ni^{2+}-NTA resin for 2 hours at 4ºC. The resin was subsequently washed with four bed volumes of each of the following: (1) 40 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1% Triton X-100; (2) 40 mM Tris-HCl [pH 8.0], 300 mM NaCl, 50 mM sodium cholate; (3) 40 mM Tris-HCl [8.0], 300 mM NaCl; and (4) 40 mM Tris-HCl [pH 8.0], 300 mM NaCl, 50 mM imidazole. MSP1E3D1 was eluted with 40 mM Tris-HCl [pH 8.0], 300 mM NaCl, 300 mM imidazole.

Peak fractions were collected and dialyzed against buffer C (20 mM Tris-HCl [pH 8.0], 100 mM NaCl) at 4°C to remove imidazole; the His tag was cleaved by incubating with TEV protease for 19 hours at 4°C. Protein purity and TEV digestion were monitored via SDS-PAGE. Undigested MSP and cleaved His tags were removed by passing the sample through a self-packed Ni^{2+}-NTA gravity column and washing with buffer C containing 25 mM imidazole. The flow-through fractions containing digested MSP were then pooled, dialyzed against buffer C to remove imidazole, concentrated and stored at -20 °C until needed. The concentrations of cleaved

MSP were measured spectrophotometrically at 280 nm using a calculated extinction coefficient of 26,600 $M^{-1}cm^{-1}$ for MSP1E3D1 and 18,200 $M^{-1}cm^{-1}$ for MSP1D1.

Phospholipid preparation

Lipids (POPC: POPG 3:2) were transferred to a small glass tube and dissolved in chloroform. The chloroform was then allowed to evaporate under a gentle stream of nitrogen gas while rotating for at least 5 hours. The thin lipid film was resuspended in buffer D (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.5 mM EDTA, 0.01% NaN_3) containing 80 mM of sodium cholate to yield a final lipid concentration of 20 mM. The suspension was subsequently vortexed and sonicated at room temperature until the solution was clear.

Empty nanodisc assembly

Purified MSP1E3D1 was added to the POPC-POPG/sodium cholate solubilized mixture, and the final MSP1E3D1 : lipid : detergent ratio was adjusted to 1 : 130 : 300 (61.5 μM : 8 mM : 18.5 mM). After the mixture was incubated on ice for 1 hour, detergent was removed by adding 80% (w/v) Bio-Beads SM-2 (Bio-Rad) and incubating on ice for 1 hour followed by gently rotating at 4°C for 4 hours. The disc preparation was filtered through 0.45 μm nitrocellulose-filter tubes, rotated at 4 °C for two additional hours, and then purified on a gel filtration column (Superdex 200 10/300 GL) prequilibrated with 20 mM Tris-HCl [pH 8.0], 100 mM NaCl. Purity was assessed by SDS-PAGE and peak fractions were collected and concentrated.

Preparation of LeuT-containing nanodiscs

To assemble LeuT into POPC/POPG nanodiscs, 5 or 10 µM of His-tagged LeuT, 100µM of untagged MSP1E3D1 and 5 mM lipids (POPC:POPG 3:2, solubilized in sodium cholate) were incubated over ice for 1 hour. The mixture volume was 500 µl and the final detergent concentration was adjusted to 18 mM. DDM and sodium cholate were subsequently removed by adding 80% (w/v) Bio-Beads SM-2 and incubating on ice for 1 hour followed by overnight incubation at 4°C. After filtering the disc preparation through 0.45 µm nitrocellulose-filter tubes to remove Bio-beads, empty nanodiscs were removed by mixing the sample with Ni^{2+}-NTA resin for 1 hour at 4°C. The resin bed and assembly mixture were equal in volume. The resin was washed with buffer E (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 15 mM imidazole,) and LeuT-containing nanodiscs were eluted with buffer E containing 300 mM imidazole.

The disc preparation was further purified on a gel filtration column (Superdex 200 10/300 GL) pre-equilibrated with 20 mM Tris-HCl [pH 8.0], 100 mM NaCl. The column had previously been calibrated with proteins of known Stokes hydrodynamic diameter: bovine thyroid thyroglobulin (17 nm), horse spleen ferritin (12.2 nm), bovine liver catalase (10.4 nm), and bovine serum albumin (7.1 nm). Fractions corresponding to the size of the LeuT-nanodisc complex (~13 nm) were collected and concentrated. Purity was assessed with SDS-PAGE, and the concentration of LeuT in the nanodisc was determined by densitometry (ImageJ) of the SDS-PAGE gel using known concentrations of DDM-solubilized LeuT. LeuT was assembled into MSP1D1 nanodiscs as delineated above except that the final LeuT : MSP : lipid : detergent molar ratio was adjusted to 0.05 : 1 : 30 : 180 (5 µM : 100 µM : 3 mM : 18 mM).

Electron microscopy of nanodiscs

Samples were prepared by conventional negative staining as described elsewhere.[5] Briefly, 5 µl of the LeuT-nanodisc complex (0.2 mg/ml) in 20 mM Tris-HCl [pH 8.0], 100 mM NaCl was lifted onto a glow-discharged, ultrathin carbon-coated copper grid. Samples were stained for 1 min with filtered 2% uranyl acetate and then air dried. Images were collected with a Tecnai T12 electron microscope operating at an acceleration voltage of 120 KV.

[^{3}H]Leu and [^{3}H]Ala binding to nanodiscs by scintillation proximity assay (SPA)

Empty or LeuT-containing nanodiscs were first pre-incubated with Cu^{2+} chelating YSi SPA beads (PerkinElmer) in buffer F (150 mM Tris-MES [pH 7.5], 50 mM NaCl, 20% glycerol) for 2 hours at 4°C to permit capture onto the bead surface. Note that empty nanodiscs had been with His-tagged MSP1E3D1 to facilitate immobilization onto the surface of the beads.

Binding was initiated by adding 100 µl of the bead-nanodisc suspension to 100 µl of buffer F containing 100 nM [^{3}H]Leu (specific activity 21.2 Ci/mmol) or 2000 nM [^{3}H]Ala (specific activity 12.0 Ci/mmol). The final concentrations of LeuT, [^{3}H]Leu and [^{3}H]Ala per reaction were 20, 50, 1000 nM, respectively. Nonspecific binding was measured in the presence of 5 mM L-Ala, and the amount of beads per well was 250 µg.

The reactions were mixed in individual wells of a 96-well white-wall clear-bottom plate (Isoplate-96, PerkinElmer), and the plate was vigorously shaken on a microplate shaker at room temperature for 30 min before reading in a photomultiplier tube MicroBeta

counter (PerkinElmer) in SPA cpm mode at 30, 120, and 900 minutes. Counts plateaued in just 30 min, indicating that equilibrium had been reached by that time.

Experiments were performed three times, each time in duplicate, with two separate LeuT-nanodisc preparations.

SPA saturation binding experiments

SPA assays were performed as described above except that the [^{3}H]Leu and [^{3}H]Ala final concentrations per well varied from 1-750 nM and 10-8000 nM, respectively. SPA with DDM-solubilized LeuT was also performed as outlined above except that buffer F was supplemented with 1 mM DDM. For all assays, nonspecific binding was measured in the presence of 5 mM L-Ala. All experiments were performed three times, each time in duplicate, with two separate LeuT-nanodisc preparations. Data were analyzed via nonlinear regression in GraphPad Prism (version 5.0).

To convert cpm to pmol, a calibration curve was generated relating cpm to known quantities of each [^{3}H]substrate. To account for the discrepancy between the counting efficiencies of SPA and cpm modes, the latter of which was used to determine counts added, a theoretical YSi bead efficiency of 60%, provided by PerkinElmer, was employed. This coefficient was then used to confirm that counts bound were less than 10% of counts added, ensuring that ligand depletion had not occurred.

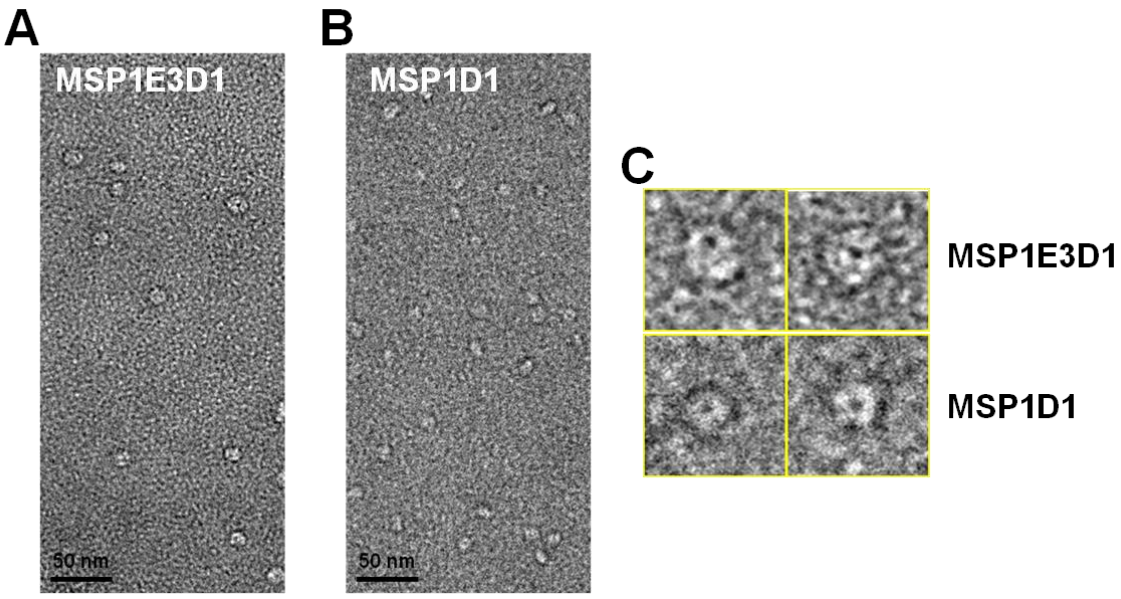

Figure S1. Transmission electron microscope images of negatively stained nanodiscs containing LeuT. (A) Nanodiscs assembled with MSP1E3D1. (B) Nanodiscs assembled with MSP1D1. (C) Single nanodisc particles assembled with MSP1E3D1 (top two particles, ~13 nm) and MSP1D1 (bottom two particles, ~10 nm). The dark spot within each nanodisc most likely corresponds to the LeuT vestibule/opening filled with stain.

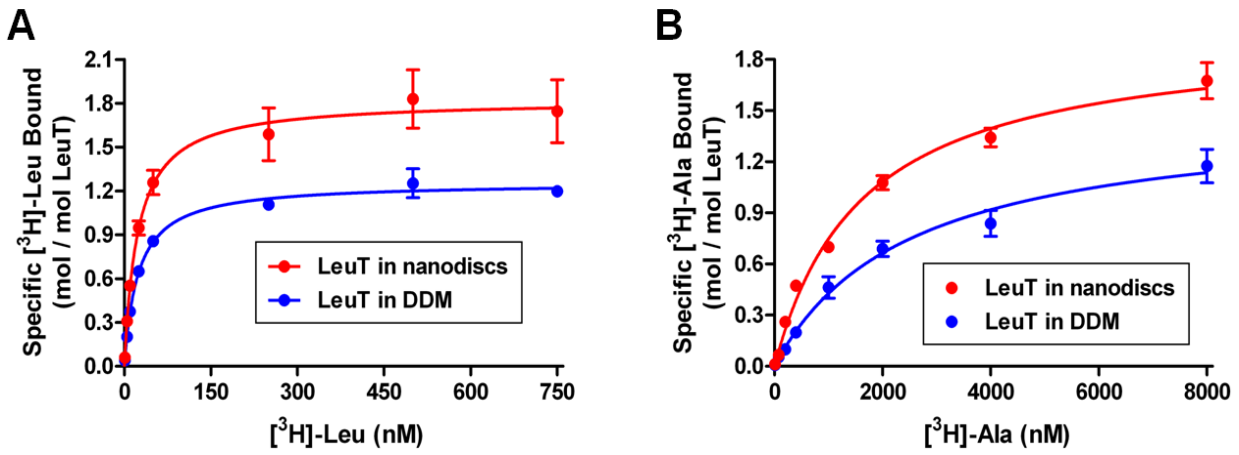

Figure S2. [^{3}H]Leu and [^{3}H]Ala binding to LeuT as measured by SPA. The y-axis is expressed as mol [^{3}H]Leu or [^{3}H]Ala bound per mol LeuT . (A) [^{3}H]Leu binding to LeuT in nanodiscs (red) versus DDM (blue). Data reveal molar binding stoichiometries of 1.82 ± 0.1 and 1.23 ± 0.02 for LeuT in nanodiscs and DDM, respectively. (B) [^{3}H]Ala binding to LeuT in nanodiscs (red) versus DDM (blue). Data show molar binding stoichiometries of 1.97 ± 0.07 and 1.48 ± 0.06 for LeuT in nanodiscs and LeuT in DDM, respectively. Data are the mean ± SD from three independent experiments, each performed in duplicate, with two separate LeuT-nanodisc preparations.

Discussion of Figure S2.

Although LeuT is clearly more active in a lipid bilayer, as would be expected for a membrane protein in a more physiologically-relevant environment, we are still not certain about the mechanistic implications for substrate binding and transport. Nevertheless, the increase in B_{max} values for both [^{3}H]Ala and [^{3}H]Leu binding with nanodisc preparations versus DDM-solubilized material, which was also observed with LeuT reconstituted into proteoliposomes or at

lower DDM concentrations (2 versus 6 mM)[6], is intriguing given the controversy over the number of substrate binding sites (one versus two) in LeuT.[7,8]

Perhaps this nanodisc system can now be employed to directly and more comprehensively address the question of LeuT-substrate stoichiometry in another model of the lipid bilayer as a complement to experiments that have already been conducted with the wild-type protein and previously-examined mutants in proteoliposomes[6] and with macromolecular crystallography in bicelles.[9]

Table S1. Equilibrium binding constants for [^{3}H]Leu and [^{3}H]Ala in the presence of DDM (bottom row) or phospholipid bilayer nanodiscs (top row).

	Leucine			**Alanine**		
	K_d **(nM)**	B_{max} **(pmole/µg LeuT)**	B_{max} **(mole/mole LeuT)**	K_d **(nM)**	B_{max} **(pmole/µg LeuT)**	B_{max} **(mole/mole LeuT)**
POPC/POPG nanodiscs	23.4 ± 3.9	30.7 ± 1.2	1.82 ± 0.07	1620 ± 125	32.7 ± 0.9	1.97 ± 0.07
DDM	23.9 ± 1.6	21.2 ± 0.3	1.23 ± 0.02	2480 ± 243	24.8 ± 1.0	1.49 ± 0.06

Data are expressed as the mean ± SD of three independent experiments, each performed in duplicate, with two separate LeuT-nanodisc preparations.

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