Osmotically-Induced Tension and the Binding of N-BAR Protein to Lipid Vesicles

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Supplementary Information

In this supplementary document, we provide additional detail on (I) the protein purification, (II) the sedimentation assay, and (III) our estimate of the change in size of the LUVs under osmotic stress.

I. Protein purification: Source and residues used, vector used, purification, characterization by SDS-PAGE, concentration assay

Plasmid pGEX-4T-2/D_Am_Bar that expresses amphiphysin N-BAR (Residues 1-357) from *Drosophila melanogaster* was obtained from the MacMahon laboratory at the Medical Research Council (MRC) Laboratory of Molecular Biology, Cambridge, UK. The plasmid expresses the protein corresponding to the N-terminal α-helix and the BAR domain of amphiphysin, which are together referred to as N-BAR^{S1}, and fused with a GST tag at the N-terminus. N-BAR region that expresses only the residues 1-256 was amplified by PCR (Reagents obtained from Agilent Technologies, CA) and cloned into pETite vector system using Expresso^{R} T7 cloning & Expression System (Lucigen Corporation, WI). The resulting plasmid (pAK811) expresses residues 1-256 with a C-terminal His-tag and with Kanamycin resistance. Cloning was confirmed by sequencing (Genewiz, South Plainfield, NJ). The constructed plasmid was transformed into Hi-Control^{TM} BL21 DE3 *E-coli* cells by heat shock. Transformed colonies were randomly selected and grown at 30 ^{o}C until the OD_{600} reached 0.7 and half of the culture was induced with IPTG (1mM) for expression analysis. Induced and un-induced cultures were grown for additional 1-2 h at 30 ^{o}C and cells were harvested by centrifugation at 13,000 rpm for 15 min in a bench-top micro centrifuge (Eppendorf). The cell pellet was resuspended in water and treated with SDSPAGE sample loading buffer. The resuspended pellets were boiled for 2-5 min and the expressed N-BAR protein band at 30 kDa was confirmed by SDSPAGE (10%). In order to facilitate the attachment of a maleimide dye only at the Cys82 position, the remaining Cys position at residue 66 was mutated to Alanine by site directed mutagenesis (Quick Change^{R} Site Directed Mutagenesis Kit, Agilent Technologies (previously Stratagene), CA). The mutation was confirmed by sequencing (Genewiz, South Plainfield, NJ).

All protein used in the binding-assay experiments was unlabeled. For the large-scale protein expression, bacteria were grown at 30 °C until OD_{600} was between 0.7-1.2, then induced with IPTG (1mM) and growth was continued for an additional 4 h at 30 °C. Cells were harvested by centrifugation at 4 °C for 30 min at 3500 rpm (Beckman Coulter Allegra^{TM} 6R bench top centrifuge) and resuspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.5). The cell

pellet was frozen and stored at -80 °C until purification. Cells were thawed, treated with lysozyme (1 mg/5 mL) and 1mM PMSF and gently shaken for 15 min at room temperature. The cells were lysed by sonication for a total of 15 min in 5-min bursts interspersed with icing. The lysate was centrifuged at 4 °C for one hour at 11,000 rpm (Sorvall^{R} RC-5B Refrigerated Superspeed Centrifuge). The supernatant was collected and ultra-centrifuged at 4 °C for one hour at 28,000 rpm (Beckman Optima^{TM} LE-80K Ultracentrifuge) and the resultant supernatant was saved for purification. His-tagged N-BAR was purified via a Ni-Chelating HisTrap^{TM} FF affinity column (GE Healthcare Life Sciences, Piscataway, NJ, cat. no. 17-5255-01). The column was equilibrated with buffer A, washed with buffer B (50mM NaH_2PO_4, 300 mM NaCl,10 mM Imidazole, pH 7.5), and fractions were eluted with buffer C (50mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 7.5). Purity of the protein was verified by the presence of a single band at 30 kDa in SDS-PAGE (10%). Pure fractions were combined and dialyzed in buffer A with several buffer exchanges to minimize the Imidazole concentration (less than 1mM). Protein concentration was determined with a Modified Lowry Protein Assay Kit and a bovine serum albumin (BSA) standard (Thermo Scientific, Rockford, IL, cat no. 23240). Before use in binding experiments, N-BAR in buffer A was diluted with deionized water to achieve a final protein concentration of 4 μM and buffer concentration of 200 mOsm, and gently centrifuged for 10 min at 10,000 rpm to remove large protein aggregates.

II. Sedimentation assay

Sedimentation assays were used to quantify the amount of N-BAR bound to the vesicles, following a procedure previously described.[S2] A fixed volume (25.7 μL) of protein solution was incubated between 5 and 10 minutes with a known quantity of LUVs in salt or glucose buffers of known osmolarity and a final protein concentration of 1 μM. Sedimentation of 75 μL samples was accomplished by centrifuging at 160,000 g for 30 min at 25°C (Beckman, Optima^{TM} TLX Ultracentrifuge). After centrifugation a 10 μL sample of the supernatant was taken and the rest was immediately removed with a pipette. The sediment (pellet) was then readily resuspended in a volume of buffer equal to that removed and a 10 μL sample was taken. Aliquots (12 μL) of both the supernatant and pellet (mixed 1:1 with SDSPAGE sample loading buffer) were separately run on SDS-PAGE (10% Acrylamide) and quantified by the gel analysis software of ImageJ.[S3] Any lanes that bled into or were contaminated by neighboring lanes of the gel were discarded.

In the data reported here, the protein:lipid ratio was 1:40. Maintaining this ratio is important for interpreting the data from the sedimentation assays. When the ratio was decreased to 1:400, the bound fraction as determined from sedimentation saturated near 100% because of the very small quantity of N-BAR remaining in the supernatant. In this regime, we were unable to detect osmotic-pressure-induced variations in binding.

III. Estimating the change of vesicle size with Δc.

We use a continuum model to find the equilibrium vesicle radius r as a function of the *initial* osmolarity difference, Δc. Over the course of the exposure to Δc (~30 min), solute molecules inside the vesicle are retained but water permeates and the vesicle volume changes.[S4,S5] The free energy change for this process has two terms: one from the osmotic pressure of the solutes which

tends to increase r, and one from the membrane stretching energy which suppresses this increase. The bending energy of a spherical vesicle is independent of size and so can be omitted from this analysis. Following the standard approach, we treat the membrane as an elastic body with area-expansion modulus k. Because our vesicles are composed of 90 mol% DOPC, we use the literature value for pure DOPC membranes, k = 265 mN/m[S6] and assume it applies here. Minimizing the free energy to find the equilibrium state, we find the fractional change in vesicle radius: $(r\text{-}r_0)/r_0 \approx r_0\, k_B T\, \Delta c\, /(4k)$, where r_0 is the initial vesicle radius and k_B is Boltzmann's constant. Corrections to this result are of order $r_0\, k_B T c_{in}/k$, which is small compared to one. For the range of parameters reported here, $(r\text{-}r_0)/r_0$ is on the order of 10^{-2}, so that it is a good approximation to replace r with r_0, and the equilibrium value of $(c_{in} - c_{out})$ with the initial value, Δc.

References

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