Supplementary figure 1

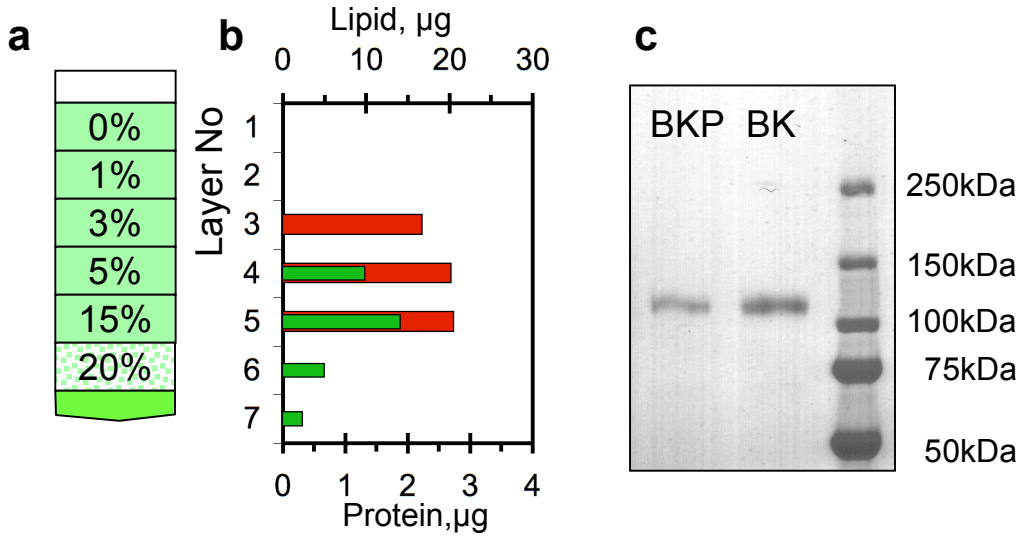

Fig. 1. Separation of BK proteoliposomes. **a**, Density gradient flotation of proteoliposomes; the weight percent Nycodenz is given for each layer. **b**, Distribution of protein (green bars) and phospholipid (red bars) in the gradient layers after flotation (18 hours at 50,000 x g). Empty liposomes are seen to float to layer 3. **c,** Coomassie-stained SDS-PAGE gel showing protein extracted from the BK proteoliposomes (BKP) as compared to the purified, solubilized BK protein before reconstitution.

Supplementary figure 2

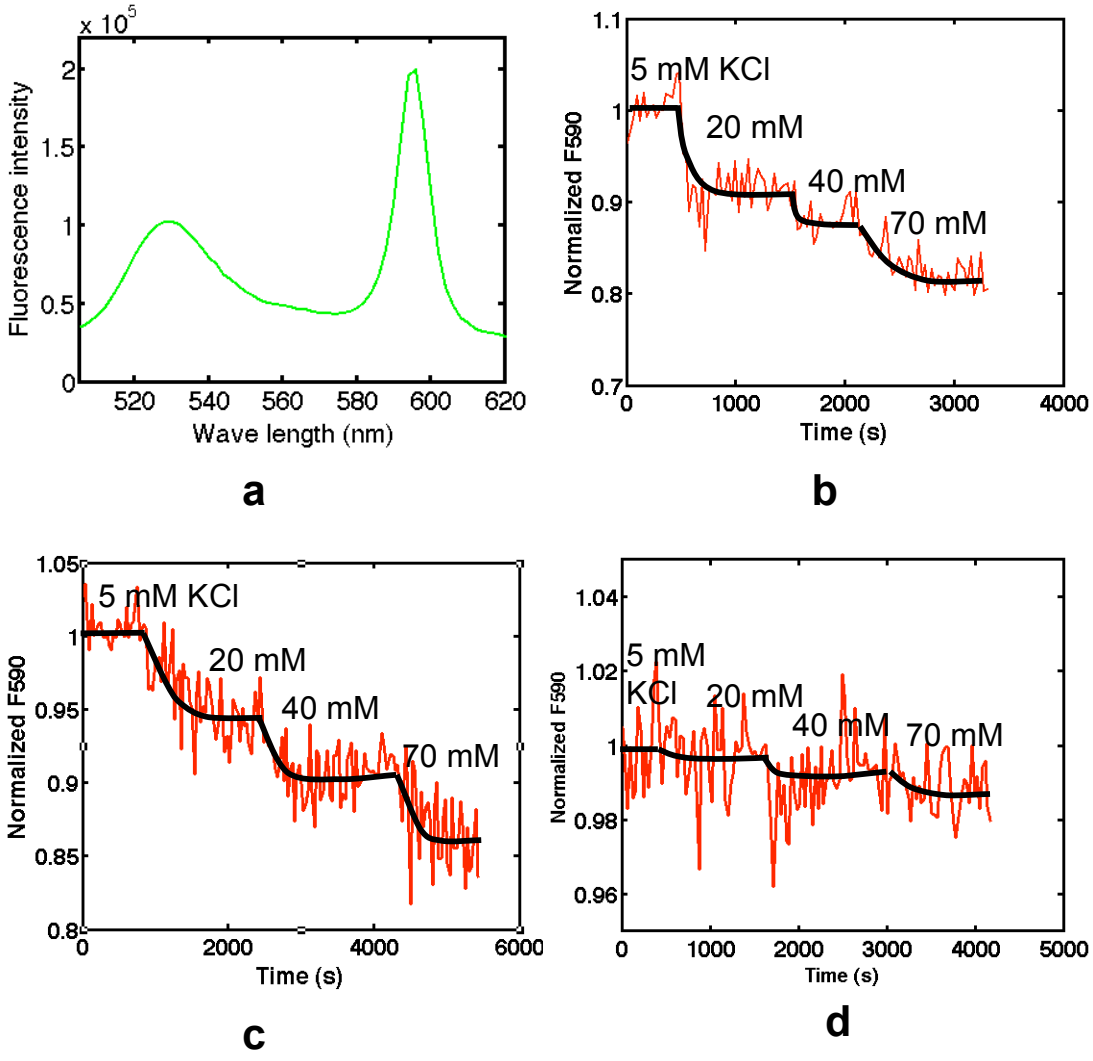

Fig. 2. Potassium flux assay. **a,** Fluorescence spectrum of JC-1 in BK proteoliposomes excited at 480 nm. The peak at 530 nm is emission from JC-1 monomers, and the emission at 590 nm comes from JC-1 aggregates. **b,** JC-1 aggregate fluorescence in the presence of POPC vesicles with valinomycin, a potassium ionophore. **c**, Fluorescence with BK proteoliposomes blocked externally by 30 μM iberiotoxin or, **d**, 30 μM iberiotoxin and 10 mM Ba^{2+}. Liposomes were loaded with 135 mM KCl, 5 mM NaCl, 20 mM Hepes and 1 mM EDTA at pH=7.4. The numbers in the figure are the external KCl concentrations, and the black solid lines depict the trend of the signal.

Supplementary figure 3

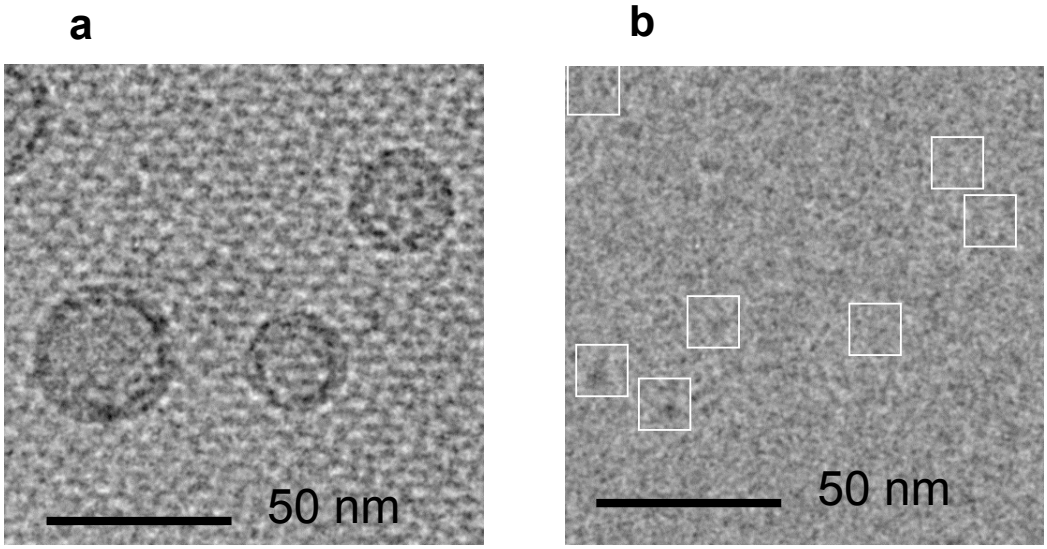

Fig. 3. Cryo-EM specimen and image processing. **a,** One fourth of a micrograph showing BK proteoliposomes tethered to a streptavidin crystal. The image was acquired with 300 keV electrons at a defocus of 2.7 µm. **b**, the same micrograph after removal of crystals and subtraction of liposome membrane model images. BK channel particles, being relatively small objects for single-particle cryo-EM imaging, have low contrast and were selected manually (white boxes).

Supplementary figure 4

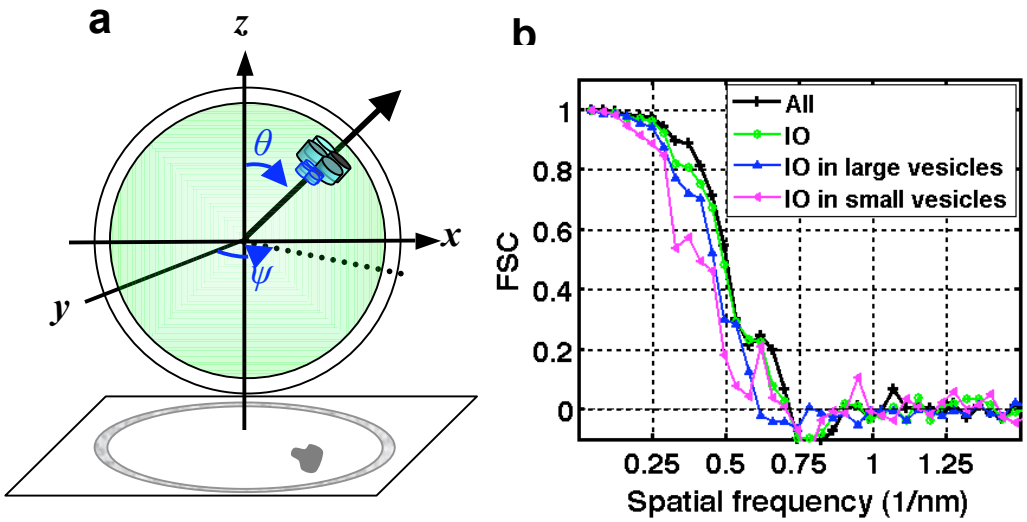

Fig. 4. Structure determination. **a**, The spherical geometry of a liposome allows estimation of the Euler angles θ and ψ from the projection image (bottom), allowing a constraint to be applied in the reconstruction process. **b**, Fourier shell correlation (FSC) computed from pairs of 3D maps reconstructed from all 8400 particle images, from 6800 images of inside-out particles, or 3400 and 3400 images of inside-out particles in large (>24.5 nm) and small (< 24.5 nm) proteoliposomes respectively.

Supplementary figure 5

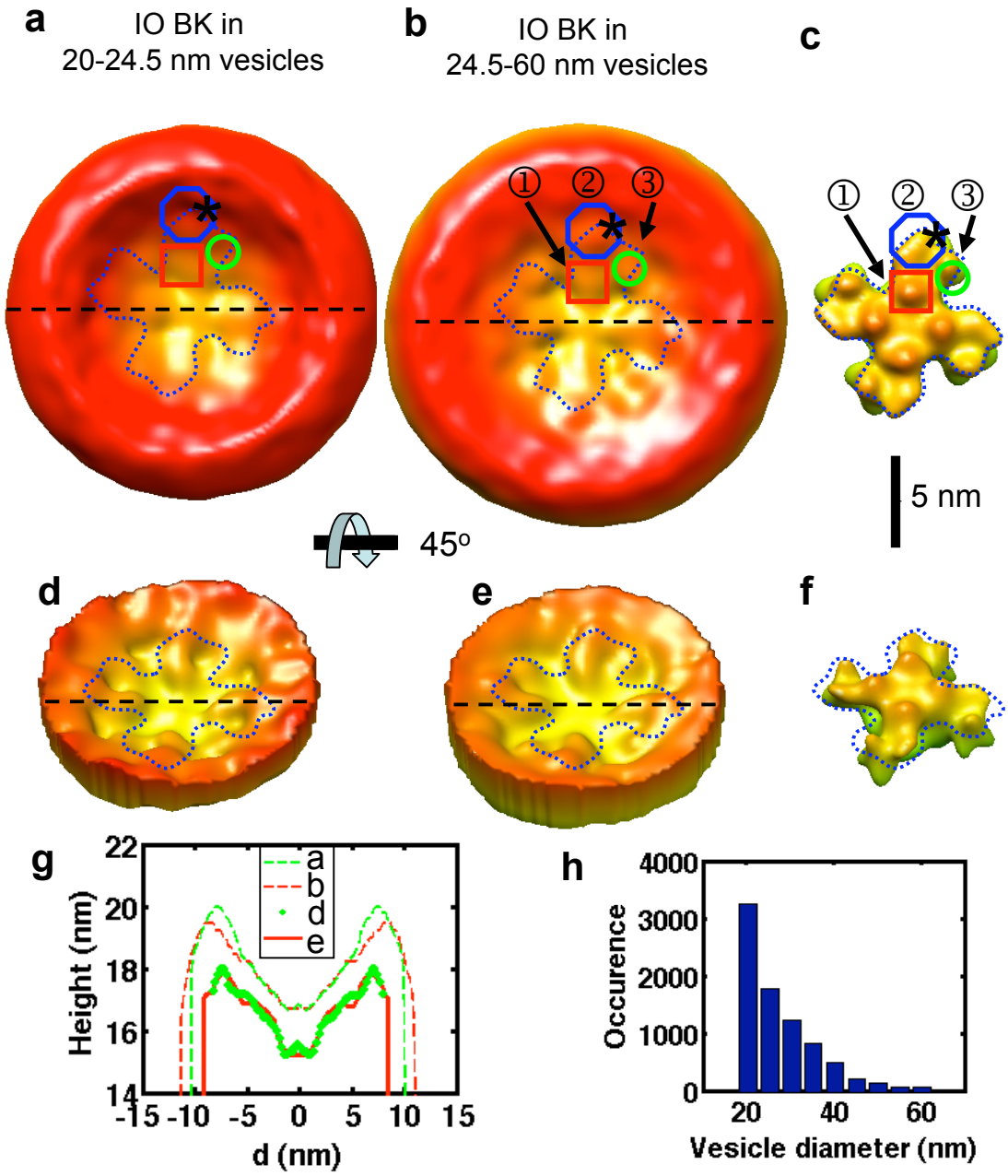

Fig. 5. Effect of vesicle size on the inside-out (IO) BK structure. Extracellular views of BK in small (**a**) and large (**b**) vesicles and the surface of Kv1.2 (**c**). Dotted curves trace the Kv1.2 VSD boundaries. The asterisk labels the expected position of S0. Also shown are oblique views of BK in small (**d**) and large (**e**) vesicles, and the corresponding view of the Kv1.2 transmembrane region (**f**). **g**: *z*-coordinate profiles of BK structures. The profile is computed along the dashed lines in parts a,b,d and e. The latter two used a higher threshold for the map surface and shows deeper structures. Note that out to a radius of 6 nm there is no difference in the height of the channel structure between small and large vesicles. **h**: The size distribution of lipid vesicles.

Supplementary figure 6

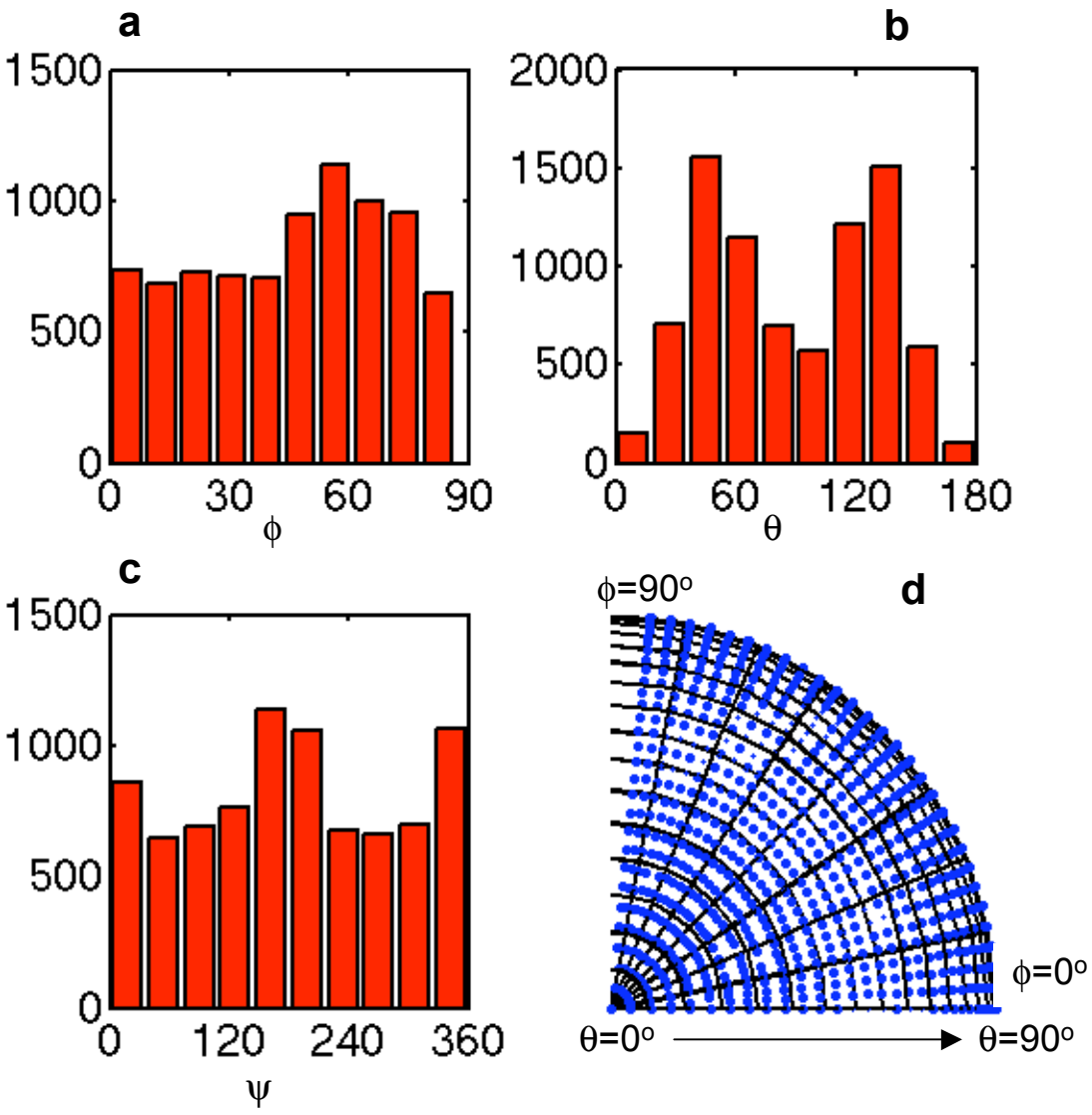

Fig. 6. Distribution of Euler angles for the entire set of 8400 particle images. **a:** Distribution of the particle self-rotation angle ϕ. **b:** Distribution of the angle θ. **c:** Distribution of the in-plane rotation angle ψ. **d**: Distribution of self-rotation angle ϕ and angle θ. Large dots indicate 10-27 particles, and small dots indicate 1-10 particles with the given angles. The distributions show that the images in the dataset cover all orientations of the BK particle.