Published in final edited form as:
Nat Methods. 2013 December ; 10(12): . doi:10.1038/nmeth.2691.

New vehicles allow detergent-free mass spectrometry of membrane protein complexes

Jonathan T.S. Hopper[1], **Yvonne Ting-Chun Yu**[2], **Dianfan Li**[3,4], **Alison Raymond**[1], **Mark Bostock**[2], **Idlir Liko**[1], **Victor Mikhailov**[1], **Arthur Laganowsky**[1], **Justin L.P. Benesch**[1], **Martin Caffrey**[3,4], **Daniel Nietlispach**[2], and **Carol V. Robinson**[1,*]

[1]Department of Chemistry, Physical and Theoretical Chemistry Laboratory, University of Oxford, Oxford OX1 3QZ [2]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA [3]School of Medicine Trinity College Dublin, Dublin, Ireland [4]School of Biochemistry and Immunology, Trinity College Dublin, Dublin, Ireland

Abstract

The rapid growth in the study of membrane protein complexes in lipid microenvironments prompts new methods for their characterization. Here we develop mass spectrometry as a method for studying membrane proteins incorporated into amphipols, bicelles and nanodiscs. We compare these detergent-free vehicles with micelles for their ability to preserve the interactions of oligomeric complexes and to stabilize proteins that require defined lipid environments.

Mass spectrometry (MS) can provide insight into the subunit stoichiometry and lipid interactions of membrane protein complexes released from detergent micelles following collisional activation of gas phase micelles [1,2]. Detergent micelles are widely used in structural biology however some detergents can promote unfolding and fail to mimic the lateral forces and curvature of the cellular membrane [3] that can be important for maintaining protein structure. These concerns prompted introduction of nanodiscs and bicelles that employ small, discoidal arrangements of phospholipid bilayers[4,5] and have demonstrated great potential for X-ray crystallography, NMR and EM[6-8]. However it can be difficult to characterise the proteins incorporated into nanodiscs and bicelles in terms of stoichiometry and observing the effects of lipid binding on these assemblies.

Using three proteins we develop an MS method that allows membrane proteins and their non-covalent complexes to be ejected from nanodiscs and bicelles. The Lactose Membrane Transport Protein (lactose permease; LacY) from *Escherichia coli* was expressed as a fusion to green fluorescent protein (GFP) to monitor assembly and purification (Supplementary Fig. 1). To test preservation of oligomeric state we use Diacylglycerol Kinase (DgkA) also from *E. coli*, a trimeric cytoplasmic membrane protein[9] with varying levels of activity and oligomeric state based on preparation[10] and presence of lipids[11]. The third protein, sensory rhodopsin II (pSRII) from *Natronomonas pharaonis*, is a seven-transmembrane (7-TM) receptor of negative phototaxis[12,13]. Since this class of membrane protein is ubiquitous, and yet notoriously unstable, finding new vehicles for their study is important.

*Correspondence should be addressed to C.V.R. (carol.robinson@chem.ox.ac.uk).

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 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

Europe PMC Funders Author Manuscripts

To compare with established methods we recorded mass spectra of DgkA, LacY-GFP and pSRII from DDM micelles, formed in solutions containing 0.02% DDM in 200mM ammonium acetate buffer. Charge states devoid of detergent were observed (Fig. 1A and Supplementary Figs. 2-6). DgkA and LacY have average charge states of 7+ and 18+ (Supplementary Fig. 5), respectively. For DgkA monomeric protein is dominant with a smaller population corresponding to dimers. Since the expected stoichiometry of DgkA is trimeric, spectra were also recorded from DM (0.2%) detergent (Supplementary Figs. 3 & 4), which yielded populations similar to those observed from DDM. To explore a detergent-free strategy, we obtained spectra of DgkA from amphipols (amphipathic polymers, with alternating hydrophilic and hydrophobic side chains)[14]. At identical protein concentrations and acceleration voltages (CE) as those used for detergent solubilized protein, we detached amphipols from the DgkA assembly and observed monomeric protein together with low populations of dimers and trimers (Fig. 1B and Supplementary Fig. 7). We conclude that although detergent and amphipols can be used for MS of membrane proteins, they are unable to promote the detection of the native trimeric form of DgkA in the gas phase.

To explore the use of bicelles to deliver complexes for MS we incorporated DgkA into DMPC- DHPC-c7 bicelles and confirmed their formation by NMR (Supplementary Fig. 8). Mass spectra acquired on an instrument modified to access higher CE, revealed intense peaks (Fig. 1C); the m/z differences due to successive lipid adducts allowed us to assign the dominant peak (~14,000 *m/z*) to the DgkA trimer (3+), in line with the crystal structure[9]. The relatively low charge states observed for the protein (2+ to 4+) (cf 7+ for monomeric DgkA from micelles and amphipols) suggest that lipids in the bicelle shield the complex from acquiring charge during ESI. This implies that the protein maintains a compact native-like state within the bilayer, prior to the application of collisional activation.

We investigated the ability of bicelles, composed of different ratios of short and long chain lipids, to deliver a 7-TM helical protein. Charge series consistent with pSRII (3+ to 2+) and ≥4 lipids were discerned (Fig. 1D). Increasing ratios of long:short chain lipids (q), from 0.1 to above 1.5, led to observation of dimeric pSRII implying that larger bicelles stabilise protein interactions, in line with other studies in which 7-TM proteins form oligomers in membrane-like environments[15]. Interactions with lipids, and 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 a possible alternative to bicelles, we next assembled MSP1D1 nanodiscs, following established protocols[16], which also provide a lipid bilayer, in this case DMPC, to solubilize the target protein. The hydrophobic perimeter is stabilized by two copies of a long α-helical membrane scaffold protein (MSP). Dynamic light scattering (DLS) measurements 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 inset). Mass spectra were recorded for ‘empty’ nanodiscs, comprising only lipids and MSPs. Efficient desolvation of nanodiscs was accompanied by lipid dissociation (Supplementary Fig. 10). At a collision energy (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 collision energy still retain an average of 119 ± 3 DMPC lipids across three charge states (Fig. 2A). Further increases in CE enabled the complete dissociation of lipids and released the MSP dimers (2+ to 4+) from nanodiscs (Fig. 2B).

A GFP fusion of LacY allowed us to monitor its incorporation into MSP1E3D1 nanodiscs through SEC and affinity purification (Supplementary Fig. 1). A series of charge states were observed for LacY (3+ to 5+, Supplementary Fig. 11) from nanodiscs (*cf* 14+ to 21+ from

DDM micelles, Supplementary Fig. 5) implying that within nanodiscs LacY is folded with extensive lipid binding. To establish if subunit interactions within complexes could survive the conditions necessary for release DgkA was reconstituted into nanodiscs containing POPC (Fig. 2C) (Supplementary Fig. 12). Mass spectra (CE = 400 V) clearly show 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 CE and yield considerably lower charge states than analogous spectra from detergent micelles, these characteristics could be exploited to determine whether or not target proteins have been incorporated into nanodiscs. With CE = 100V and DgkA-containing nanodiscs no charge states for protein were observed, only lipid clusters (Supplementary Fig. 13). When DgkA in a DM micelle was added to the suspension of nanodiscs, however, under the same MS conditions, monomeric DgkA with high charge states was readily observed. This control experiment confirms that DgkA is released from micelles but retained within nanodiscs (at a CE = 100 V) and also establishes the absence of detergent-solubilised DgkA in our nanodisc preparations.

To investigate the wider applicability of nanodiscs we examined other lipids and proteins. Nanodiscs with pSRII and DMPC lipids were prepared and formation of nanodiscs was confirmed by TEM (Fig. 2D). Using similar activation conditions to those applied to DgkA and LacY containing-nanodiscs we observed lipid clusters consisting of DMPC lipids with charge state peaks readily discerned for pSRII and the MSPs. Very low charge states were observed for pSRII (2+ to 3+) similar to those observed for DgkA and LacY. Interestingly the presence of MSPs and lipid clusters, which are of higher intensity than for DgkA, may imply that to liberate pSRII requires more extensive disruption of the nanodisc structure.

The low charge states observed for proteins ejected from nanodiscs or bicelles minimize the potential for coulomb-induced structural unfolding in the gas phase. Both nanodiscs and bicelles maintain the native stoichiometry of DgkA, a greater population remaining trimeric assemblies after dissociation from bicelles compared with nanodiscs (Figs. 1C vs. 2C). Interestingly larger bicelles, presumably with greater lateral forces, were able to maintain dimeric forms of pSRII. While it is not clear if this dimeric form is physiologically relevant, formation of pSRII dimers in lipid bilayers is enhanced compared with K_D values of pSRII dimers in detergent micelles[17].

Through comparison of four different vehicles for introducing three membrane proteins into the mass spectrometer we have shown that while micelles and amphipols are compatible with electrospray, in line with previous studies [18], the dominant charge states for monomeric LacY and DgkA suggest that the native oligomeric states may not be preserved. Stepwise dissociation of nanodiscs in the gas phase reported here, in which first lipids and subsequently MSPs are released, as well as the lipid clusters reported previously [19], highlight the ability to deliver gas phase protein complexes from lipidic environments. While gas phase dissociation of micelles occurs more readily than 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 within the mass spectrometer, in well-defined environments akin to the membrane, is however a tantalising prospect that will in turn have impact on downstream structural biology approaches.

Online Methods

Diacylglycerol Kinase (DgkA) Expression/Purification

The *dgkA* gene was synthesised (Genscript, Piscataway, NJ) based on the DNA sequence of *dgkA* in *E. coli* K12 and was cloned into pTrcHisB (Cat. V360-20, Invitrogen, Carlsbad, CA) using NcoI and EcoRI. The amino acid sequence of the expressed, wild-type (WT) protein is the same as that reported for pSD0005[21], where the N-terminal methionine is replaced by a hexa-His tag-containing decapeptide (MGHHHHHHEL) to facilitate purification. DgkA production and purification, primarily from inclusion bodies, was carried out following published procedures with minor modifications. Briefly, WH1061 an *E. coli* strain lacking the *dgkA* gene cells carrying the pTrcHisB-DgkA plasmid and grown in Luria-Bertani broth were induced at an absorbance of 600 nm of 0.6 for 3 h at 37 °C with 1 mM IPTG. Biomass was harvested by centrifugation at 5,000 g for 15 min at 4 °C. The cell pellet from 1 L of culture was suspended in 0.1 L of Lysis Buffer (0.2 mM TCEP, 0.3 M NaCl, 0.2 mg/mL lysozyme, 50 mg/mL DNAase, 10 μM BHT, 1 mM PMSF (toxic), 0.2 mM EDTA, 5 mM MgCl2, 75 mM Tris/HCl pH 7.8) and gently stirred at 25 °C for 30 min. The suspension was cooled on ice and cells were broken using a probe sonicator at 4 °C for 10 min with a 0.3 s on / 0.7 s off duty cycle and a power setting of 30 % (Model HD2200, Probe KE76. Bandelin, Berlin, Germany). The zwitterionic detergent, Empigen BB, was added to the cell lysate at 3 %(w/v) to solublize the protein from inclusion bodies and membrane fragments. All subsequent steps were carried out at 4 °C unless otherwise noted. After 30 min of mixing at 10 rpm on a Stuart SB3 rotator (Bibby Scientific Ltd., Staffordshire, UK), the solublized protein was separated from cell debris and insoluble material by centrifuging the detergent-treated lysate at 9,000 g for 10 min. To the supernatant was added 4 mL Ni-NTA resin pre-equilibrated with 1.5 %(w/v) Empigen BB in Buffer A (0.3 M NaCl, 0.2 mM TCEP, 40 mM HEPES pH 7.5). After 30 min incubation in the SB3 rotator, the resin was placed in a gravity flow column (Cat. 732-1010, Bio-Rad, Hercules, CA). The resin was washed with 50 mL 10 μM BHT, 3 % (w/v) Empigen BB in Buffer A, followed by a 50 mL wash with 40 mM imidazole and 1.5 % (w/v) Empigen BB in Buffer A. The Empigen BB detergent was exchanged to DM by washing the column with 60 mL of 0.25 %(w/v) DM in Buffer B (50 mM LiCl, 0.2 mM TCEP, 20 mM HEPES pH 7.5). Protein was eluted with 0.25 M imidazole/HCl pH 7.5 and 0.5 %(w/v) DM in Buffer C (1 mM TCEP, 50 mM LiCl, 10 mM HEPES pH 7.5) and concentrated to 12 mg/mL using a concentrator (Amicon Ultracel-50 membrane, Cat. UFC905008, Millipore, Billerica, MA). The sample was then subjected to a size exclusion gel filtration on a Superdex 200 16/60 column equilibrated with Gel Filtration Buffer (0.25 %(w/v) DM, 0.1 M NaCl, 1 mM TCEP, 10 mM Tris/HCl pH 8.0) attached to an AKTA FPLC system (GE Healthcare). The protein was eluted at 76.2 mL as a symmetric peak. Peak fractions were pooled (8 mL total) and concentrated to 12 mg/mL using a spin concentrator, as above. The protein was aliquoted into PCR tubes at 10 μL/tube (0.2 μL PCR tube, Cat. 72.737.002, Sarstedt, Numbrecht, Germany), flash-frozen in liquid nitrogen and stored in −80 °C. Protein concentration was determined by measuring absorbance at 280 nm ($\varepsilon_{1\ mg/ml} = 2.1$) in a NanoDrop 1000 spectrometer (Thermo Fisher Scientific Inc., Wilmington, DE). Protein purity was estimated at >95 % based on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The typical yield was 15 mg of purified DgkA per liter of culture.

Sensory Rhodopsin II (pSRII) Expression/Purification

Expression of $pSRII_{1-241}$[22] used the psopII gene from *Natronomonas pharaonis* plus a sequence encoding a C-terminal hexahistidine-tag inserted into the pET-28b(+) vector and transformed into the *E. coli* expression strain BL21 Tuner (DE3). Cells were grown in M9 minimal medium at 37 °C, and expression induced at an OD600 of 1.0 with 1 mM IPTG and 10 μM all-trans-retinal added, and temperature 25 °C. Cells were supplemented with 10 μM

all-trans-retinal every 2 h for 10 h, after which cells were resuspended and lysed using an Emulsiflex (Avestin, Inc.). Crude membranes were collected by ultracentrifugation (100,000g for 90 min at 4 °C) and resuspended in one-fortieth of the original culture volume using buffer S (50 mM MES-NaOH, pH 6.5, 300 mM NaCl, 5 mM imidazole). The detergent n-dodecyl-β-D-maltoside (DDM) was added to a final concentration of 1.5% (w/v), and the mixture was incubated overnight. Insoluble material was removed by ultracentrifugation (100,000 g, 1 h, 4 °C). Solubilized pSRII was purified with Ni-NTA affinity beads (Novagen) in buffer S. The beads were washed with 30 column volumes of the same buffer containing 0.06% (w/v) DDM. The protein was eluted in 50 mM Tris-HCl, pH 7.0, 300 mM NaCl, 0.1% DDM containing 150 mM imidazole, and the solution was exchanged into phosphate buffer N7 (50 mM NaCl, 20 mM sodium phosphate, 0.05% NaN3) by repeated rounds of concentration and dilution with a final volume of 0.5 ml. For the preparation of NMR samples, the protein was mixed with Ni-NTA beads as before and after removal of the flow-through, washed with 50 column volumes of buffer N7 containing 0.6% (w/v) 1,2-diheptanoyl-sn-glycero-3-phosphocholine (c7-DHPC). The protein was eluted in the buffer N6 (50 mM NaCl, 50 mM sodium phosphate, pH 6, 0.05% azide) containing 300 mM imidazole and 0.1% c7-DHPC, and the eluted solution was repeatedly concentrated and diluted in a concentrator (10-kDa cutoff, Vivaspin 20) with 60 ml buffer N6 containing 0.06% (w/v) DHPC to a final volume of 0.45 ml. The protein concentration and purity were controlled by measuring the absorbance at 498 nm using a UV-Vis spectrophotometer (molar extinction coefficient at 498 nm is 48,000 $M^{-1}cm^{-1}$[23,24].

Lactose Permease (LacY) Expression/Purification

A LacY-GFP plasmid was prepared as previously described for other bacterial membrane proteins[25]. The plasmid was transformed into *E. coli* BL21 (DE3) Gold cells (Agilent) and smeared onto LB agar plates supplemented with ampicillin. Following overnight incubation at 37 °C, colonies were used to inoculate LB media (4 × 50 mL) supplemented with ampicillin and incubated overnight at 37 °C with agitation. 12 × 1 L LB media, in 2 L conical flasks, were inoculated with the overnight cultures (3 × 1L media inoculated with 10mL of an overnight culture). Cells were induced with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) once an OD_{600} of between 0.6-0.8 was reached. Expression was allowed to proceed at 37 °C for approximately 3 h before being harvested by centrifugation (5,000 g for 10 minutes) at 4 °C. Pellets were stored at −80 °C. Pellets were thawed and re-suspended in buffer (50mM TRIS pH 8, 300 mM sodium chloride). Cells were lysed with several passes through a microfluidizer (M-110PS, Microfluidics) and cell debris removed by centrifugation (20,000 g for 30 min). Membranes were collected by centrifugation (100,000 g for 2 h). Membranes were then re-suspended in buffer (150 mM NaCl, 50 mM Tris, 5 mM β-mercaptoethanol, 20 % glycerol at pH 7.4). LacY was extracted from membranes by adding DDM to the re-suspension to a final concentration of 2% and the tube was rotated at 4 °C overnight. The following day, the sample was centrifuged (20,000 g for 30 min.) and the soluble fraction was isolated and loaded to a pre-equilibrated (300 mM NaCl, 20 mM Imidazole, 0.025% DDM, 50 mM Tris at pH 8.0) HISTrap 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 (50 mM TRIS pH 7.4, 150 mM sodium chloride, 10% glycerol, 0.025% DDM).

Membrane Scaffold Protein (MSP1D1 and MSP1E3D1) Expression/Purification

Plasmids were purchased from Addgene (plasmid 20061), made available courtesy of S. Sligar. Plasmid was transformed into BL21 Gold (DE3) cells and plated onto LB agar media supplemented with Kanamycin. Following overnight incubation, colonies were used to inoculate 50mL of Luria Broth (LB) media containing Kanamycin and incubated overnight at 37°C and 230rpm agitation. 9mL of the overnight cultures were used to inoculate 1 L of

LB and cells were induced with 1mM IPTG at an absorbance of 0.6 (600 nm) and allowed to express at 37°C for 3 hours. Cells were harvested by centrifugation at 5,000 x g 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 (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.; 3 sec.pulse at 60 % amplitude with 5 sec. rest times). Cell debris was removed by centrifugation (20,000 × g for 30 min.) and solubilised material was loaded to a 5 mL Ni-NTA column equilibrated with Buffer (300 mM NaCl, 20 mM imidazole, 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 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 IMAC was performed on the dialysed sample to remove TEV and cleaved His6-tags. Successful 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. DM solubilized DgkA was diluted in the A8-35 stock to give a final protein concentration of between 20 – 200 μM and a 1:4 weight:weight ratio between protein/amphipol. Following 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 described by [18], 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. 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 a 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 speed vac overnight to remove the residual solvent. Lipids were re-suspended in MSP buffer (0.1M NaCl, 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 RT for 15 min. The appropriate amount of target protein, purified MSP and supplement of detergent (required to maintain the detergent concentration above the 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 HISTrap columns (Fisher, Loughborough) were used. Since target proteins contained His6 tags, only material incorporating the target protein is retained by the column. This is illustrated using the GFP fusion construct of LacY (Supplementary Fig. 1).

Elution of enriched target protein was achieved by adding MSP buffer containing 100 mM imidazole (typically 200 μL 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 prior to MS into 200 mM ammonium acetate using biospin-6 columns (Bio-Rad).

Preparation of DMPC/DHPC Bicelles

Bicelles were prepared using a recently published protocol[26] 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 rotating 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_2HPO_4, 44 mM NaH_2PO_4, pH 6.0) to a concentration of 20 mg/ml. Vortex mixing was used to re-dissolve 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% of octylglucoside for a further 15 min. Purified protein, solubilised 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 Absorbents (Bio-Rad, Hertfordshire or Amberlite XAD-2, SUPELCO) were added stepwise, to avoid protein aggregation from rapid detergent removal. Typically 30 mg were added per mg of detergent and rotated for 2 h. A second aliquot of beads were added and incubated overnight followed by a final quantity the following day with a 2 h incubation time. The resulting proteolipsomes were collected by ultacentrifugation at 50,000 × g (Beckman optima, MLA130 rotor) for 1 h. The supernatant was discarded before re-suspending the pellet in buffer supplemented with DHPC, to initiate bicelles formation at RT, so that the final DMPC:DHPC molar ratio was 1:3 (we assume 90% recovery of the DMPC lipid). Final lipid ratios of the bicelles were confirmed by the lipid terminal methyl peaks in 1H NMR spectra.

Transmission Electron Microscopy (TEM) of Nanodiscs

TEM images of nanodiscs were obtained using the negative stain method with uranyl acetateNanodiscs prepared for MS were diluted 50 to 100 fold in MSP buffer. Carbon-coated copper TEM grids (Electron Microscopy Sciences, Hatfield, PA, USA) were pre-treated 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. Staining of the nanodiscs was achieved 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, 46-67 k magnification, defocusing = −1μm) using FEI Eagle 4k × 4k CCD camera.

Nuclear Magnetic Resonance (NMR) of Bicelle Samples

1D ^{1}H NMR 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[27]. The self-diffusion coefficient D_s was calculated according to:

Europe PMC Funders Author Manuscripts

$$I(g_2) = I(g_1) \exp\left[-\gamma^2 \delta^2 \left(g_2^2 - g_1^2\right) D_s \left(\Delta - {}^{\delta}/_{3} - {}^{\tau}/_{2}\right)\right]$$

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), γ is the proton gyromagnetic ratio (γ = 26.752 rad $s^{-1}T^{-1}$), δ is the gradient length (δ = 2 ms), Δ is the diffusion time (Δ = 0.6 s), and τ is the delay between bipolar encoding and decoding gradients.

In order to obtain the viscosity η of different solutions, the water diffusion coefficient $D_{w,293}$ was measured by NMR at 293 K and adjusted using the formula:

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

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

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

$$D_s = k_B \mathrm{T}/6\pi\eta R_s$$

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

Electrospray Ionization – Mass Spectrometry

Aliquots were desalted/ 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[28]. 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 × 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 high mass modifications described previously[29], 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[30,31], membrane proteins studied here are electrosprayed from aqueous/buffered solvents using membrane mimics to solubilise the hydrophobic, folded conformations of these molecules. Solutions were prepared as follows:

Europe PMC Funders Author Manuscripts

Detergent and Amphipol solutions

For detergent containing solutions, ammonium acetate used in the desalt step prior to 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[21]. Following 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 solubilised 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 and 1.5 kV).

Spectra presented are smoothed using Masslynx and baseline minimisation where required was performed using Massign[20].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to A. Borysik and N. Morgner for assistance acquiring DLS data and assigning mass spectra using *Mas*sign [20]. We are particularly grateful to D. Reilly, responsible for the high collision energy modifications to the mass spectrometer and to C. Sanders for WH1061 an *E. coli* strain lacking the *dgkA* gene. Also thanks to E. Reading and J. Marcoux for discussions and to M. Smikle for help generating figures. We thank A. Siebert for electron microscopy support. The OPIC electron microscopy facility was founded by a Wellcome Trust JIF award (060208/Z/00/Z) and is supported by a WT equipment grant (093305/Z/10/Z). J.T.S.H and C.V.R acknowledge funding from the Medical Research Council and an European Research Council advanced grant (IMPRESS). D.L and M.C acknowledge Science Foundation Ireland (grants 07/IN.1/B1836 and 12/IA/1255) and the National Institutes of Health (grants GM75915, P50GM073210, U54GM094599). M.J.B would like to thank Biotechnology and Biological Sciences Research Council for a studentship. Y.T.C.Y thanks the Cambridge Overseas Trust and the Taiwan Ministry of Education for a studentship. A.L is a Nicholas Kurti Junior Research Fellow of Brasenose College. C.V.R is a Royal Society Professor.

References

1. Barrera NP, et al. Nat. Meth. 2009; 6:585–587.
2. Barrera NP, Zhou M, Robinson CV. Trends Cell Biol. 2013; 23:1–8. [PubMed: 22980035]
3. Botelho AV, et al. Biophys. J. 2006; 91:4464–4477. [PubMed: 17012328]
4. Denisov IG, et al. J. Am. Chem. Soc. 2004; 126:3477–3487. [PubMed: 15025475]
5. Whiles JA, et al. Bioorganic Chem. 2002; 30:431–442.
6. Ujwal R, Bowie JU. Methods. 2011; 55:337–341. [PubMed: 21982781]
7. Yu TY, et al. Biochim. Biophys. Acta-Biomembr. 2012; 1818:1562–1569.
8. Duerr UHN, Gildenberg M, Ramamoorthy A. Chem. Rev. 2012; 112:6054–6074. [PubMed: 22920148]
9. Li D, et al. Nature. 2013; 497:521–524. [PubMed: 23676677]
10. Van Horn WD, Sanders CR. Annu. Rev. Biophys. 2012; 41:81–101. [PubMed: 22224599]
11. Bohnenberger E, Sandermann H. Eur. J. Biochem. 1983; 132:645–650. [PubMed: 6303781]
12. Gordeliy VI, et al. Nature. 2002; 419:484–487. [PubMed: 12368857]

13. Gautier A, et al. Nat. Sruct. Mol. Biol. 2010; 17:768–774.
14. Gorzelle BM, et al. J. Am. Chem. Soc. 2002; 124:11594–11595. [PubMed: 12296714]
15. Huang J, et al. Nat. Struct. Mol. Biol. 2013; 20:419–425. [PubMed: 23435379]
16. Bayburt TH, Sligar SG. FEBS Lett. 2010; 584:1721–1727. [PubMed: 19836392]
17. Kriegsmann J, et al. Biochim. Biophys. Acta. 2009; 1788:522–531. [PubMed: 19094962]
18. Leney AC, et al. Anal. Chem. 2012; 84:9841–9847. [PubMed: 23072351]
19. Marty MT, et al. Anal. Chem. 2012; 84:8957–8960. [PubMed: 23061736]
20. Morgner N, Robinson CV. Anal. Chem. 2012; 84:2939–2948. [PubMed: 22409725]

References

21. Zhou YF, Bowie JU. J. Biol. Chem. 2000; 275:6975–6979. [PubMed: 10702260]
22. Shimono K, et al. FEBSLett. 1997; 420:54–56.
23. Ikeura Y, et al. Photochem. Photobiol. 2003; 77:96–100. [PubMed: 12856889]
24. Mironova OS, et al. FEBS Lett. 2005; 579:3147–3151. [PubMed: 15919078]
25. Laganowsky A, et al. Nat. Protoc. 2013; 8:639–651. [PubMed: 23471109]
26. Morrison EA, Henzler-Wildman KA. Biochim. Biophys. Acta. 2012; 1818:814–820. [PubMed: 22226849]
27. Wu DH, Chen AD, Johnson CS. J. Magn. Reson. 1995; 115:260–264.
28. Hernandez H, Robinson CV. Nat. Protoc. 2007; 2:715–726. [PubMed: 17406634]
29. Sobott F, et al. Anal. Chem. 2002; 74:1402–1407. [PubMed: 11922310]
30. Ho JTC, et al. Anal. Biochem. 2008; 376:13–24. [PubMed: 18294946]
31. Weinglass AB, et al. EMBO J. 2003; 22:1467–1477. [PubMed: 12660154]

Europe PMC Funders Author Manuscripts

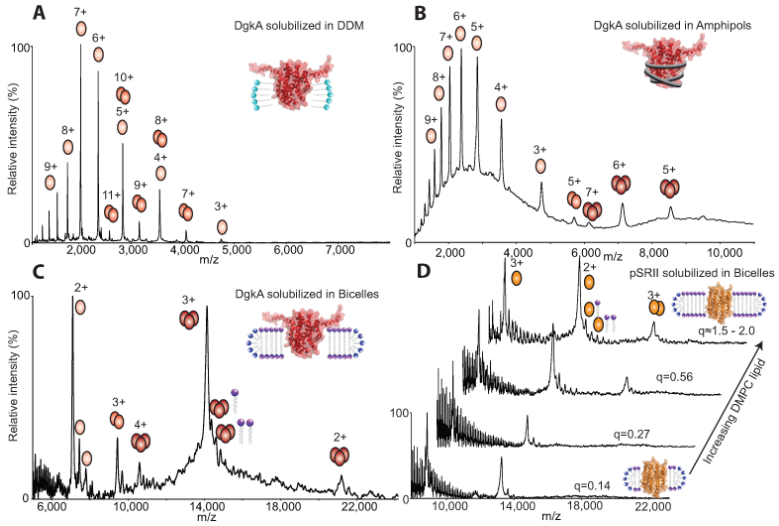

Figure 1.

Comparison of mass spectra of DgkA and pSRII from micelles, amphipols and bicelles. (A) Mass spectrum of DgkA (60 μM) in DDM micelles formed in a 0.02% DDM solution yields primarily monomeric protein. (B) DgkA (200 μM) reconstituted into A8-35 amphipol, showing essentially monomeric protein with small populations of dimer and trimer. (C) DgkA ejected from a lipid bicelle (DMPC and DHPC, q=0.27) showing essentially trimeric DgkA. (D) pSRII in bicelles of increasing size indicated by the q values. The top spectrum is beyond the limits of solution NMR hence the q value can only be estimated. A small population of dimer is present at higher q values.

Europe PMC Funders Author Manuscripts

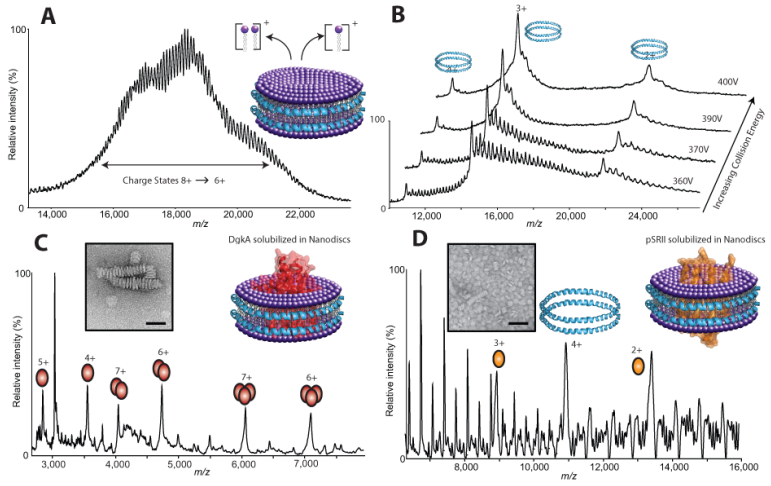

Figure 2.

Mass spectra of empty nanodiscs and those containing DgkA and pSRII. (A) Mass spectrum of empty DMPC nanodiscs at 200 V, resulting in dissociation of some charged lipid clusters (low m/z region, not shown) and residual charge reduced nanodiscs, manifested as a series of overlapping charge states containing varying numbers of lipids. Spacing between the peaks allows the charge states to be discerned (6+ to 8+) and possessing an average of 119 ± 3 DMPC lipids calculated form three charge states. B) Increasing the collision energy to 360 – 400 V results in dissociation of the lipid clusters and loss of the intact MSP dimer. (C) ESI-MS of DgkA liberated from nanodiscs reveals a substantial population of trimer. The initial reconstitution solution contained a total of 77 μM DgkA, however, < 50% is incorporated into isolated nanodiscs (supplementary Fig. 12). (D) Mass spectrum of pSRII liberated from nanodiscs with MSP and charge states assigned to monomeric pSRII. Insets show EM images confirming the formation of nanodiscs (scale bars = 20 mM).

Europe PMC Funders Author Manuscripts