Insights into Membrane Protein-Lipid Interactions from Free Energy Calculations

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Supplementary methods

CGMD simulations

All systems were solvated with Martini 2.2 waters and Na^+ and Cl^- ions to a neutral charge and 0.15 M. Additional elastic bonds of 1,000 kJ mol^{-1} nm^{-2} were applied between all backbone beads within 1 nm. Electrostatics were described using the reaction field method, with a cut-off of 1.1 nm using a potential shift modifier, and van der Waals interactions were shifted from 0.9-1.2 nm. Bonds were constrained using the LINCS algorithm.

For initial set up, self-assembly simulations were run to allow the membrane lipids to assemble into a bilayer around the protein. These were run over 100 ns in the NPT ensemble, with V-rescale temperature coupling at 323 K [1] and semi-isotropic Berendsen pressure coupling at 1 bar [2]. Following this, the systems are then simulated for an extended period (>3 μs) to permit the formation of specific protein-lipid interactions. These and all subsequent simulations used V-rescale temperature coupling at 323 K [1] and semi-isotropic Parrinello-Rahman pressure coupling [3], unless specified otherwise. All simulations were run using Gromacs 2018 [4,5] unless otherwise specified

Potential of mean force calculations

Steered MD simulations were run to allow construction of the 1D CV. For these, an umbrella pulling force of 1,000 kJ mol^{-1} nm^{-2} was applied along the desired CV (e.g. along the x-axis), with a rate of 0.01 nm ns^{-1}, defined in the mdp file as:

```
pull                = yes
pull_ngroups        = 2
pull_ncoords        = 1
pull_group1_name    = CDL
pull_group2_name    = Protein
pull_coord1_type    = umbrella
pull_coord1_groups  = 1 2
pull_coord1_rate    = 0.00001
pull_coord1_k       = 1000
pull_coord1_start   = yes
pull-nstfout        = 50
pull_coord1_geometry = direction
pull-coord1-vec     = 1 0 0
```

The collective variable (CVs) were defined as follows: Kir2.2, between PIP_2 and residues Arg 186 on the adjacent protein chain and Lys 189 on the furthest protein chain, and RP2, PO3 and GL1 beads of PIP_2; AAC, between the protein centre of mass (COM) and GL0, PO1, GL1, GL2, PO2, GL3 and GL4 beads of CDL (using the mapping from Dahlberg et al [6]); LeuT, between Arg 506 and Asn 509 and GL0, PO1, GL1, GL2, PO2, GL3 and GL4 beads of CDL. Umbrella potentials were applied to each independent simulation, using the code as described above but with pull_coord1_rate set to 0.

To prevent any rotational movement of the proteins in the PMF calculations, *xy* positional restraints of 100 kJ mol^{-1} nm^{-2} were applied to 3-4 backbone beads in each system: Kir2.2, Leu 85 in each subunit; AAC, Ile 78, Leu 127, Ile 185 and Leu 278; LeuT, Ile 48 and Pro 200 in monomer 1 and Val 54 and Phe 203 in monomer 2.

Free energy perturbation

For each FEP calculation, the target lipid was alchemically perturbed into a bulk lipid. For PIP_2 to POPC, this involved a removal of beads PO1, PO2, RP1, RP2 and a conversion of bead RP3 from Martini type SP1 to Q0, including changing the charge from 0 to +1. The charge was also removed from 5 sodium beads to keep the system neutral at each λ state.

For CDL to POPC (mitochondrial) and POPE (bacterial), we initially tried splitting the CDL molecule into two separate residues, and growing in and converting the head group beads accordingly. This is the simplest path to follow, however removal of the bond between the residues proved difficult to remove without incurring very high energies, and the energies we obtained were highly variable. Instead, we chose to fully remove two of the acyl CDL chains (i.e. beads PO2, GL3, GL4, C1C, C2C C3C, C4C, C1D, C2D, D3D, C4D, C5D), and changed bead GL0 from type Nda to either Qd (for POPE) or Q0 (for POPC), switching from a 0 to +1 charge in both cases.

We used a dual topology approach, and FEP calculations were run using the following section added to the production mdp file:

```
free_energy             = yes
init_lambda_state       = 0     ; change for each window
delta_lambda            = 0
calc-lambda-neighbors   = -1    ; Need -1 for MBAR
; init_lambda_state          0   1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19
20
coul_lambdas            = 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.10 0.20 0.30 0.40 0.50
0.60 0.70 0.80 0.90 1.00
vdw_lambdas             = 0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90 1.00 1.00 1.00 1.00 1.00 1.00
1.00 1.00 1.00 1.00 1.00
sc-alpha                = 0.5
sc-coul                 = no
sc-power                = 1
sc-sigma                = 0.3
nstdhdl                 = 100
```

Absolute binding free energy calculations

The details of the restraints used on the $A_{2A}R$-cholesterol ABFE calculations are outlined below.

In the free cholesterol simulations, two restraints were applied to the cholesterol. The first of these (θ) was a flat-bottomed restraint of 1,000 kJ mol^{-1} nm^{-2} keeping the cholesterol molecule within 1 radian of perpendicular to the membrane. This was described in Plumed 2.4, using the following syntax:

```
theta: ZANGLES ATOMS1=4655,4662 MEAN
LOWER_WALLS ARG=theta.mean AT=2.14 KAPPA=1000 EXP=2 OFFSET=0 LABEL=thetuawall
```

Another flat-bottomed restraint of 1,000 kJ mol^{-1} nm^{-2} (z) kept the inner most bead of cholesterol (C1) within 1.4 nm above and 0.4 nm below the centre of the membrane, as below:

```
lig: CENTER ATOMS=4662 NOPBC
leaflet: CENTER ATOMS=1-4654 NOPBC
d1: ZDISTANCES ATOMS1=lig,leaflet MEAN
UPPER_WALLS ARG=d1.mean AT=0 KAPPA=1000 EXP=2 OFFSET=0 LABEL=uwall
LOWER_WALLS ARG=d1.mean AT=-1 KAPPA=1000 EXP=2 OFFSET=0 LABEL=lwall
```

Together, the θ and z restraints form the = restraints. These restraints were then transferred to a restraint (o) in the gas phase, where the cholesterol COM was kept within 0.8 nm of its starting point (defined using a fixed point based on the input coordinates), using the following Plumed syntax:

```
ref: FIXEDATOM AT=5.75,3.97,4.97
d1: DISTANCES ATOMS1=lig,ref MEAN
UPPER_WALLS ARG=d1.mean, AT=0.8 KAPPA=100 EXP=2 LABEL=comwall
```

In the gas phase and receptor-bound simulations, the cholesterol was kept within a 0.2 nm RMSD of a reference position using a flat-bottomed restraint of 100 kJ mol^{-1} nm^{-2} (^). In the gas phase simulation, the reference used was simply the starting coordinates for first and last bead, and were imposed using Gromacs with the following section added to the mdp file:

```
; define groups
pull_ngroups          = 2
pull_group1_name      = a_1
pull_group2_name      = a_8
; define coord1
pull_ncoords          = 2
pull_coord1_type      = flat-bottom
pull-coord1-init      = 0.2
pull-coord1-groups    = 0 1
pull-coord1-origin    = 5.5330  4.0380  4.3150
pull_coord1_geometry  = distance
pull_coord1_dim       = Y Y Y
pull_coord1_k         = 0
pull_coord1_kB        = 100
pull_coord1_rate      = 0
; define coord2
pull_coord2_type      = flat-bottom
pull-coord2-init      = 0.2
pull-coord2-groups    = 0 2
pull-coord2-origin    = 6.1230  3.8280  5.6620
pull_coord2_geometry  = distance
pull_coord2_dim       = Y Y Y
pull_coord2_k         = 0
pull_coord2_kB        = 100
pull_coord2_rate      = 0
```

These could then be turned on over 6 steps (λ = 0, 0.05, 0.1, 0.2, 0.5 and 1) using restraint FEP:

```
free_energy           = yes
init_lambda_state     = 0     ; change for each window
delta_lambda          = 0
calc-lambda-neighbors = -1
; init_lambda_state     0    1    2    3    4    5
restraint-lambdas     = 0.00 0.05 0.10 0.20 0.50 1.00
```

```
couple-moltype          = CHL2
nstdhdl              = 100
couple-lambda0          = vdw-q
couple-lambda1          = vdw-q
```

In the receptor-bound system, the RMSD reference was to two dummy particles which updated position in relation to the binding site as the receptor diffuses through the membrane, which was defined separately in the mdp file as:

```
; DBC restraints
pull                 = yes
; define groups
pull_ngroups           = 6
pull_group1_name       = RES_1
pull_group2_name       = RES_2
pull_group3_name       = Dum1
pull_group4_name       = Dum2
pull_group5_name       = a_1
pull_group6_name       = a_8
; define coord1
pull_ncoords           = 8
; coord1
pull_coord1_type       = constraint
pull-coord1-groups     = 1 3
pull_coord1_geometry   = distance
pull_coord1_dim        = Y N N
pull_coord1_k          = 100
pull_coord1_rate       = 0
pull-coord1-start      = yes
…
Repeat for x,y and z coords for groups 1-3 and 2-4.
```

where RES_1 and RES_2 are reference positions on the receptor, Dum1 and Dum2 are the dummy particles and a_1 and a_8 are the first and last beads of the cholesterol molecule. Note that the dummy particles have the same initial coordinates as the a_1 and a_8 beads. As the receptor diffuses in the membrane, the dummy particles move with it without introducing additional energy into the system. The RMSD restraints can then be established between the cholesterol particles and the dummy particles, using the following mdp setting:

```
; coord7
pull_coord8_type       = flat-bottom
pull-coord8-init       = 0.2
pull-coord8-groups     = 3 5
pull_coord8_geometry   = distance
pull_coord8_dim        = Y Y Y
pull_coord8_k          = 100
pull_coord8_rate       = 0
; repeat for groups 4-6
```

Restraint FEPs were then computed as described above.

$\Delta\Delta G_{bind}$ values were calculated using the thermodynamic cycle in Supporting Figure 3, and Supporting Equation 2, adapted from Salari et al [7], and outlined below, with the values used in this study in brackets. Term 1 can be calculated as the product of number of potential ligands (1) and the ratio of available

volume to the ligand in the free and bound simulations (1.01). Term 3 can be calculated from the values used for the θ, z and o restraints (see Supporting Figure 3 and Supporting Equation 3). Term 2 is the FEP of the cholesterol molecule from the bulk membrane, fitted for the mole fraction of cholesterol present in the membrane (see Salari et al [7]). Term 4 is the FEP of applying the ^ restraints in the gas phase. Term 5 is the inverse of FEP of decoupling the cholesterol from the bound state of the receptor.

WTMetaD

To prevent any rotational movement of the proteins and increase the resolution of the 2D FES, 1,000 kJ mol^{-1} nm^{-2} *xyz* positional restraints were used on a set of backbone residue beads: Kir2.2, Thr 142 in each subunit; AAC, Ala 75 and Ala 273; LeuT, Ala 22, Ala 105 and Ala 407 each monomer; $A_{2A}R$, Ala 15, Ala 134 and Ala 243.

The CVs were defined along the *xy* axes of the protein's geometric centre to the ROH bead of cholesterol, GL0 bead of CDL and the head group of PIP_2 (RP1, RP2, RP3, PO1, PO2, PO3). The restraints were imposed using Plumed 2.4, using the following syntax from the LeuT WTMetaD simulations:

```
# atom selections
ligand: CENTER ATOMS=2305 NOPBC
protein: CENTER ATOMS=1-2304 NOPBC
protein1: CENTER ATOMS=1-1152 NOPBC
protein2: CENTER ATOMS=1153-2304 NOPBC

# CVs
d1: DISTANCE ATOMS=protein,ligand COMPONENTS
d2: DISTANCES GROUPA=protein1,protein2 GROUPB=ligand LOWEST LOWMEM

# restraints
UWALL: UPPER_WALLS ARG=d2.lowest AT=4.5 KAPPA=1000 EXP=5 OFFSET=0

#WTMetaD
METAD ARG=d1.x,d1.y SIGMA=0.01,0.01 HEIGHT=1 PACE=50 BIASFACTOR=8 TEMP=310
GRID_MIN=-9,-9 GRID_MAX=9,9 WALKERS_N=20 WALKER_ID=0 WALKERS_DIR=../LEUT_hills/
WALKERS_RSTRIDE=100 LABEL=metad
```

To prevent the Cholesterol molecule flip-flopping, a set of additional restraints were applied in addition to the upper walls described above, using the following syntax:

```
# atom selections
lipid: CENTER ATOMS=675-5289 NOPBC

# CVs
d3: DISTANCE ATOMS=lipid,lig COMPONENTS
angle: ZANGLES ATOMS1=lig,12696 MEAN

# restraints
LWALL: LOWER_WALLS ARG=d3.z AT=1 KAPPA=1000 EXP=3 OFFSET=0
UWALLAN: UPPER_WALLS ARG=angle.mean AT=1 KAPPA=1000 EXP=3 OFFSET=0
```

To compare the 2D landscape, with the 1D PMFs, we converted the WTMetaD FES to a 1D CV binned to 0.05 Å slices limited to ±0.5 or ±1 nm perpendicular to the CV, within each slice the energy minima was recorded. Each bin of the 1D FES was averaged via bootstrapping with replacement (10,000 iterations) with error reported as the bootstrapped standard deviation. The final reported free energy was recovered from the 1D landscape, with the error reported being the sum of the bulk and energy minima standard deviation. (e.g. Supporting Figure 4).

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