Detergent-free mass spectrometry of membrane protein complexes

Jonathan T S Hopper1, Yvonne Ting-Chun Yu2, Dianfan Li3,4, Alison Raymond1, Mark Bostock2, Idlir Liko1, Victor Mikhailov1, Arthur Laganowsky1, Justin L P Benesch1, Martin Caffrey3,4, Daniel Nietlispach2 & Carol V Robinson1

We developed a method that allows release of intact membrane protein complexes from amphipols, bicomplexes and nanodiscs in the gas phase for observation by mass spectrometry (MS). Current methods involve release of membrane protein complexes from detergent micelles, which reveals subunit composition and lipid binding. We demonstrated that oligomeric complexes or proteins requiring defined lipid environments are stabilized to a greater extent in the absence of detergent.

MS can provide insight into the subunit stoichiometry and lipid interactions of membrane protein complexes released from detergent micelles after collisional activation1-2. Briefly, micromolar concentrations of membrane proteins are introduced from solutions containing detergent above the critical micelle concentration. Detergent micelles are widely used in structural biology; however, some detergents can promote unfolding, and they do not mimic the lateral forces and curvature of the cellular membrane3 that can be important for maintaining protein structure. These concerns prompted the introduction of nanodiscs and bicomplexes that employ small, discoidal arrangements of phospholipid bilayers4,5 and have demonstrated great potential for X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and electron microscopy6-8. However, it can be difficult to characterize the proteins incorporated into nanodiscs and bicomplexes in terms of stoichiometry and to observe the effects of lipid binding on these assemblies.

We report an MS method that allows membrane proteins and their noncovalent complexes to be ejected from amphipols, nanodiscs and bicomplexes into the gas phase for MS. We demonstrate the approach using three proteins. We expressed Escherichia coli LacY as a GFP fusion to monitor its assembly and purification (LacY-GFP; Supplementary Fig. 1). To test the preservation of oligomeric state, we analyzed E. coli diacylglycerol kinase (DgkA), a trimeric cytoplasmic membrane protein9 whose activity and oligomeric state varies based on its preparation10 and the presence of lipids11. The third protein we studied, sensory rhodopsin II (pSRII) from Natronomonas pharaonis, is a seven-transmembrane (7-TM) receptor of negative phototaxis12,13; this protein represents a ubiquitous yet notoriously unstable class of membrane protein.

To compare our approach with the established detergent-based method, we recorded mass spectra of DgkA, LacY-GFP and pSRII in detergent micelles, formed in solutions containing 0.02% n-dodecyl-β-D-maltopyranoside (DDM) or 0.2% n-dodecyl-β-D-maltoside (DM) in 200 mM ammonium acetate buffer. We observed peaks that correspond to charge-state distributions of the respective proteins, devoid of detergent (Fig. 1a and Supplementary Figs. 2-6). DgkA, pSRII and LacY-GFP were released from micelles possessing average charge states of 7+, 7+ and 8+ and 9+, respectively, indicative of compact structures. Unfolded proteins, with larger surface areas and/or more exposed basic residues, result in a wider charge-state distribution centered around higher charge. For DgkA, we observed the monomeric protein to be dominant, with a smaller population corresponding to dimers. However, the expected stoichiometry of DgkA is trimeric, indicating that detergent micelles are inadequate for preserving this complex in the gas phase.

To explore a detergent-free strategy, we obtained spectra of DgkA incorporated into amphipols (amphiphilic polymers, with alternating hydrophilic and hydrophobic side chains)14. At identical protein concentrations and collision energies (CEs) (defined here as the acceleration voltage and not corrected for charge state) as those used for detergent-solubilized protein, we observed the detachment of amphipols from the DgkA assembly. However, we observed mainly monomeric protein together with small populations of dimers and trimers (Fig. 1b and Supplementary Fig. 7). We concluded that although amphipols can be used for MS of membrane proteins, they cannot promote the detection of the native trimeric form of DgkA in the gas phase.

To explore the use of bicomplexes to deliver complexes for MS, we incorporated DgkA into 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)-1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC)-c7 bicomplexes and confirmed formation of the bicomplex-DgkA assembly by NMR spectroscopy (Supplementary Fig. 8). Mass spectra acquired on an instrument modified to access higher CEs revealed intense peaks (Fig. 1c); the m/z differences resulting from successive lipid adducts allowed us to assign the dominant peak (~14,000 m/z) to the DgkA trimer (3+), in line with the crystal structure9. The relatively low charge states observed for the protein (2+ to 4+ compared to 7+ for monomeric DgkA from micelles and amphipols) suggest that lipids in the bicomplex shield the complex from acquiring charge during electrospray ionization.

1Department of Chemistry, Physical and Theoretical Chemistry Laboratory, University of Oxford, Oxford, UK. 2Department of Biochemistry, University of Cambridge, Cambridge, UK. 3School of Medicine Trinity College Dublin, Dublin, Ireland. 4School of Biochemistry and Immunology, Trinity College Dublin, Dublin, Ireland. Correspondence should be addressed to C.V.R. (carol.robinson@chem.ox.ac.uk). RECEIVED 22 MAY; ACCEPTED 29 AUGUST; PUBLISHED ONLINE 13 OCTOBER 2013; DOI:10.1038/NMETH.2691
This implies that the protein maintains a compact native-like state in the bilayer before the application of collisional activation.

We next investigated the ability of bicelles composed of different ratios of short and long chain lipids to deliver a 7-TM helical protein for MS. We observed a charge series consistent with that of pSRII (3+ to 2+) and up to four lipids (Fig. 1d). Increasing ratios of long:short chain lipids (q), from 0.1 to > 1.5, allowed the detection of dimeric pSRII, which implied that larger bicelles stabilize protein interactions, in line with other studies in which 7-TM proteins had been observed to form oligomers in membrane-like environments. Our observation of the higher-order oligomeric states of DgkA and pSRII demonstrate that bicelles enable subunit and lipid interactions to be maintained in the gas phase.

As an alternative to using bicelles, we next assembled membrane scaffold protein 1E3D1 (MSP1E3D1) nanodiscs (following established protocols), to solubilize the target protein. MSP1E3D1 nanodiscs also provide a lipid bilayer environment, in this case DMPC. The hydrophobic perimeter of a nanodisc is stabilized by two copies of a long α-helical membrane scaffold protein (MSP).

Measurements of dynamic light scattering revealed homogeneous size distributions (Supplementary Fig. 9), and transmission electron microscopy (TEM) showed uniformly sized disc structures, some in face-to-face stacked arrangements, of the correct geometry (Fig. 2c). We initially recorded mass spectra for ‘empty’ nanodiscs that comprised only lipids and MSPs. Activation and desolvation of nanodiscs was accompanied by some lipid dissociation (Supplementary Fig. 10). At a CE of 200 V, spacing between the lipid peaks was used to assign charge states (6+ to 8+). Based on this assignment, nanodiscs raised to this CE still retained an average of 119 ± 3 (calculated within 95% confidence limits; s.e. × 1.96) DMPC lipids across three charge states (Fig. 2a). Additional increases in CE enabled the complete dissociation of lipids and released the MSP dimers (2+ to 4+) from nanodiscs (Fig. 2b).

LacY-GFP allowed us to monitor its incorporation into membrane scaffold protein 1E3D1 (MSP1E3D1) nanodiscs through size exclusion chromatography and affinity purification (Supplementary Fig. 1). We observed a series of charge states for LacY-GFP (3+ to 5+, Supplementary Fig. 11) from nanodiscs (compare to 14+ to 21+ from DDM micelles, Supplementary Fig. 5), implying that in nanodiscs LacY is folded and undergoes extensive lipid binding. To establish whether subunit interactions in complexes could survive the conditions necessary for release, we reconstituted DgkA into nanodiscs containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; Fig. 2c and Supplementary Fig. 12). The initial reconstitution

Figure 1 | Comparison of mass spectra of DgkA and pSRII from micelles, amphipols and bicelles. (a-c) Mass spectra of DgkA (60 μM) in DDM micelles formed in a 0.02% DDM solution (a). DgkA (200 μM) reconstituted into Aβ-35 amphipol (b) and DgkA ejected from a lipid bicelle (DMPC and DHPC, q = 0.27) (c). Charge states and oligomerization state are indicated above the peaks. (d) Mass spectra of pSRII in bicelles of increasing size indicated by the q values. The top spectrum is beyond the limits of solution NMR spectroscopy; hence the q value can only be estimated. Insets, schematics of pSRII solubilized in bicelles of varying bilayer size.

Figure 2 | Mass spectra of empty nanodiscs and those containing DgkA and pSRII. (a) Mass spectrum of empty DMPC nanodiscs at 200 V. Inset, illustration of an empty nanodisc, with dissociation of some lipids. (b) Mass spectra of empty nanodiscs at CEs of 360–400. Schematics label the peaks corresponding to free, intact MSP dimers, with no lipids remaining. (c,d) Mass spectra of DgkA liberated from nanodiscs (c) and pSRII liberated from nanodiscs with MSP (d), and charge states assigned to the monomers and oligomers indicated by schematics.Insets, electron micrographs, confirming the formation of nanodiscs. Scale bars, 30 nm.
solution contained a total of 77 μM DgkA, but <50% was incorporated into isolated nanodiscs (Supplementary Fig. 12). Mass spectra (CE of 400 V) clearly showed the presence of well-defined trimers as well as monomers and dimers of DgkA.

Given that spectra of proteins from nanodiscs or bicelles require higher CEs and yield considerably lower charge states than analogous spectra from detergent micelles, these characteristics could be exploited to determine whether target proteins have been incorporated into nanodiscs. For DgkA-containing nanodiscs subjected to a CE of 100 V, we observed no charge states for the protein, only for lipid clusters (Supplementary Fig. 13). But when we added DgkA incorporated in a DM micelle to the suspension of nanodiscs under the same MS conditions, we readily observed monomeric DgkA with high charge states. This control experiment confirmed that DgkA was released from micelles but retained in nanodiscs (at a CE of 100 V) and also established the absence of detergent-solubilized DgkA in our nanodisc preparations.

To investigate the broad applicability of nanodiscs for MS analysis of membrane protein complexes, we examined other lipids and proteins. We prepared nanodiscs containing pSRII and DMPC lipids, and confirmed formation of nanodisc structures by TEM (Fig. 2d). Using similar activation conditions to those applied to nanodiscs containing DgkA and LacY-GFP, we observed lipid clusters consisting of DMPC lipids with charge state peaks readily discerned for pSRII and the MSPs. We observed very low charge states for pSRII (2+ to 3+) similar to those observed for DgkA and LacY-GFP. The presence of MSPs and lipid clusters in the pSRII spectrum, which are of higher intensity than that of DgkA, may imply that the liberation of pSRII requires more extensive disruption of the nanodisc structure.

The low charge states observed for proteins ejected from nanodiscs or bicelles, compared with those from micelles, minimize the potential for coulomb-induced unfolding in the gas phase. Both nanodiscs and bicelles maintained the native trimeric stoichiometry of DgkA, though a larger population of trimeric assemblies was preserved after dissociation from bicelles compared with nanodiscs (Figs. 1c and 2c). Larger bicelles, presumably with greater lateral forces that effectively ‘compress’ the protein subunits along the plane of lipid bilayer, could maintain dimeric forms of pSRII. Although it is not clear whether this dimeric form is physiologically relevant, formation of pSRII dimers in lipid bilayers is enhanced compared with dissociation constant (K_D) values of pSRII dimers in detergent micelles17.

By comparing four different vehicles for introducing three membrane proteins into the mass spectrometer, we showed that although micelles and amphipols are compatible with electrospray studies, in line with previous observations18, the dominant charge states for DgkA suggest that the native oligomeric states may not be preserved. Stepwise dissociation of nanodiscs in the gas phase, in which first lipids and subsequently MSPs are released, as well as the lipid clusters as reported previously19, highlight the ability to deliver gas-phase protein complexes from lipidic environments. Although gas-phase dissociation of micelles occurs more readily than that of nanodiscs and bicelles, the lipidic microenvironment is important for maintaining subunit interactions within DgkA complexes. Both nanodiscs and bicelles have the ability to preserve protein-lipid interactions, although the range of lipids that can be explored is limited for bicelles. The potential for examining subunit and lipid interactions in the mass spectrometer, in well-defined environments akin to the membrane, is a tantalizing prospect that we believe will have an impact on downstream structural biology approaches.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.T.S.H. and C.V.R. designed the project, J.T.S.H. and A.R. performed all MS experiments and molecular biology excluding those detailed hereafter. Y.T.-C.Y., M.B. and D.N. provided pSRII protein and carried out bicelle NMR spectroscopy experiments. D.L. and M.C. provided DgkA protein, I.L. and V.M. performed EM experiments. J.L.P.B. was involved in the experimental design. A.L. provided molecular biology advice and assistance. J.T.S.H. and C.V.R. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Membrane protein expression and purification. DgkA and pSRII were expressed as reported previously20,21. A LaC-Y-GFP plasmid was prepared as described elsewhere for other bacterial membrane proteins22. Briefly, the plasmid was transformed into E. coli BL21 (DE3) Gold cells (Agilent) and smeared onto LB agar plates supplemented with ampicillin. After overnight incubation at 37 °C, colonies were used to inoculate LB medium (4 x 50 ml) supplemented with 100 µg/ml ampicillin and incubated overnight at 37 °C with agitation. Twelve 2-l conical flasks, each with 1 l of LB, were inoculated with 10 ml of an overnight culture. Cells were induced with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) once an OD600 of 0.6–0.8 was reached. Expression was allowed to proceed at 37 °C for ~3 h before cells were harvested by centrifugation (5,000 g for 10 min) at 4 °C. Pellets were stored at −80 °C. Pellets were thawed and resuspended in buffer (50 mM Tris pH 8 and 300 mM sodium chloride). Cells were lysed with several passes through a microfluidizer (M-110PS, Microfluidics), and cell debris removed by centrifugation (20,000g for 30 min). Membranes were collected by centrifugation (100,000g for 2 h). Membranes were then resuspended in buffer (150 mM NaCl, 50 mM Tris, 5 mM β-mercaptoethanol and 20% glycerol at pH 7.4). LaC-Y was extracted from membranes by adding DDM to the resuspension to a final concentration of 2%, and the tube was rotated at 4 °C overnight. The following day, the sample was centrifuged (20,000g for 30 min), and the soluble fraction was isolated and loaded to a preequilibrated (300 mM NaCl, 20 mM imidazole, 0.025% DDM, 50 mM Tris at pH 8.0) HiTrap column. Sample was eluted using an imidazole gradient to a final imidazole concentration of 500 mM. Peak fractions were pooled and injected onto a Superdex 200 column equilibrated in solution containing 50 mM Tris, pH 7.4, 150 mM sodium chloride, 10% glycerol and 0.025% DDM.

Membrane scaffold protein (MSP1D1 and MSP1E3D1) expression and purification. Plasmids were purchased from Addgene (plasmid 20061), made available courtesy of S. Sligar. Plasmid was transformed into BL21 Gold (DE3) cells, which were plated onto LB agar medium supplemented with 30 µg/ml kanamycin. After overnight incubation, colonies were used to inoculate 50 ml of Luria Broth (LB) medium containing kanamycin and incubated overnight at 37 °C and 230 r.p.m. agitation. 9 ml of the overnight cultures were used to inoculate 1 l of LB, and cells were induced with 1 mM IPTG at an absorbance of 0.6 (600 nm) and allowed to express protein at 37 °C for 3 h. Cells were harvested by centrifugation at 5,000g for 10 min, and the pellet was frozen at ~80 °C.

Lysis buffer was prepared containing NaCl (300 mM), imidazole (20 mM), Tris (50 mM), Triton X (1%), pH 8.0 and EDTA-free protease inhibitor tablet (Roche). 50 ml of lysis buffer was used to resuspend cells harvested from 2 l of culture. Cell suspension was lysed by sonication (5 min total; 3-s pulse at 60% amplitude with 5 s rest times). Cell debris was removed by centrifugation (20,000g for 30 min) and solubilized material was loaded to a 5 ml Ni-NTA column equilibrated with buffer containing 300 mM NaCl, 20 mM imidazole and 50 mM Tris at pH 8.0. Protein was eluted using a gradient ramp up to 500 mM imidazole. Eluted material was added to TEV protease and loaded into 10-kDa molecular weight cutoff (MWCO) dialysis cassettes. The cassette was gently stirred overnight in dialysis buffer (4 l) consisting of Tris (20 mM), imidazole (20 mM), NaCl (150 mM) and β-mercaptoethanol (5 mM) at pH 8.0. Reverse immobilized metal ion affinity chromatography was performed on the dialyzed sample to remove TEV protease and cleaved His6 tags. Expression and purification was confirmed using MS (Supplementary Fig. 14).

Reconstitution of membrane proteins into Amphipol A8-35. Stock solution of the A8-35 (Affymetrix) was prepared at 150 mg/ml in 200 mM ammonium acetate at pH 8.0. DMSO-solubilized DgkA was diluted in the A8-35 stock to give a final protein concentration of 20–200 µM and a 1:4 weight:weight ratio of protein:amphipol. After a 30-min incubation, bio-beads (40 mg) were added and the solution was stored overnight. Samples were washed using Vivaspin (10 kDa MWCO; Millipore) columns to remove free amphipol. In a separate experiment DgkA was refolded into amphipol, as described18, to assess whether this procedure would promote the formation of the expected trimeric stoichiometry.

Reconstitution of membrane proteins into nanodiscs. Reconstitution procedures for nanodisc samples have been described previously and a protocol is available at http://sligarlab.life.uiuc.edu/MS. The sensitivity and low sample requirements of MS allowed reconstitution procedures to be conducted on a smaller scale. Typically, 100-µl reconstitution mixtures were prepared. Briefly, lipid stocks (POPC and DMPC) were prepared in chloroform at 100 mM. The required amount was then transferred to 2 ml glass vial, and the chloroform was evaporated using a gentle stream of nitrogen to leave a thin film of lipids on the container walls. Vials were placed in a vacuum concentrator overnight to remove the residual solvent. Lipids were resuspended in MSP buffer (0.1 M NaCl and 20 mM Tris, pH 7.4) supplemented with sodium cholate (100 mM) to give a final lipid concentration of 50 mM, and the samples were sonicated at room temperature (RT) for 15 min. The appropriate amount of target protein, purified MSP and supplement of detergent (required to maintain the detergent concentration above the critical micelle concentration (cmc) in the reconstitution mixture) were added. Ratios were determined by performing SEC to judge disc homogeneity (Supplementary Figs. 1 and 12). Mixtures were incubated for 1 h at RT for DMPC discs and on ice for POPC discs. Bio-beads were then added and allowed to incubate overnight. Samples were removed from the Bio-beads using gel-loading tips and loaded to approximately 400 µl of Ni resin (Qiagen) pre-equilibrated in MSP buffer (Supplementary Fig. 1A). For larger quantities, 1 ml HiTrap columns (Fisher) were used. As target proteins contained His6 tags, only material incorporating the target protein was retained by the column. This was illustrated using the GFP fusion construct of LaC-Y (Supplementary Fig. 1).

Enriched target protein was eluted by adding MSP buffer containing 100 mM imidazole (typically 200 µl was added), and samples were further purified using size-exclusion chromatography (Superdex 200/75 column; Supplementary Figs. 1 and 12). Fractions corresponding to correctly formed nanodiscs were pooled and concentrated using Vivaspin columns (Millipore) with 100 kDa MWCO. Concentrated samples were washed with MSP buffer to remove the high concentration of imidazole for storage. Aliquots were desalted before MS analysis into 200 mM ammonium acetate using biospin-6 columns (Bio-Rad).
Preparation of DMPC and DHPC bicelles. Bicelles were prepared using a published protocol\textsuperscript{23} above the phase transition temperature of the lipid used; 23 °C for DMPC used here. Briefly, the appropriate amount of DMPC lipid was weighed and dissolved in chloroform. The correct volume was then aliquoted into a glass vial and slowly dried while it rotated in a stream of nitrogen to produce a thin lipid film on the walls of the vial. The vial was then placed into a vacuum desiccator for at least 1 h to remove residual solvent. The DMPC lipid was then rehydrated in phosphate buffer (50 mM NaCl, 6 mM Na\textsubscript{2}HPO\textsubscript{4} and 44 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 6.0) to a concentration of 20 mg/ml. Vortex mixing was used to redissolve the lipid, and samples were sonicated at high power in a water bath for 15 min. The resulting lipid vesicles were incubated with 0.51% (wt/vol) of octylglucoside for a further 15 min. Purified protein, solubilized in detergent, was added to the lipid solution at a molar ratio of 1:130 protein: DMPC and incubated for 1 h. Bio-beads SM-2 Absorbsents (Bio-Rad or Amberlite XAD-2, SUPELCO) were added stepwise, to avoid protein aggregation from rapid detergent removal. Typically 30 mg were added per milligram of detergent and rotated for 2 h. A second aliquot of beads was added and incubated overnight followed by a final quantity the following day with a 2-h incubation. The resulting proteoliposomes were collected by ultracentrifugation at 50,000g (Beckman optima, MLA130 rotor) for 1 h. The supernatant was discarded before resuspending the pellet in buffer supplemented with DHPC, to initiate bicelle formation at RT, so that the final DMPC:DHPC molar ratio was 1:3 (we assumed 90% recovery of the DMPC lipid). Final lipid ratios of the bicelles were confirmed by the lipid terminal methyl peaks in \textsuperscript{1}H NMR spectra.

Transmission electron microscopy of nanodiscs. TEM images of nanodiscs were obtained using the negative stain method with uranyl acetate. Nanodiscs prepared for MS were diluted 50- to 100-fold in MSP buffer. Carbon-coated copper TEM grids (Electron Microscopy Sciences) were pretreated in a glow-discharge chamber in oxygen (15 s). A 10-µl droplet of the sample solution was pipetted onto the grid. A time period of 30–60 s was allowed for binding nanodiscs to the carbon surface before excess solution was removed by blotting. Nanodiscs were stained by pipetting 7–10 µl droplets of 2% uranyl acetate solution on the grids and followed by immediate blotting. TEM analysis of the samples was carried out on the same or following day at a Tecnai T12 transmission electron microscope (80 kV acceleration voltage, 46,000–67,000 magnification, defocusing = −1 µm) using an FEI Eagle 4k × 4k charge-coupled device (CCD) camera.

Nuclear magnetic resonance spectroscopy of bicelle samples. One-dimensional \textsuperscript{1}H NMR spectroscopy experiments were recorded at 308 K on a Bruker DRX500 spectrometer equipped with a 5 mm TXI/xyz-gradient RT probe. Spectra were processed with the XWIN-NMR software package, and q values for bicelles determined by integrating the methyl-signals from DHPC and DMPC signals.

Diffusion rates. The properties of the isotropic bicelle-reconstituted membrane proteins were determined by performing a bipolar pulse pair longitudinal Eddy current delay (BBP-LED) NMR experiment\textsuperscript{24}. The self-diffusion coefficient $D_s$ was calculated according to

$$I(g_2) = I(g_1)\exp(-\gamma^2\delta^2(g_2^2 - g_1^2)D_s(\Delta - \delta/3 - \tau/2))$$

where $I(g_1)$ and $I(g_2)$ are the peak intensities at different gradient strengths $g_1$ and $g_2$ ($g_1 = 3.5$ G/cm and $g_2 = 31.1$ G/cm), $\gamma$ is the proton gyromagnetic ratio ($\gamma = 26.752$ rad s$^{-1}$ T$^{-1}$), $\delta$ is the gradient length ($\delta = 2$ ms), $\Delta$ is the diffusion time ($\Delta = 0.6$ s) and $\tau$ is the delay between bipolar encoding and decoding gradients.

To obtain the viscosity $\eta$ of different solutions, the water diffusion coefficient $D_{w,293}$ was measured by NMR spectroscopy at 293 K and adjusted using the formula

$$\eta = (D_{w,293}/D)(T/293)\eta_{w,293}$$

where $\eta_{w,293}$ is the viscosity of water at 293 K. $T$ is the temperature in Kelvin.

The Stoke-Einstein formula was used to calculate the hydrodynamic radius $R_h$ for a qualitative comparison of the bicelle solutions

$$D_s = k_BT/6\pi\eta R_h$$

where $k_B$ is the Boltzmann constant. Although this equation is correct for spherical molecules, for the measured bicelles entities the calculated $R_h$ values are only a qualitative approximation.

Electrospray ionization–mass spectrometry. Aliquots were desalted and buffer-exchanged into 200 mM ammonium acetate solutions using biospin-6 (Bio-Rad) columns supplemented with the appropriate solubilizing agent (see below). Gold-coated nanospray capillaries were prepared in-house, using a procedure previously published\textsuperscript{25}. Typically 2–3 µl of solution were loaded into a capillary and mounted on the static nanospray block of a quadrupole–time-of-flight (qTOF) mass spectrometer modified as described below. The source backing pressure was raised above $7 \times 10^{-3}$ mbar to improve the transmission of high-mass species, and sample cone voltage was maintained between 100–200 V.

Modifications to a Q-ToF2 (Micromass, Waters) were conducted by Mass Spec Service Solutions Ltd. (MSSS) to facilitate the transmission of high-mass molecules and to allow higher energy collisional activation. In addition to modifications required for the transmission of high-mass complexes described previously\textsuperscript{26}, the collision energy range was doubled to 400 V by effectively dropping all voltages after the collision cell by 200 V. The pusher interval (time between pushes) was extended, allowing more high-mass ions to fill the pusher before the subsequent ‘push’.

Preparation of membrane proteins. Unlike previous MS studies on LacY and 7-TM receptors\textsuperscript{27,28}, membrane proteins studied here are electrosprayed from aqueous/buffered solvents using membrane mimics to solubilize the hydrophobic, folded conformations of these molecules. Solutions were prepared as follows.

Detergent and amphipol solutions. For detergent-containing solutions, ammonium acetate used in the desalting step before MS analysis (see above) was supplemented with the appropriate detergent (DM for DAGK and DDM for LacY and pSRII) maintained at 2 × critical micelle concentration (CMC; 0.2% and
0.02%, respectively). Detergents were selected based on previous data. DgkA was shown to be very stable in both DM and DDM. After reconstitution of proteins into amphipols, aliquots were desalted using biospin-6 (Bio-Rad) columns into 200 mM ammonium acetate.

**Bicelle and nanodisc preparations.** Reconstitution of detergent-solubilized protein solutions into bicelles and nanodiscs are described above. The MS analysis of bicelles and nanodiscs was conducted on a Q-TOF employing increased energy regimes, allowing a collision voltage of up to 400 V to be applied to the collision cell, necessary for the observation of naked protein signals, without lipid adducts. Although these analyses provided reasonable ion current at similar capillary voltages, as used for detergent and amphipol solutions, improved spectra were obtained at minimum voltages (usually between 1.0 kV and 1.5 kV).

Spectra presented are smoothed using Masslynx and baseline minimization where required was performed using Massign29.

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