The role of interfacial lipids in stabilizing membrane protein oligomers

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Oligomerization of membrane proteins in response to lipid binding has a critical role in many cell-signalling pathways but is often difficult to define or predict. Here we report the development into the regulatory roles of lipids. The advent of methods using mass spectrometry to characterize membrane proteins individually, including G-protein-coupled receptors.

Figure 1 | Plot of buried surface area and number of salt bridges for oligomeric α-helical membrane proteins and native mass spectra.

Protein oligomers are represented by circles colour-coded according to the number of salt bridges they form, where maximum number of salt bridge is designated to 100% and 0 is marked as 0% and are grouped by oligomeric state (pentamers have an oligomeric state ≥5). A random horizontal jitter has been applied to all points to aid visualization. NhaA and LeuT (outlined in red) are two of the weakest oligomers, having some of the lowest buried surface areas and forming no salt bridges. The 12 proteins for which mass spectra have been recorded are outlined in green. Illustrated are mass spectra of the trimeric ammonia channel AmtB, tetrameric aquaporin AqpZ and pentameric ion channel ELIC. A larger buried surface area than LeuT and NhaA but weaker than the other 12 oligomeric proteins.

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present in the MS/MS spectrum originate from dimeric LeuT parent binding of two phospholipids. It is important to emphasize that all ions bling us to distinguish the binding of one cardiolipin from the potentialomer retaining one cardiolipin molecule (Extended Data Fig. 3), ena-the apo and lipid-bound states, with one cardiolipin molecule and up to
the dimer in a bilayer revealed binding of cardiolipins and phospholipids at the dimer interface. Each cardiolipin interacts with both mono-
dimers (green/grey) with a mass 7.4 kDa greater than the amino acid sequence mass. c, MS/MS of 23+ charge state of LeuT reveals monomers with cardiolipin (CDL, purple head-group) and phospholipid (blue head-group) retained. Masses of the bound lipids are marked black (phospholipids) and red (cardiolipin). Inset, molecular dynamics simulation of LeuT in an E. coli lipid bilayer revealing possible binding sites of interfacial cardiolipin. The cardiolipin phosphate groups (orange) interact closely with positively charged residues (K376, H377, R506; blue) at the dimer interface. Interactions are shown (yellow dotted lines) with distances measured in ångströms (red).

recovered a significant population of the dimer (Extended Data Fig. 2d). These observations imply that the additional mass associated with the dimer is comprised of lipid.

Conventional lipid-identification experiments require the extraction of lipids from the proteo-micelle solution or cellular environment, followed by either a chromatographic or mass spectrometry step. These approaches report on the entire set of lipids present but fail to distinguish endogenous lipids bound to the membrane protein of interest from those in bulk solution. Simultaneous identification of the bound lipids and the oligomeric state of the protein requires a tandem mass spectrometry (MS/MS) platform, akin to protocols developed to sequence peptides in top-down proteomics. MS/MS in its current form cannot be applied to membrane proteins directly since the activation energy available in the collision cell is used to liberate membrane proteins from detergent micelles. To overcome this problem, we developed an instrument platform in which the high energy applied at the source region removes the detergent micelle before entry into the collision cell, enabling isolation of discrete lipid-bound complexes in the quadrupole for subsequent MS/MS and lipid identification (Fig. 2a). Using this platform, we isolated the 23+ charge state of the dimer that incorporates the 7.4 kDa of additional mass (Fig. 2c). Activation of this species in the collision cell yields monomeric LeuT in the apo and lipid-bound states, with one cardiolipin molecule and up to three phospholipids. Increasing the collision energy results in the monomer retaining one cardiolipin molecule (Extended Data Fig. 3), enabling us to distinguish the binding of one cardiolipin from the potential binding of two phospholipids. It is important to emphasize that all ions present in the MS/MS spectrum originate from dimeric LeuT parent ions with the additional 7.4 kDa, which can now be assigned to three phospholipids and one cardiolipin per subunit (3.7 kDa or 7.4 kDa per dimer).

Production of wild-type LeuT in a cardiolipin-deficient E. coli strain yielded exclusively delipidated monomeric LeuT (Extended Data Fig. 4), confirming that cardiolipin is essential for dimerization. Performing coarse-grained molecular dynamics (CGMD) simulation of the LeuT dimer in a bilayer revealed binding of cardiolipins and phospholipids to the dimer interface. Each cardiolipin interacts with both monomeric units, with its bi-phosphate head group binding to basic residues (K376, H377 and R506) on either side of the dimer interface (Fig. 2c and Extended Data Figs 4, 5). These results help to identify the residues that form critical contacts with lipid head-groups to confer the specificity of these interactions. Substitution to alanine of either the dimer-interface residues or the basic residues that bind to cardiolipin, abolished dimer formation (Extended Data Fig. 4). We conclude that LeuT has a weak dimer interface that is stabilized by cardiolipin, which bridges the interface and is augmented by six phospholipids.

The bacterial sugar transporter SemiSWEET from V. splendidus is a functional dimer. A combination of the buried surface area and absence of salt-bridge interactions between monomers makes SemiSWEET an example of an oligomer with stability higher than LeuT but considerably lower than the 12 strong oligomers considered above. The mass spectrum of SemiSWEET shows the presence of both monomer and dimer species (Extended Data Fig. 6a). To investigate whether the presence of monomers is a consequence of lipid removal during protein purification and whether the monomers are in equilibrium with the dimers, we prepared two mass-distinct forms of
SemiSWEET (with or without a deca-His tag, Extended Data Fig. 6). A time-course mass spectrometry experiment, subsequent to mixing of these two mass-distinct forms in equal ratios, revealed the rapid appearance of heterodimeric peaks, consistent with a solution-phase monomer–dimer equilibrium (Extended Data Fig. 6). High-energy MS/MS of SemiSWEET also identified endogenous bound lipids, a significant proportion of which were cardiolipin (Extended Data Fig. 6c). Upon the addition of increasing amounts of cardiolipin, we observed preferential lipid binding to the dimer and a subsequent shift in the equilibrium towards the dimeric population (Fig. 3). By contrast, addition of phosphatidylglycerol revealed no such preference towards any oligomeric forms (Extended Data Fig. 6d). We therefore conclude that the preferential binding of lipid to the dimer drives the equilibrium towards the functionally relevant state of the protein.

Given our emerging hypothesis—that lipids are critical for stabilizing weak dimer interfaces—we sought to compare proteins with weak interfaces that might require lipids for dimerization to homologues with higher interface strengths. One such pair of proteins is the NhaA and NapA Na\(^+\)/H\(^+\) antiporters, from E. coli and T. thermophilus, respectively (Fig. 1). Although the oligomeric stability of dimeric NhaA is comparable to that of LeuT, our interface analysis suggests that dimeric NapA is likely to be more stable (Fig. 4). The mass spectrum of NhaA reveals an ensemble of lipid-bound dimeric species (Fig. 4a) and a complete absence of delipidated dimer. Performing MS/MS on the lipid-bound NhaA dimer leads to stepwise losses of cardiolipin and yields monomers with one cardiolipin bound as well as an apo NhaA dimer that readily dissociates (Extended Data Fig. 7). The appearance of monomeric NhaA is coincident with the loss of the second cardiolipin, suggesting that cardiolipin stabilizes the dimer structure. Molecular dynamics simulations of NhaA in a lipid box reveal that cardiolipin can bind at the interface, further supporting the observed role of cardiolipin in stabilization (Fig. 4a and Extended Data Fig. 5). Mass spectrometry analysis of the homologous NapA revealed a marked contrast; the NapA dimer is completely lipid-free, confirming its intrinsic stability in the absence of interfacial lipids. This is consistent with the known higher stability of the proteins of thermophiles than their non-thermophilic homologues. In NapA, an additional N-terminal helix that is absent from NhaA strengthens the protein–protein interface, essentially removing the requirement for lipids to stabilize dimer formation. These two proteins, which were purified from identical membranes and share the same fold and physiological role, demonstrate that membrane proteins can either acquire additional structural elements to ensure greater contacts between subunits or recruit lipids to preserve their oligomeric state.

We anticipate that the ability to form a stable interface or to recruit lipids to preserve oligomeric state might be a general phenomenon that exists in other membrane protein systems such as G-protein-coupled receptors (GPCRs)\(^{21}\). When estimating the oligomeric strength of the two possible interfaces of \(\mu\)-opioid receptor, we see that the transmembrane helix 5 (TM5)–TM6 and TM1/TM2–H8 interfaces have buried surface areas of 1,585.6 Å\(^2\) and 588.0 Å\(^2\), respectively, both without salt bridges. We therefore estimate that the TM1/TM2–H8 interface is considerably weaker\(^{22}\) (Fig. 4b). However, the tighter interface (TM5–TM6) restricts the conformational flexibility required to attain

**Figure 3** | Mass spectrum recorded for SemiSWEET and the effect of cardiolipin on the monomer–dimer equilibrium. Mass spectrum of SemiSWEET following incubation with cardiolipin (purple head-groups). Plot of cardiolipin concentration against the percentage of monomer or dimer observed in mass spectra at various cardiolipin concentrations (bars represent \(n=5\) data points denoted with black dots, error bars denote s.d.).
the agonist-bound state. The weaker interface contains a cavity, akin to that found in NhaA, wherein a side chain of a fatty acid has been modelled in the crystal structure. The weak dimeric interfaces involving TM1/TM2–H8 can also be constructed for many other GPCRs, including the β1 adrenergic receptor and κ-opioid receptors, which have buried surface areas of 833.6 Å² and 1025.1 Å², respectively, both without salt bridges (Fig. 4b). These very low oligomeric strengths are consistent with observations of transient oligomeric states, leading us to speculate that much of the controversy surrounding the oligomeric state of GPCRs stems from their ability to exist in multiple forms, with different interfaces modulated by interfacial lipids, analogous to the monomer–dimer equilibrium shown here for SemiSWEET.

Although the intrinsic stability of the oligomers correlates with lipid binding to stabilize interfaces, a key question arises with respect to function. For both SemiSWEET and NhaA, the existence of a stable dimeric state is thought to be critical for their mechanistic pathways. Under conditions of extreme stress, the dimer of NhaA is more functionally active than the monomer. By analogy with LeuT, dimerization of the homologous eukaryotic dopamine and serotonin transporters (DAT and SERT, respectively) might also be anticipated in vivo. Sequence alignment and superposition of the structures of LeuT and SERT reveal that the cardiolipin-binding residues identified here are conserved in all biogenic amine transporters (Extended Data Fig. 8). A key difference between the X-ray crystallographic structures of LeuT and SERT arises in the C-terminal helix of SERT, which orients away from the subunit interface, preventing dimerization in the crystal form. Nevertheless, the functional significance of dimers (and possibly higher oligomers) of SERT is well documented.

Overall our data show how lipid-binding at interfaces stabilizes weak oligomers and provide direct and compelling evidence that altering the lipid composition in solution can propagate changes in oligomeric state. In the cellular environment, such mechanisms are likely to be employed to regulate the abundance of functional forms of membrane proteins. As new structures of membrane proteins emerge, the approach described here can help to resolve conflicts in oligomeric state and contribute to our understanding of their functional relevance—important considerations for the design of bio-therapeutics and for drug targeting.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Molecular cloning and plasmid construction. LeuT was expressed from a pET-16b vector, containing a thrombin-cleaveable C-terminal 8×His tag. The SemiSWEET plasmid was transformed into BL21 DE3 E. coli (Novagen), and expressed and purified as reported previously. In brief, multiple colonies were used to inoculate 100 ml of terrific broth (TB) and were grown overnight at 37°C. A total of 10 ml of overnight culture was used to inoculate each of 6 l of TB, which were allowed to grow at 37°C until the OD600 reached 1.5. Cells were centrifuged at 4,000 rpm for 10 min at 4°C, resuspended in lysis buffer solution (300 mM sodium chloride, 20 mM Tris, pH 7.4) supplemented with protease inhibitors (Roche) and used for a M-110 P6 microfluidic chip (Microfluidics), and the cell debris was pelleted by centrifugation at 20,000 g for 20 min at 4°C. Membranes were pelleted by centrifugation of the supernatant at 100,000 g for 2 h at 4°C and subsequently resuspended in ice-cold buffer solution (100 mM sodium chloride, 20 mM Tris, 20% glycerol; pH 8.0) and homogenized using a PowerElveHjem Teflon pestle and glass tube. n-dodecylβ-D-maltopyranoside (DDM) was added to resuspended membranes to a final concentration of 2% (w/v) and the suspension incubated with gentle agitation for 15 h at 4°C. Insoluble material was pelleted by centrifugation at 20,000 g for 30 min and the supernatant filtered through a 0.22-μm filter. LeuT was purified by immobilized-metal ion-affinity chromatography using a HiTrap HP 5 ml column (GE healthcare) equilibrated with buffer A (150 mM sodium chloride, 10 mM potassium chloride, 20 mM Tris, 20 mM imidazole, 10% glycerol, 0.02% DDM; pH 8.0) and eluted with buffer B (150 mM sodium chloride, 10 mM potassium chloride, 20 mM Tris, 500 mM imidazole, 10% glycerol, 0.02% DDM; pH 8.0). The eluted protein was transferred to a dialysis cassette (100 kDa molecular weight cut-off) and dialysed against a buffer (150 mM sodium chloride, 20 mM Tris, 10% glycerol; pH 8.0) with 0.02% DDM overnight. For the next HC-1 column (GE Healthcare), equilibrated in a buffer (150 mM sodium chloride, 10 mM potassium chloride, 20 mM Tris, 10% glycerol; pH 8.0) with 1% octylglucoside. Peak fractions containing octylglucoside-solubilized LeuT were concentrated as above and used for further study. All protein concentration measurements were carried out using a UV/Vis spectrophotometer (DS-11 +, DeNovix). The mass addition to the wild-type LeuT was observed over seven different preparations.

The V. splendidus SemiSWEET plasmid was transformed into BL21 DE3 E. coli (Novagen), and expressed and purified as reported previously. In brief, multiple colonies were used to inoculate 100 ml of LB and grown overnight at 37°C. A total of 10 ml of overnight culture was used to inoculate each of 6 l of LB, which were allowed to grow at 37°C until the culture reached an OD600 of 0.8. IPTG was added to a final concentration of 0.2 mM, and the culture grown for 15 h at 22°C. Cell harvesting, resuspension, lysis, membrane isolation and detergent extraction steps were identical to the LeuT purification. SemiSWEET was first purified by IMAC using a HiTrap HP 5 ml column (GE healthcare) equilibrated with buffer A (150 mM sodium chloride, 20 mM Tris, 20 mM imidazole, 10% glycerol, 1 mM DTT, 0.02% DDM; pH 8.0) and eluted with Buffer B (150 mM sodium chloride, 20 mM Tris, 500 mM imidazole, 10% glycerol, 1 mM DTT; 0.02% DDM; pH 8.0). The eluted protein and HC-3 protein (Novagen) were transferred to a dialysis cassette (300 Da MWCO, Chromatography, and dialysed against buffer A (150 mM sodium chloride, 20 mM Tris, 10% glycerol, 1 mM DTT; pH 8.0) with 0.02% DDM overnight. For preparation of 10× His-tagged SemiSWEET, no HRV 3C protease was added to the dialysis cassette. A 50-kDa MWCO concentrator was used to concentrate the dialysed protein. SemiSWEET was then injected onto a Superdex 200 Increase GL 10/300 column (GE Healthcare), equilibrated in a buffer solution (150 mM sodium chloride, 20 mM Tris, 10% glycerol, 1 mM DTT, 0.02% DDM; pH 8.0) with 0.5% C8E4. Peak fractions containing C8E4-solubilized SemiSWEET were concentrated as above and used for further study. NapA, NhaA, AqpZ, AmtB and ELIC were expressed as described previously.

Non-denaturing mass spectrometry. Samples were prepared for non-denaturing mass spectrometry by buffer-exchange into mass spectrometry buffer (200 mM ammonium acetate, 2 mM ethylene glycol, 2×Sodium chloride, 1 mM DTT, 1 mM EDTA, 0.5 mM ATP, 1 mM MgCl2, 0.25 mM detergent of interest; pH 7.4) using a centrifugal buffer exchange device (Micro Bio-Spin, Biorad). For SemiSWEET experiments, 1 mM DTT was also added to the mass spectrometry buffer.

Mass spectrometry measurements were performed on a Synapt G1(Waters) with a 2×-z-spray source, using nanoelectrospray capillaries prepared in-house. The source pressure was set to 4–7 mBar, with a capillary voltage of 1.4–1.7 kV, capillary nanoflow of 0.05–0.2 mBar and argon as collision (trap) gas at flow rate of 1.5–8.0 ml min⁻¹. Other parameters, including the sample and extraction cone and trap bias voltages, collision voltages and quadrupole profile were optimized for maximal ion intensity and minimal dissociation of the target membrane protein complex. Data were processed using Masslynx software.

High-energy non-denaturing mass spectrometry. To allow the use of higher voltages on the extraction cone in the source region of the Synapt G2, the configuration file for the extraction cone was modified to increase the maximum voltage setting from 10 kV to 200 V. However, altering this setting alone would restrict the maximum sample cone voltage that could be accessed owing to the limits imposed by the power supplies. To overcome this limitation, the capability to drive the sample cone voltage from an external supply was implemented. This took the form of a patch cable introduced between the instrument lens control PCB and the source ion block. The ion block contains both heater elements and a thermocouple in addition to supporting the extraction cone and these were decoupled with the patch cable to prevent possible electrical breakdown with use of higher cone voltages. The extraction cone was patched directly through from the lens PCB while the sample cone voltage was decoupled and a new wire connection made to an external power supply.

Delipidation. Purified, octylglucoside-solubilized LeuT was incubated in 2% neopentyl glycol overnight at 4°C. Subsequently, the sample was passed through a Superdex 200 Increase GL 10/300 column (GE Healthcare) equilibrated in 200 mM ammonium acetate with twice the critical micelle concentration of neopentyl glycol, to remove the excess octylglucoside and neopentyl glycol. This sample was subjected to mass spectrometric analysis. The delipidated LeuT in neopentyl glycol was re-exchanged back in octylglucoside using the above protocol with the 200 mM ammonium acetate containing 1% octylglucoside. E. coli polar lipid stocks were made from powder (Avanti Polar Lipids Inc.) at a concentration of 10 mg ml⁻¹ using previously published methods and subsequently diluted 50 times in 200 mM ammonium acetate solution containing 1% octylglucoside. A dicylo-cardiolipin stock (10 mg ml⁻¹ in 200 mM ammonium acetate) was prepared as described previously, aliquots of this stock were diluted 50 times in 200 mM ammonium acetate solution containing 1% octylglucoside for each mass spectrometry experiment.

Preparation and titration of phospholipids. Purified SemiSWEET–His8 in mass spectrometry buffer was diluted to an oligomer concentration of 20 μl. A cardiolipin stock (10 mg ml⁻¹ in 200 mM ammonium acetate) was prepared as described previously, aliquots of this stock were diluted 50 times in 200 mM ammonium acetate solution containing 1% octylglucoside for each mass spectrometry experiment.

Data was acquired for 100 scans, which were summed in Masslynx software and processed using UniDec deconvolution software. Relative monomer and dimer abundances were calculated by taking the sum of the respective deconvoluted intensities in all lipidation (and non-lipidated) states, normalized to the total ion counts for each mass spectrometry experiment. Diluted SemiSWEET and cardiolipin solutions were then mixed 1:1 and incubated on ice for 5 min (for the measurement without cardiolipin, SemiSWEET was mixed with mass spectrometry buffer). Following this, 2 μl of the mixture was used for each mass spectrometry measurements at the 4 lipid concentrations.

Mass spectrometry measurements were performed on a Synapt G1 as described above. The source pressure was set to 4.2 mBar, capillary nanoflow 0.1 mBar, trap collision voltage 50 V, transfer collision voltage 10 V and collision gas flow rate 1.8 ml min⁻¹.

Subunit exchange. Purified, C8E4-solubilized SemiSWEET and SemiSWEET–His10 were separately buffer-exchanged into mass spectrometry buffer as described above, then concentrated to 40 μl using a 30-kDa MWCO concentrator.
SemiSWEET and SemiSWEET–His\textsubscript{10} were then mixed briefly on ice and 3 μl of this equimolar mixture was immediately transferred to a nanoelectrospray capillary for mass spectrometry data acquisition. Data was acquired continuously for 10 min.

Data were processed using Xcalibur software (Thermo Scientific) as follows: spectra were extracted from summation of the chromatogram in 30-s scan windows centred on each minute (for example, 0.75–1.25 min for the 1-min time point). Relative abundances of the SemiSWEET and SemiSWEET–His\textsubscript{10} homodimers and the SemiSWEET–SemiSWEET–His\textsubscript{10} heterodimer were calculated for each time point using UniDec. A plot of relative homodimer abundance and heterodimer abundance against time was generated using SigmaPlot.

Mass spectrometry measurements were performed on a modified Q-Exactive orbitrap mass spectrometer (Thermo Fisher)\textsuperscript{39} modified and optimized for non-denaturing mass spectrometry of membrane protein complexes. Spectra were acquired in ‘Native Mode’ with maximum radio frequency applied to all ion optics, –3.2 kV to the central electrode of the Orbitrap and with ion trapping in the higher-energy collisional dissociation (HCD) cell. Ions were generated in positive ion mode from a static nanospray source using gold-coated capillaries prepared in-house. Transient times were 64 ms and AGC target was 1 × 10\textsuperscript{6}. Spectra were acquired with 1 microscan, a noise-level parameter set to 3 and HCD cell voltage of 75 V; no in-source activation was applied. The collision gas was argon and pressure in the HCD cell was maintained at approximately 1 × 10\textsuperscript{−9} mbar.

**Molecular dynamics simulations.** All molecular dynamics simulations were performed using GROMACS v5.1.2 (ref. 39). The MemProtMD pipeline\textsuperscript{40} was used with the Martini 2.2 force field\textsuperscript{41} to run five repeats of a 1-μs CGMD simulation of the dimeric protein complexes. The last 800 ns of each of these simulation trajectories were considered for further analysis. The proteins were centred within the simulation system to permit the assembly and equilibration of 10% cardiolipin either with a 20%:70% ratio of 1-palmitoyl, 2-oleyl phosphatidylglycerol (POPG) to 1-palmitoyl, 2-oleyl phosphatidylethanolamine (POPE), or with 90% 1-palmitoyl, 2-oleyl phosphatidy choline (POPC) bilayers. Systems were neutralized with a 150 mM concentration of NaCl. All simulations were performed at 323 K, with protein, lipids and solvent separately coupled to an external bath using the velocity-rescale thermostat\textsuperscript{42}. Pressure was maintained at 1 bar with a semi-isotropic compressibility of 5 × 10\textsuperscript{−8} us the Berendsen barostat\textsuperscript{43}. All bonds were constrained with the P-LINCS algorithm\textsuperscript{44}. Electrostatics was measured using the ‘reaction field’ method\textsuperscript{45}, while a Verlet cut-off scheme to permit GPU calculation was employed for the martini coarse-grained protein force field. J. Chem. Theory Comput. 9, 687–697 (2013).


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Extended Data Figure 1 | Mass spectra of LeuT recorded with increasing collision voltages and of a LeuT fusion protein construct.

a, Mass spectra of LeuT, liberated from octylglucoside micelles, (green/grey spheres, most abundant charge state highlighted in pale blue), show that the 7.4-kDa lipid adduct (blue/purple head groups) is retained throughout the trap collision energy range (white, blue arrow) of the mass spectrometer.

b, Mass spectra of LeuT expressed as a fusion protein with eYFP (LeuT–eYFP yellow circles), liberated from octylglucoside micelles, show that the dimer is similarly associated with a 7.4-kDa adduct.
Extended Data Figure 2 | Mass spectra of LeuT following incubation with delipidating detergents and *E. coli* polar lipids. **a**, Mass spectrum of LeuT liberated from octylglucoside micelles (green head groups) shows low-abundance delipidated monomers (green spheres, 59.3 kDa) and high-abundance lipid-bound dimers (green/black spheres, 126.0 kDa). **b**, Mass spectrum of LeuT after incubation with neopentyl glycol (NG, orange head-groups) shows only delipidated monomers. **c**, Mass spectrum of LeuT in octylglucoside (OG), after incubation with neopentyl glycol, shows only delipidated monomers. **d**, Mass spectrum recorded after incubation of delipidated LeuT monomers, in octylglucoside, with *E. coli* polar lipids (blue/purple head-groups) shows delipidated monomers and lipid-bound dimers. **e**, Mass spectrum recorded after adding dilsyocardioliopin (blue head-groups) to delipidated monomeric LeuT in octylglucoside (c) shows no dimerization in the presence of this lipid.
Extended Data Figure 3 | High-energy MS/MS experiment of the 23+ charge state of dimeric LeuT, with the 7.4-kDa adduct, as a function of collision voltage. Three satellite peaks represent the lipid-bound states arising through the dissociation of the monomer. The naked monomer is highlighted in blue, while the three satellite peaks are assigned to one phospholipid, one cardiolipin and three phospholipid-bound species (red, green and yellow, respectively). Under higher energy, only the cardiolipin-bound species remains, discounting the mathematical possibility of two phospholipid-bound species. Inset shows the isolated 23+ charge state of the lipid bound dimer. Presence of bound cardiolipin at a higher energy, over that of phospholipid, indicates a higher binding energy of cardiolipin over the latter, potentially owing to greater ionic and hydrophobic interactions.
Extended Data Figure 4 | Site-directed mutagenesis of selected residues at the LeuT dimer interface, resulting mass spectra and molecular dynamics simulations. a, Mass spectrum of LeuT F488A/Y489A, liberated from octylglucoside micelles, reveals monomeric LeuT (green spheres). Inset shows the LeuT dimer interface, with key π-stacking interactions (yellow dotted lines, distances labelled in red) and between aromatic residues (purple). When residues F488 and Y489 (orange arrows) are mutated to alanine, the π-stacking interactions are abolished and LeuT cannot dimerize. b, Molecular dynamics simulations of LeuT in an E. coli lipid bilayer reveal possible binding sites of interfacial phospholipids and cardiolipin (upper panel, viewed from cytoplasmic side of membrane). The cardiolipin phosphate groups (orange) interact closely with positively charged residues (K376, H377, R506; blue) at the dimer interface. Phosphoethanolamine (PE) and phosphatidylglycerol (PG) also bind at the dimer interface. c, Mass spectrum of LeuT expressed in a cardiolipin-deficient E. coli strain (BKT22), liberated from octylglucoside micelles, shows monomeric LeuT, implying that cardiolipin is required for LeuT dimerization. d, Mass spectrum of LeuT K376A/H377A, liberated from octylglucoside micelles, shows monomeric LeuT.
Extended Data Figure 5 | CGMD simulations on LeuT and NhaA dimer. a, Particle densities from five repeats of 1-μs CGMD simulations for cardiolipin around LeuT. The surface densities represent the most occupied positions from the simulations of the phosphate (orange), glycerol (red) and alkyl tails (purple) particles of cardiolipin. The proposed binding sites at the interface are the only places where cardiolipin shows considerable population density. b–d, Comparative particle densities of cardiolipin (b), phosphatidylglycerol (c) and phosphoethanolamine (d) at the LeuT dimeric interface, summed over the simulations show no or minimal densities for phosphatidylglycerol and phosphoethanolamine at the cardiolipin-binding site. Together, a–d show that the proposed binding sites of cardiolipin at the interface are sites of specific bindings. e, Dimeric structure of LeuT with modelled APT (aminopentanetetrol, aminophospholipids) classes of lipid present in *A. aeolicus*. The lipid was drawn in ChemDraw and subsequently modelled by superimposition onto cardiolipin to give the cardiolipin-bound dimeric structure. The favourable van der Waals distances show that it is capable of bridging the dimeric entity through the same sets of residues that were found to be critical towards cardiolipin binding, in an endogenous environment lacking cardiolipins. f, Particle densities from five repeats of 1-μs CGMD simulations for cardiolipin (phosphate group in orange, glycerol in red and alkyl tails in purple) and POPG (in blue) around NhaA dimer interface. As before, the density of cardiolipin is considerably higher than that of phosphatidylglycerol. However, unlike LeuT, here the difference between the density of cardiolipin and phosphatidylglycerol is lower, suggesting this site has less exclusivity towards cardiolipin than that in LeuT. Indeed, mass spectrometry analysis shows a heterogenous distribution of lipids with dimeric NhaA, with mostly cardiolipin but some amount of bound phospholipids.
Extended Data Figure 6 | Mass spectra of His-tagged and unmodified SemiSWEET and identification of endogenous and exogenous lipid binding. **a**, Mass spectrum of unmodified SemiSWEET, liberated from tetraethyleneglycolmonoctyl ether (C₈E₄) micelles, reveals SemiSWEET monomers and dimers (black spheres). **b**, Mass spectrum of deca-His tagged SemiSWEET, liberated from C₈E₄ micelles, reveals SemiSWEET monomers and dimers (green spheres). **c**, High energy MS/MS of unmodified SemiSWEET, liberated from dodecylmaltoside (DDM) micelles, allows isolation of the 6⁺ charge state (black spheres) of the SemiSWEET monomer (black spheres) bound to endogenous lipids. Fragmentation of the lipid-bound species leads to loss of either cardiolipin (1,470 ± 26 Da, purple head-groups), one or two neutral phospholipids (each 756 ± 22 Da, blue head-groups), or a positively charged phospholipid. Trap collision voltages are shown in white inside the blue arrow. **d**, Mass spectrum of deca-His SemiSWEET, liberated from C₈E₄ micelles and incubated with phosphatidylglycerol (blue head-groups). Phosphatidylglycerol binds to both monomers and dimers (dotted boxes highlight lipid-bound peaks) without substantial preference. **e**, Mass spectrum recorded after incubation in solution of an equimolar ratio of deca-His tagged and untagged SemiSWEET (green and black spheres, respectively), liberated from tetraethyleneglycolmonoctyl ether (C₈E₄) micelles. Plot of the percentage abundance of hetero- and homodimers over time (inset), SemiSWEET heterodimers (red trace, peaks highlighted red in mass spectrum) and homodimers (black trace), revealing the solution-phase monomer–dimer equilibrium (PDB accession number: 4QND).
Extended Data Figure 7 | Mass spectrum and high-energy MS/MS of NhaA at a range of collision voltages. a, Mass spectrum of NhaA, liberated from C₈E₄ micelles, reveals NhaA monomers (green spheres) bound to cardiolipin (purple head-groups) and an ensemble of NhaA dimer species in different lipidation states (highlighted in green). b, MS/MS of the 15⁺ charge state (green) of the NhaA dimer (green/black spheres) bound to two cardiolipin molecules liberated from C₈E₄ micelles. Increasing collision voltage applied to the 2× cardiolipin-bound species leads either to loss of 1 cardiolipin to form NhaA dimers bound to 1 cardiolipin (40 V) or to loss of 2 cardiolipin molecules to form delipidated NhaA dimers, with concomitant generation of NhaA monomers (70 V) and further dissociation of NhaA dimers into monomers (120 V). Trap collision voltages are depicted in white, inside the blue arrow.
Extended Data Figure 8 | Sequence and structure alignment of LeuT with other eukaryotic biogenic transporters.  

a, The basic residues of LeuT that are involved in lipid binding (red box) are conserved across the BATs.  
b, Two views of the superimposed structures of LeuT (PDB accession number: 2A65, black) and SERT (PDB accession number: 5I6Z, light blue) show the differences in the dimer interface.  

Dimer interface helices are highlighted with arrows and coloured (LeuT, green; SERT, red); basic residues responsible for lipid binding in LeuT are shown in yellow mesh. One of the interface helices in SERT swings away from the interface, negating the possibility of lipid-induced oligomerization, analogous to that proposed for LeuT.
## Extended Data Table 1 | Summary of the mass spectral analysis of membrane proteins forming strong oligomers

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<tr>
<th>Name</th>
<th>Expected Oligomeric State</th>
<th>Observed Oligomeric State</th>
<th>Expected Mass (kDa)</th>
<th>Observed Mass kDa (Reference)</th>
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<tbody>
<tr>
<td>AqpZ</td>
<td>4</td>
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<td>98.8 x 10^3</td>
<td>98.9 x 10^3 (This work)</td>
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<tr>
<td>MscS</td>
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<td>7</td>
<td>223.7 x 10^3</td>
<td>224.3 x 10^3 (15)</td>
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<tr>
<td>MscL</td>
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<td>5</td>
<td>85.2 x 10^3</td>
<td>85.5 x 10^3 (12)</td>
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<tr>
<td>ELIC</td>
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<td>185.6 x 10^3</td>
<td>185.7 x 10^3 (This work)</td>
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<tr>
<td>AmtB</td>
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<td>126.7 x 10^3 (This work)</td>
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<tr>
<td>NapA</td>
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<td>2</td>
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<tr>
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<tr>
<td>MexB</td>
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<tr>
<td>FocA</td>
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<td>158.6 x 10^3 (56)</td>
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<tr>
<td>AcrB</td>
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<td>342.9 x 10^3</td>
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<tr>
<td>BtuC₂D₂</td>
<td>2 (membrane dimer)</td>
<td>2</td>
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