Supporting Information

Specific Lipid Binding of Membrane Proteins in Detergent Micelles Characterized by NMR and Molecular Dynamics

Linlin Zhao §*, Shuqing Wang ‡*, Changqing Run §, Bo OuYang §, James J. Chou §, †

§ State Key Laboratory of Molecular Biology, National Center for Protein Science Shanghai, Shanghai Institute of Biochemistry and Cell Biology, Shanghai Science Research Center, Chinese Academy of Sciences, Shanghai 200031, China.
‡ School of Pharmacy, Tianjin Medical University, Tianjin 300070, China.
† Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA.

Supplementary Methods
Protein expression, purification and reconstitution
Molecular dynamics simulations
Supplementary References

Figure S1. Purification and NMR characterization of the yAAC3 NMR sample

Figure S2. Complete residue-specific chemical shift perturbation of yAAC3 induced by cardiolipin and estimation of $K_D$

Figure S3. Close-up views of the yAAC3 regions that showed NOEs to the cardiolipin head groups

Figure S4. Time trajectory of the molecular dynamics simulation of cardiolipin binding to yAAC3 in POPC bilayer

Figure S5. Close-up view of the middle transmembrane region of yAAC3 for showing residues in this region that showed NOEs with the terminal methyl groups of the cardiolipin acyl chains
Supplementary Methods

Protein expression, purification and reconstitution

A gene encoding residues 1–307 of the yeast ADP/ATP carrier 3 (yAAC3) with an N-terminal 6His tag was synthesized by GenScript and was subsequently cloned into the pET21a expression vector. The plasmid containing the yAAC3 insertion was transformed into the E. coli BL21(DE3) expression strain. The cells were grown at 37 °C in M9 minimal medium to optical density (O.D.) of ~0.8. After induction with 0.5 mM IPTG, the protein was expressed for 4 h at 37 °C. yAAC3 was expressed in inclusion bodies. The cells were lysed in lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 30 mM lysozyme and 1 mM β-mercaptoethanol (BME). After centrifugation at 20,000g for 30 min, the pellet containing the inclusion body was collected and solubilized with solubilization buffer containing 1.67% (w/v) N-lauroylsarcosine (sarkosyl), 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM BME and 0.05% polyethylene glycol 3500 (PEG). After solubilization at 4 °C for 16 h, the solution was centrifuged at 20,000g for 30 min to remove the insoluble debris. The supernatant containing the solubilized yAAC3 was rapidly diluted three-fold with refolding buffer containing 6 mM dodecylphosphocholine (DPC), 20 mM HEPES, pH 7.4, 150 mM NaCl and 20 mM imidazole and was then passed through Ni-NTA resin (Thermo Scientific) pre-equilibrated with the refolding buffer. The protein was eluted in elution buffer containing 3 mM DPC, 20 mM HEPES, pH 7.4, 150 mM NaCl and 200 mM imidazole. The yAAC3 eluate was dialyzed against ion exchange buffer containing 20 mM HEPES, pH 7.4, and 30 mM NaCl for 4 h, followed by purification using a Hi-trap Q HP column (GE Life Sciences). Finally, the misfolded yAAC3 was removed by passing the sample through a Superdex 200 size-exclusion column (GE Life Sciences) in FPLC buffer containing 30 mM MES, pH 6.0, 20 mM NaCl, 5 mM BME and 3 mM DPC. The homogenous yAAC3 fractions were pooled and concentrated to produce the final NMR sample containing 0.8 mM yAAC3, 120 mM DPC, 5 mM BME, 30 mM MES, pH 6.0, and 20 mM NaCl.

Molecular dynamics simulations

The molecular dynamics (MD) simulations were carried out using Desmond 4.5 package and OPLS-AA 2005 force field in a neutral POPC (1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine) bilayer with appropriate number of counter ions to balance the net charge of the system solvated in 0.15 M NaCl. The yAAC3 position and orientation in lipid bilayer were defined using the Orientations of Proteins in Membranes (OPM) database. The protein, three cardiolipins (CLs) and POPC bilayer were embedded in a periodic orthorhombic box (~14 × 14 × 14 Å³) containing the explicit simple point charge (SPC) water molecules, which ensured that the entire surface of the yAAC3-CL complex was covered by the lipid bilayer and water molecules. Nose-Hoover temperature coupling and Martina-Tobias-Klein method with isotropic scaling were used to control the simulation temperature (310 K) and atmospheric pressure (1 atm). The particle-mesh Ewald method (PME) was used to calculate long-range electrostatic interactions with grid spacing of 0.8 Å. A RESPA integrator was used and the real-space part electrostatic and van der Waals interactions were cut off at 9 Å.
The simulation consisted of two MD stages. The first stage was a restrained MD simulation performed using the default ‘membrane relax’ protocol provided in Desmond, which slowly equilibrated the system without deviating too much from the initial protein and ligand coordinates. The initial coordinates of yAAC3 and the three CL head groups were downloaded from protein data bank (PDB ID: 4C9Q). Since the CL acyl chains are missing in the crystal structure, the acyl chains were built from the head groups such that their positions are consistent with the NOE data in Fig. 2. In the restrained simulation, the protein backbone and the CL head group atoms were restrained with a force constant of 100 kJ/mol Å\(^2\) and the side chains of the protein and lipids were allowed to move freely. Equilibrium was reached after 10 ns as indicated by trajectory lines in Fig. S4a. After equilibration, the second stage, which was unrestrained MD, was performed by running 40 ns of NPT (amount of substance (N), pressure (P) and temperature (T) were conserved) production simulation without any restriction. The configurations and interval energy were recorded every 2 ps.

Figure S1. Characterization of the yAAC3 NMR sample.  (a) Gel-filtration profile of the purified full length yAAC3 from Superdex 200 10/60 Hiload column (GE Health) in 30 mM MES, pH 6.0, 5 mM BME, 3 mM DPC and 20 mM NaCl.  (b) SDS-PAGE analysis of the gel-filtration peak in (a) showing the high purity of the yAAC3 sample.  (c) The $^1$H-$^{15}$N TROSY-HSQC spectrum of 0.5 mM U-$[^2]$H, $^{15}$N, $^{13}$C] yAAC3 sample in the presence of 75 mM DPC recorded at 30 °C and 600 MHz $^1$H frequency.
Figure S2. Chemical shift perturbation of yAAC3 induced by cardiolipin (CL) addition.

(a) The residues with assigned and analyzable peaks in the 3D TROSY-HNCO spectra
during cardiolipin titration (indicated by white spheres).

(b) Examples of resonance-specific chemical shift changes caused by CL titration. The peaks are from 2D $^1$H-$^{15}$N TROSY-HSQC spectra recorded with 0.1 mM U-[$^2$H, $^{15}$N, $^{13}$C] yAAC3 solubilized in 50 mM DPC at 30 °C and 600 MHz $^1$H frequency. The spectra were recorded in the presence of 0 mM (red), 0.1 mM (orange), 0.2 mM (green), 0.3 mM (blue), and 0.4 mM (purple) CLs.

(c) Residue-specific chemical shift changes at 3:1 [CL]:[yAAC3] ratio measured using the 3D TROSY HNCO spectrum. Only resolved peaks were measured and displayed. The chemical shift change ($\Delta \delta_{\text{comb}}$) were calculated using the formula:

$$
\Delta \delta_{\text{comb}} = \sqrt{\left(\omega_H \Delta \delta_H\right)^2 + \left(\omega_C \Delta \delta_C\right)^2 + \left(\omega_N \Delta \delta_N\right)^2},
$$

where $\Delta \delta_H$, $\Delta \delta_C$, and $\Delta \delta_N$ are chemical shift changes (in ppm) in the $^1$H, $^{13}$C and $^{15}$N dimensions, respectively, and $\omega_H = 1.00$, $\omega_C = 0.35$ and $\omega_N = 0.15$ are normalization factors.

(d) Estimation of the apparent $K_D$ for examples in Fig. 1a using binding curve simulations. Binding affinity simulation curves for 0.1 mM AAC titrated with substrates with affinities of 20, 50, 100, and 200 $\mu$M are colored in red, orange, green, and blue, respectively. The following equation was used for calculating the substrate bound fraction of AAC

$$
\frac{[PS]}{[P_{\text{total}}]} = \frac{([P_{\text{total}}] + [S_{\text{total}}] + Kd) \pm \sqrt{([P_{\text{total}}] + [S_{\text{total}}] + Kd)^2 - 4[P_{\text{total}}][S_{\text{total}}]}}{2[P_{\text{total}}]}. 
$$

$P_{\text{total}}$ represent the total amount of AAC used. $S_{\text{total}}$ represents amount of substrate titrated. PS represents the complex. The x-axis indicates the total substrate concentration used for titration. The y-axis indicates the theoretical percentage of bound fraction. A 0.1 mM AAC sample was titrated with 0, 0.1, 0.2, 0.3, and 0.4 mM CL. The corresponding bound fractions as measured from chemical shifts for residue A91, A148, and E268 were plotted as blue dots, which suggest apparent $K_D \sim 100, 100, \text{and } 20 \mu$M, respectively.
Figure S3. Close-up views of the yAAC3 regions that showed NOEs to the cardiolipin (CL) head groups. The orientation of each region is the same as those in Fig. 2b. The gray spheres indicate protein residues that showed no lipid NOEs. The spheres with colors represent protein residues that showed NOEs to the head group of CL (blue), acyl chain (orange), the terminus of acyl chain (red). Residues that showed CL head group NOEs are labeled by their residue numbers. The CL head group from the crystal structure (PDB ID: 4C9Q) is shown as purple sticks.
Figure S4. Time trajectory of the molecular dynamics (MD) simulation of cardiolipin (CL) binding to yAAC3 in POPC bilayer. (a) The RMSD value of heavy atoms between the sampled conformations and the starting conformation of yAAC3 and CLs during the 40 ns MD production simulation. The whole system reached the equilibrium state after 10 ns of simulation. (b) The monitored distances (during the simulation in (b)) between the yAAC3 backbone nitrogen atoms of residues indicated by red balls in Fig. 2b and the carbon atoms of the terminal methyl groups of CL acyl chains. The time trajectories indicate that the conformations of CLs relative to yAAC3 were stable during the MD simulation. The monitored distances between carbon and nitrogen atoms were within the $^{1}$H-$^{1}$H distances of NOE detection (3-5 Å) plus 2 Å.
**Figure S5.** Close-up view of the middle transmembrane region of yAAC3 for showing residues in this region that showed NOEs with the terminal methyl groups of the cardiolipin (CL) acyl chains. Residues showing the methyl NOE are indicated by red spheres and those showing CL head group NOEs are indicated by blue spheres. The three bound CLs from molecular dynamics simulation are distinguished by different colors, with the CL in the region being examined shown in yellow and others shown in purple. The red spheres are all close to an acyl chain terminus, providing an explanation for the unusual methyl NOEs observed around the middle of the transmembrane helices.