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Lipid bilayer preparations of membrane proteins for oriented and magic-angle spinning solid-state NMR samples

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Abstract

Solid-state NMR spectroscopy has been used successfully for characterizing the structure and dynamics of membrane proteins as well as their interactions with other proteins in lipid bilayers. such an environment is often necessary for achieving native-like structures. sample preparation is the key to this success. Here we present a detailed description of a robust protocol that results in high-quality membrane protein samples for both magic-angle spinning and oriented-sample solid-state NMR. the procedure is demonstrated using two proteins: CrgA (two transmembrane helices) and rv1861 (three transmembrane helices), both from *Mycobacterium tuberculosis*. the success of this procedure relies on two points. First, for samples for both types of NMR experiment, the reconstitution of the protein from a detergent environment to an environment in which it is incorporated into liposomes results in ‘complete’ removal of detergent. second, for the oriented samples, proper dehydration followed by rehydration of the proteoliposomes is essential. By using this protocol, proteoliposome samples for magic-angle spinning NMR and uniformly aligned samples (orientational mosaicity of <1°) for oriented-sample NMR can be obtained within 10 d.

INTRODUTION

The structural characterization of small helical membrane proteins is particularly challenging, as their tertiary structural stability is low because of the hydrophobic amino acid composition and the uniform low dielectric environment of the membrane interstices resulting in only van der Waals interactions and a few weak electrostatic interactions to stabilize the structure[1,2]. Increasingly it is recognized that this class of proteins needs to be characterized in a sample environment that accurately mimics the native membrane environment, properties such as hydrophobic thickness, lateral pressure profile, dielectric

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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gradient, water concentration gradient and lipid order parameter gradient[3]. Forty years ago, Christian Anfinsen recognized that protein structure is the result of interactions within the protein and between the protein and its environment[4]. This is especially important for small helical membrane proteins that have a higher fraction of interactions with their environment than within the transmembrane domain of the protein.

Membrane proteins constitute ~30% of all expressed genes in bacteria, in yeast, and in the human genome, yet less than 2% of the protein structures in the Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do) are integral membrane proteins. Although two-thirds of the putative helical membrane proteins from *M. tuberculosis* have fewer than four transmembrane helices (currently estimated at 769 proteins), this class of proteins is even more severely under-represented in the PDB[5]. Indeed, the vast majority of the helical membrane proteins in the PDB are large oligomeric membrane proteins. β-barrel integral membrane proteins are another class of important membrane proteins. Although the techniques discussed in this protocol may apply for these proteins as well, the protocols have not been tested on β-barrel protein in this report.

The main structural techniques being used today to determine structural models for membrane proteins suffer from poor membrane-like environments. With X-ray crystallography, the high concentration of protein results in marked crystal contacts that can overpower the weak interhelical interactions that stabilize transmembrane domains. Recent efforts to crystallize small helical membrane proteins using a lipidic cubic phase may prove to be effective[6]; although a demonstration with the peptide gramicidin did not yield the native conformation[7], a recent structure of DgkA seems to be native like[8]. For liquid-state NMR, the results have been mixed because of the use of detergent micelles for solubilizing the protein, which poorly mimics the properties of the native membrane[3,8–10].

Consequently, there is interest in a technique that can characterize small helical membrane proteins in a more native-like environment. Much effort has been devoted to the development of solid-state NMR (ssNMR) technology for characterizing these structures with atomic resolution in lipid environments[11–16]. Magic-angle spinning (MAS) spectroscopy of small helical membrane proteins has improved substantially, but the uniform helical structures and the uniform low dielectric environment in the membrane interstices result in a poor dispersion of the resonances for individual amino acid types, especially in the side chains. As MAS structures have been dependent on collecting large numbers of qualitative intra- and interhelical distances, it has been difficult to characterize structures with this approach alone. However, the helical backbone structure can be characterized, as can the helical tilt and rotation angles at high resolution for each helix, by using oriented-state (OS) NMR. OS NMR information greatly limits the tertiary conformational space for packing the helices, which can then be solved using sparse distance restraints from MAS ssNMR spectroscopy[17,18].

Currently, we are working with eight helical membrane proteins in our laboratory. Here we present the step-by-step sample preparation protocols for OS and MAS ssNMR for two small helical membrane proteins from *M. tuberculosis*, H37Rv strain: CrgA (two transmembrane helices) and Rv1861 (three transmembrane helices) (Table 1)[19–21]. In

addition, we provide some data for gramicidin A that we use as a test sample. Once conditions for protein expression and purification have been optimized, the success of the sample preparation protocols is so robust that OS and MAS NMR samples can routinely be achieved in 10 d (Fig. 1).

Experimental design

Membrane protein overexpression

A wide variety of expression strategies for membrane proteins are available[22,23]. We typically express membrane proteins with histidine affinity tags, which when overexpressed at 37 °C are typically observed in both membranes and inclusion bodies[24–30]. Expression at lower temperature often favors insertion in the cellular membrane. Purification and reconstitution of these proteins into a functional state can often be achieved from both inclusion bodies and from membrane extracts. Such overexpression of small helical proteins in *E. coli* typically yields 10–30 mg l^{-1} of protein using defined medium appropriate for isotopic labeling (Box 1, Supplementary Methods, Supplementary Fig. 1)[27,30].

Membrane protein purification

Whether the membrane protein is expressed in inclusion bodies or in cellular membranes can influence the choice of detergents and denaturants to be used[31]. During the cell lysis process, cellular membrane and inclusion body solubilization requires harsh detergents, such as Empigen (*N*,*N*-dimethyl-*N*-dodecylglycine betaine). Although we favor Empigen in most cases, it is important to note that the best detergent is protein specific and there is no general solution to the problem of protein stabilization in detergent solution. If the protein is expressed only in the inclusion bodies, it may be more challenging to refold the protein into a functional state—this is especially true for larger membrane proteins. For refolding proteins from inclusion bodies, urea is often used along with detergent, but other denaturants such as guanidinium chloride can be used and can make a difference for certain proteins. The goals for this step are to solubilize the overexpressed protein, to separate it from the bulk cellular environment and to prepare the protein for nickel affinity–mediated protein purification. Subsequently, the protein is washed on the column to remove impurities, and the harsh detergent is exchanged for one that lacks a net charge or may have a higher CMC (such as octyl glucopyranoside (OG, 10–20 mM), decyl dimethyl glycine (DDGly, 19 mM), dodecyl phosphocholine (DPC, 1.5 mM) or decyl maltopyranoside (DM, 1.8 mM)), which is more amenable for reconstitution. However, the detergent must be selected carefully so that the protein remains soluble and does not aggregate. DPC, DDGly, OG and DM are some of the detergents that have allowed us to achieve high levels of protein purification and effective reconstitution. These detergents are either uncharged or zwitterionic and ideally have short acyl chains (≤12 carbons). Before performing any large-scale protein purification with a particular detergent, a small-scale purification screening step with various detergents having different CMCs is recommended. Once the protein is purified and solubilized in an appropriate detergent, it is ready for lipid reconstitution.

Membrane protein reconstitution

The goal of the reconstitution procedure is to replace the detergent micelle environment with a more native-like liposome environment. We exclusively use detergent-mediated reconstitution methods, although it is important to note that organic solvent–mediated or mechanical reconstitution protocols exist in the literature[32,33]. The reconstitution procedure takes four steps. First, a suspension of liposomes is formed either from an organic solvent–prepared lipid film or from a lipid powder by extensive bath sonication in buffer. Next, the liposomes are solubilized with a detergent that is both easily removed and that keeps the membrane protein soluble. Then the protein is mixed with the lipid and detergent mixture and allowed to equilibrate, forming mixed micelles of membrane protein, detergent and lipid. Finally, the detergent is removed either by dialysis or by reconstitution using methyl β-cyclodextrin (MβCD)[34]. The cyclodextrin-detergent complex disrupts detergent monomer-monomer association and micelle formation[35]. Alternatively, the detergent removal can also be done by using hydrophobic polystyrene resins such as Bio-Beads, Calbiosorb or Amberlite XAD2 during dialysis[36,37]. In the present study, we have used MβCD for reconstituting CrgA because of its high solubility in DPC and strong affinity for the detergent. An overview of this procedure can be seen in Figure 2. For Rv1861, we have used dialysis to remove the DDGly detergent. The modest affinity of the DDGly for liposomes and the high CMC of the detergent permit efficient detergent removal from the proteoliposomes[31]. Others have had success with a detergent-absorbing column[38]. Final proteoliposome samples contain virtually no detergent. If residual detergent is suspected, evaporative light-scattering technology coupled with HPLC[39] can be used to monitor the presence of detergent in the sample. For the samples exemplified here, the concentration of detergent was below the limit of detection and is many orders of magnitude less than the protein concentration.

It is important to mimic the physical properties of the lipid bilayer environment for the membrane protein. Synthetic lipid environments can mimic many of the physical properties of the native membranes. We have used one of three binary mixtures of lipid: DMPC/DMPG (1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol)), POPC/POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-*rac*-glycerol)) and DOPC/DOPE (1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) for reconstitution[14]. These mixtures are all net negatively charged and contain both ‘cylindrical’ (PC head group) and ‘conical’ lipid head groups (PG or PE head group) at a 4:1 molar ratio (Table 2)[40]. Myristoyl lipids have traditionally been used for sample alignment because these bilayers are easier to uniformly orient, but efforts are being made to use more native and native-like lipids with unsaturated acyl chains and with a somewhat larger hydrophobic thickness, such as POPC/POPG and DOPC/DOPE. The experimenter is free to choose lipids that are required for any protein system, but care must be taken to maintain a bilayer environment. Lipid compositions of mainly conical lipids, such as PG, probably will not form well-oriented samples with a high degree of order. The quality of the sample can be assessed using the protocol described here. For MAS spectroscopy, we have used additional environments such as cellular membranes[41], and Hong and colleagues[42] have recently used a complex mixture of sphingomyelin, cholesterol, DPPC

and DPPE to model the Influenza viral coat. K^+ channel functional assays suggest that DOPE/DOPS (1,2-dioleoyl-sn-glycero-3-[phospho-L-serine]) liposomes with a 9:1 (mol/mol) ratio are more appropriate for structural studies and have therefore been used for MAS samples[43]. Here we focus on lipid choices that are optimal for both MAS and OS samples.

A benefit of this protocol is that the proteoliposomes obtained are readily used in other biochemical and biophysical experiments, such as channel conductance measurements[43,44] or enzymatic assays[45], as well as in secondary structure determination by circular dichroism (CD) spectrometry of membrane proteins[46].

Although the spectroscopist desires as much protein as possible in the samples, the molar ratio of protein to lipid is dependent on the protein and on whether an OS or MAS sample is being prepared. For OS samples, a high degree of alignment is aided by using a relatively high mole fraction of lipid (e.g., protein-to-lipid molar ratio, 1:80 or 1:100), and in MAS samples the protein fraction can be increased to 1:30 without increasing the ^{13}C linewidths[17,47]. Currently, we are using ~5–10 mg of membrane protein per sample for proteins of molecular weights ranging from 10 to 20 kDa.

ssNMR sample preparation

OS samples contain several thousand planar hydrated lipid bilayers aligned mechanically between each pair of glass surfaces, such that the bilayer normal and glass slide normal are parallel. A stack of 35–40 glass slides are used for each OS sample in a square-bottomed rectangular glass sample cell[14,48]. Once the liposomes are pelleted, they are resuspended with buffer and bath-sonicated to homogenize the preparation, and then aliquots are spread as uniformly as possible on each glass slide. To achieve optimal alignment of the bilayers, we present two protocol options for the slide-supported samples. In the first protocol, applied to CrgA, the individual glass slides were partially dehydrated at 37 °C and 16% relative humidity (RH) for ~20 min. The glass slides are then stacked, rehydrated and inserted into the sample cell, in which rehydration at ~96% RH and 37 °C is completed before sealing the cell (Fig. 3). In the second protocol used for Rv1861, the glass slides are dehydrated at ~96% RH and 20 °C for ~24 h. Approximately 2 μl of autoclaved water is added to each slide before stacking, and the slides are rehydrated, inserted into the sample cell and further rehydrated before sealing the cell. Final hydration levels for both protocols are ~40–50% by weight of water. The sample is sealed with a plastic plug and a wax seal. The final sample should be clear. Although the protocol does not directly address variations in ionic strength or pH for the proteins discussed here, the methodology used to assess the quality of the samples applies equally well over a wide range of sample conditions. Unless they are needed for specific proteins, divalent cations should be avoided, as they induce bilayer fusion and could interfere with alignment of the sample.

These mechanically aligned samples offer a variety of advantages as well as some disadvantages relative to bicelle preparations that are magnetically aligned membrane protein preparations of lipid and detergent[49]. The mechanically aligned samples display less motional averaging, with order parameters ~1.0, compared with the bicelle preparations in which the spin interactions are typically scaled by an order parameter of 0.8. Order parameters range from zero (no alignment of the proteins with respect to each other) to one

(indicating total alignment of the proteins with respect to each other). Here, alignment means uniform orientation of the time-averaged rotation axis of the protein in the lipid bilayer and, in turn, the lipid bilayer normal is aligned parallel to the magnetic field in OS ssNMR. The detergent in bicelle samples also raises a concern for the interfacial and water-soluble domains of the protein because of the high monomeric concentration of most detergents[50]. The mechanically aligned samples can be observed over a broad range of temperature and pH with a wider variety of lipid preparations than can be accommodated by the bicelle preparations. They also have longer sample stability (up to 1 year) when compared with bicelle samples[49].

For MAS experiments, the reconstitution process mimics the OS preparations, but these preparations are less sensitive to bilayer curvature and require less lipid than OS samples[51]. Proteoliposomes are concentrated into a dense lipid, protein and buffer pellet that is packed into a small rotor for MAS experiments[52]. We describe here an in-house MAS sample transfer protocol to minimize sample loss (Box 2, Fig. 4). Variations in ionic strength and pH from the values used in this protocol are anticipated to have no effect on the samples. The quality of these samples is evaluated as presented herein.

ssNMR spectroscopy

We prepared ^{15}N- and ^{13}C-labeled membrane protein samples for both OS and MAS ssNMR experiments. OS NMR is mainly ^{15}N spectroscopy to avoid the large homonuclear interactions present in uniform ^{13}C-labeled proteins. Polarization inversion spin exchange at the magic angle (PISEMA)- and SAMPI4-class separated local field experiments[16,53] are performed on the OS samples and used to correlate the anisotropic 1H-^{15}N dipolar couplings with ^{15}N chemical shift interactions, resulting in orientational restraints. The resonances form PISA (polar index slant angle) wheel patterns in the separated local field spectra for helical membrane proteins[54,55]. The helical tilt angle (τ) relative to the bilayer normal and the rotation angle (ρ) about the helix, as well as kinks and bends in the transmembrane helices, can be uniquely characterized[56]. Structures have been characterized primarily with these orientational restraints, such as gramicidin A, Vpu, phospholamban, MerF, CXCR1, the M2 conductance domain and M28 from the acetylcholine receptor[14,48,57–59].

Although OS NMR provides orientational restraints, the characterization of multihelix proteins requires distances between the helices to define the helix packing arrangement. MAS spectroscopy provides such distance restraints as relative restraints that fix one site relative to another site in the protein. Inter-residue distances can be obtained from ^{13}C-^{13}C homonuclear recoupling experiments, such as 2D dipolar-assisted rotational resonance (DARR)[60,61]. Because the backbone structure is well defined by the orientational restraints, only sparse distance restraints are needed to define the tertiary or quaternary helical structure in the transmembrane domains. Consequently, it is not necessary to achieve a complete set of resonance assignments to achieve a few distances between each pair of helices. A distance can be measured between any two uniquely assigned resonances. To enhance the search for such sparse restraints, we and various groups have developed labeling strategies such as individual amino acid-specific ^{13}C enrichment, 2-^{13}C, 1,3- ^{13}C glycerol-labeling[62] and reverse-labeling strategies[14]. Recently, the rotational alignment of transmembrane

proteins has been used in MAS experiments to measure dipolar couplings[63–66]. The samples described here are well suited to these experiments and the methods are highly complementary.

By combining OS and MAS ssNMR structural restraints, the limitations of these individual methods for membrane protein structure determination can be overcome. OS restraints can provide high-resolution helical structures and the orientation of individual helices or helical fragments with respect to the bilayer normal. For the packing of the transmembrane helices, sparse, MAS-derived, inter-helical distance restraints are adequate[67]. Thus, with the use of absolute orientational and sparse distance restraints, high-resolution membrane protein structures can be achieved.

Limitations

The main limitations for the protocol are threefold. Obtaining enough isotopically labeled protein from *E. coli* can be an issue for certain proteins. Often other *E. coli* strains or other expression systems, such as yeast or insect cells, can be used to increase the protein yields[68,69]. For some proteins, achieving high protein-to-lipid ratios in liposomes that are desirable is not possible leading to poor signal to noise and increased spectrometer usage. Sometimes, other lipids can be added or used to increase reconstitution efficiency and can enhance signal to noise in the NMR experiments[14,70]. For membrane proteins with three or more transmembrane helices, spectral crowding can be an issue complicating the measurement of the structural restraints. Increased dimensionality of the experiments and well-thought-out labeling strategies can be used to minimize the spectral complexity[15].

MATERIALS

REAGENTS

- ▲ **CRITICAL** We have included the source of our reagents and the brands of instrumentation that we have used. This is not intended to imply that these sources and brands are the only ones that can be used to conduct this protocol.
- pET29b and pET16b vectors (EMD Millipore, cat. nos. 69872-3 and 69662-3, respectively)
- BL21(DE3) RP codon plus competent cells (Agilent Technologies, cat. no. 230250)
- Harvested cell resuspension buffer (buffer T80, see Reagent Setup)
- Cell lysis buffer (see Reagent Setup)
- Wash buffers (see Reagent Setup)
- Elution buffer (see Reagent Setup)
- Empigen BB detergent (*N*,*N*-dimethyl-*N*-dodecylglycine betaine: Empigen, 35% (wt/vol) active substance in water; Sigma-Aldrich, cat. no. 66455-29-6)
- *n*-Dodecylphosphocholine (DPC, >99%; Affymetrix, cat. no. F308S)

- *n*-Decyl-*N*,*N*-dimethylglycine, Anagrade (DDGly, >99%; Affymetrix, cat. no. D352)
- SDS, 20% (wt/vol) (TEKnova, cat. no. S0295)
- Tris (Amresco, cat. no. 77–86-1)
- HEPES (Amresco, cat. no. 7365-45-9)
- 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, >99% pure, supplied as a 1% (wt/vol) solution in chloroform; Avanti polar lipids, cat. no. 850457C) ! **CAUTION** Chloroform solvent is hazardous. Work in a fume hood as appropriate.
- 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-*rac*-glycerol) (POPG, >99%, pure, supplied as a 1% (wt/vol) solution in chloroform; Avanti polar lipids, cat. no. 840457C) ! **CAUTION** Chloroform solvent is hazardous. Work in a fume hood as appropriate.
- 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC, >99%; Avanti polar lipids, cat. no. 850345P)
- 1,2-Dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG, >99%; Avanti polar lipids, cat. no. 840445P)
- Methyl-β-cyclodextrin (MβCD; Sigma-Aldrich, cat. no. 332615)
- Potassium sulfate (K_2SO_4; EMD Chemicals, cat. no. P1595-1)
- Sample sealing wax: for samples running under 25 °C, sticky wax from Mole Hollow Candles; for samples running above 25 °C, bee wax from Hampton research
- Parafilm 'M' laboratory film (Pechiney Plastic Packaging)
- Sodium hydroxide or hydrochloric acid
- Imidazole
- Sodium chloride

EQUIPMENT

- Glass slides: 5.7 mm × 12.0 mm × 0.06–0.08 mm for OS preparations (Marienfeld)
- Rectangular glass cells: 4.3 mm × 6.4 mm × 20 mm inner dimension (New Era Enterprises). Sample caps designed and made in house are used for OS sample sealing (Fig. 3)
- MAS rotors, 3.2-mm (Revolution NMR)
- Dialysis bag (Spectrum Laboratories)
- Erlenmeyer flasks, 2 liters (Pyrex)
- Multitron shaker incubator with temperature control (ATR Biotech)
- French pressure cell press (Thermo Spectronic)

- NanoDrop 2000 spectrophotometer (Thermo Scientific)
- Avanti J-26XP centrifuge, Optima XL-80K ultra centrifuge and Optima TLX ultra centrifuge (Beckman Coulter)
- AKTAXpress FPLC (GE Healthcare)
- His Trap FF nickel column (GE Healthcare, cat. no. 17–5255-01)
- Bath sonicator (Branson Ultrasonics)
- Vortex (Barnstead International)
- pH meter (Fisher Scientific)
- SpeedVac connected to a Heto cooling trap for removing chloroform from the lipid samples (Savant Instruments)
- NMR Instruments used for the examples here include: Magnex 600 MHz magnets with a Bruker Avance II console and an NHMFL900-MHz, 105-mm-bore magnet with a four-channel Bruker Avance 900-MHz console. We have also used NHMFL-engineered low-electric-field probes for OS and MAS spectroscopy[71]

REAGENT SETUP

Detergents—Prepare 10% (wt/vol) DPC and DDGly detergent stocks by dissolving the powdered detergents in autoclaved water; follow this by filter sterilization. These detergent stocks can be stored at −20 °C for 1 year. Purchase ready-made 20% (wt/vol) SDS stock. SDS stock can be stored at room temperature (23 °C) for 3 years.

Buffer stock solutions—Prepare 1 M Tris (pH 8.0) and HEPES (pH 7.5) stock solutions by dissolving the powder; follow this by filter sterilization. Make subsequent dilutions for other uses. Adjust the desired pH by adding sodium hydroxide or hydrochloric acid. These solutions can be stored at room temperature for 1 year, after which the pH value should be checked and adjusted as necessary.

Buffer T80 preparation—Mix 500 mM sodium chloride with 40 mM Tris or 75 mM HEPES (pH 7.5). This buffer can be stored at 4 °C for 1 year, after which the pH value should be checked and adjusted as necessary.

Cell lysis buffer preparation—Mix 20 mM Tris (pH 8.0) with 8 M Urea. Add 1% (vol/vol) Triton X-100 and mix the solution gently to avoid froth formation. This solution can be stored at 4 °C for 1 year.

Washing step 1 buffer preparation—Prepare the buffer by mixing 300 mM sodium chloride, 20 mM Tris (for CrgA) or 75 mM (pH 7.5), HEPES (for Rv1861), 40 mM imidazole and 0.7% (vol/vol) Empigen detergent. This buffer can be stored at 4 °C for 6 months.

Detergent exchange buffer preparation (wash 2)—For CrgA, mix 100 mM sodium chloride, 20 mM Tris (pH 7.5), 60 mM imidazole and 0.2% (wt/vol) DPC detergent to the

final concentration. For Rv1861, mix 20 mM HEPES (pH 7.5), and 1.1% (wt/vol) DDGly detergent. This buffer can be stored at 4 °C for 6 months.

Membrane protein elution buffer preparation—For CrgA, mix 100 mM sodium chloride, 20 mM Tris (pH 7.5), 500 mM imidazole and 0.4% (wt/vol) DPC detergent. This buffer can be stored at 4 °C for 6 months. For Rv1861, mix 300 mM sodium chloride, 20 mM HEPES (pH 7.5), 500 mM imidazole 1.1% (wt/vol) DDGly detergent. This buffer can be stored at 4 °C for 6 months.

Lipid film preparation—All lipid stocks can be stored in −20 °C. Owing to the hygroscopic nature of lipids, the lifetime of the stock depends on the number of times the container is opened. After five to ten openings, discard the lipid stock. Prepare lipid films from the chloroform-dissolved lipids. Remove the bulk chloroform with nitrogen gas and then leave them overnight in a SpeedVac. Dissolve powdered lipids directly into buffer for liposome preparations.

Hydration chamber preparation—Dissolve potassium sulfate in 50 ml of dH_2O to form a saturated solution. Transfer the solution to a glass desiccator (1 liter) and equilibrate jt overnight at either 20 °C or 37 °C to achieve ~96% RH inside the chamber. The saturated solution should be changed once per month.

OS glass slide washing—Wash the new glass slides in benzene:chloroform (4:1 (vol/vol)) mixed solvent. Rinse them thoroughly in autoclaved water, and then dry them. This step is important for obtaining a uniform distribution of liposomes on the glass slides for OS sample preparation. Slides can be stored at room temperature indefinitely.

PROCEDURE

Reconstitution of purified membrane protein into liposomes ● TIMING ~1–4 d

1| The goal is to eliminate detergent from the sample and replace it with lipids. Reconstitute membrane proteins in liposomes by one of two methods: (option A) MβCD-mediated reconstitution (Fig. 2) and (option B) dialysis-mediated reconstitution. The outcome of these two methods is the same. MβCD-mediated reconstitution takes 1 d, whereas the dialysis method takes 3 or more days, depending upon the detergent CMCs, detergent affinity for liposomes and the choice of dialysis membrane pore size. A pore size of one-half the molecular weight of the protein is recommended to minimize lipid loss while maximizing the detergent removal rate. Although the dialysis method is slower, it is more effective for high-CMC detergents (e.g, DDGly), whereas MβCD is useful for detergents with low CMCs (e.g., SDS) that are not easily removed by dialysis.

A. reconstitution using MβcD as used for CrgA ● TIMING ~1 d

i. Pipette lipids appropriate for protein from chloroform stock lipid solutions into a clean 10-ml glass round-bottomed flask. Here we use a 4:1 molar ratio of POPC and POPG for CrgA (Table 2).

ii. Remove bulk chloroform by evaporating the mixture under a low-N_2 gas flow with gentle rotation forming a semitransparent film. Remove the traces of chloroform by vacuum evaporation overnight.

iii. Dissolve the lipid film completely into 3 ml of 5 mM Tris-HCl (pH 8.0) by repeated pipetting up and down.

iv. Add 300 μl of 20% (wt/vol) SDS (0.3% final working concentration), and bath-sonicate the mixture for 10 min at room temperature until it is clear like water (Fig. 2, step 3). Use glass vials rather than plastic tubes during bath sonication for faster results.

! **CAUTION** Gently pipette up and down when adding detergent because lipid and detergent solutions froth easily and can result in the loss of sample.

v. Add 10–12 mg of purified protein to the lipid-detergent mixture. Mix the proteoliposome solution for 30 min at room temperature with gentle rocking.

vi. Add MβCD (1:3 molar ratio, detergent: MβCD) and 20 ml of 5 mM Tris-HCl (pH 8.0) buffer to the mixture, and then incubate the mixture for 10 min on a rocker at room temperature. This step turns the clear solution translucent without protein precipitation (Fig. 2, step 4).

vii. Centrifuge the mixture at 223,000(7 for 3 h at 8 °C. Record the $0D_{280}$ of the supernatant before discarding (Fig. 2, step 5).

▲ **CRITICAL STEP** If the supernatant contains protein, centrifuge it for a longer period of time. For DOPC/DOPE, it takes 6 h to pellet everything, whereas for POPC/POPG or DMPC/DMPG it takes 1–2 h.

viii. Dissolve the proteoliposome pellet into 20 ml of 5 mM Tris-HCl (pH 8.0) by bath sonication (Fig. 2, step 6). If the proteoliposome pellet is going to be used for a MAS sample, make the solution 0.01% (wt/vol) sodium azide, and then centrifuge the pellet again at 223,000*g* for 2 h at 8 °C to remove any residual MβCD in the supernatant and to concentrate the proteoliposomes. The final pellet is transparent (Fig. 2, step 7), and the SDS-PAGE gel shows a highly purified protein with no aggregation (Fig. 5a,b).

? **TROUBLESHOOTING**

B. Reconstitution by dialysis used for Rv1861 ● TIMING ~3 d

i. Weigh DMPC/DMPG (4:1 molar ratio) powdered lipids in a clean glass tube (Table 2). Dissolve the lipids in 2 ml of 10 mM HEPES (pH 7.5), using bath sonication until the solution is clear.

ii. Add DDGly detergent to a final concentration of 2.5% (wt/vol) into the liposome preparation, and mix the detergent lipid preparation gently by pipetting up and down to obtain a clear solution.

! **CAUTION** Gently pipette up and down when adding detergent because lipid and detergent solutions froth easily, potentially resulting in the loss of sample.

iii. Add 10–12 mg of protein to the lipid-detergent solution, and then add 10 mM HEPES buffer to a final volume of 8 ml. Add DDGly detergent to a final concentration of 2.0% (wt/vol) in 8 ml.

iv. Incubate the solution for 12–24 h at 37 °C (mixing it by gentle inversion 1–3 times during this interval), and transfer it into a dialysis tube with a 6–8 kDa molecular weight cutoff (MWCO) for conventional dialysis[14,21,33].

! **CAUTION** Choose the dialysis membrane MWCO such that it is no more than half of the expected protein-mixed micelle molecular weight.

v. Start dialysis at 37 °C against 2 liters of 10 mM HEPES buffer (pH 7.5), and continue this process by changing the dialysis buffer after the first 2 h and then twice daily for 3 d.

vi. At the end of day 3 when the solution is translucent, prepare the sample for centrifugation. If you are going to use the proteoliposome pellet for a MAS sample, make the solution 0.01% (wt/vol) in sodium azide and then centrifuge the sample at 223,000*g* for 1.5 h at 8 °C to concentrate the proteoliposomes. The final pellet is transparent, and the SDS-PAGE gel shows a highly purified protein with minimal aggregation (Fig. 5c).

? **TROUBLESHOOTING**

ssNMR sample preparation ● TIMING ~4–5 d

2| Prepare two types of ssNMR samples for membrane protein structure determination studies using both (option A) OS preparation and (option B) MAS sample preparation. Follow Figure 3 for step-by-step OS ssNMR sample preparation and Figure 4 for transferring the final proteoliposome pellet into a MAS 3.2-mm rotor (Box 2).

A. OS ssNMR sample preparation ● TIMING ~3–5 d

i. Add dilute buffer solution (5–10 mM) using Tris-HCl or HEPES with 0.01% (wt/vol) sodium azide to the final pellet to make a final volume of 1.2 ml, and then homogenize the solution by bath sonication. (If the pellet is easily dissolved, bath sonication can be avoided). Use this homogeneous proteoliposome suspension for glass-supported OS ssNMR sample preparation.

! **CAUTION** The final solution should be a low-viscosity, translucent fluid without any precipitation. If detergent is not removed completely, the proteoliposome solution may look opaque and sticky after sonication (Fig. 5d). Remove residual detergent by either adding MβCD to the solution or by dialyzing for a longer period of time.

■ **PAUSE POINT** The resuspended pellet for OS sample preparation can be stored above or below the phase transition of the lipids. In our experience, membrane proteins are very stable in lipid environments provided that the detergent has been completely removed.

ii. Spread 32 μl of the final proteoliposome solution onto each of 35–40 glass slides (5.7 mm × 12.0 mm × 0.06–0.08 mm). Each glass slide will have 1.2–1.5 mg of protein-lipid mixture.

? **TROUBLESHOOTING**

iii. Follow either of the two options described below for dehydrating the proteoliposome preparation. Both options have been successful for achieving good OS samples. We recommend dehydration in a 96% humidity chamber as a good first approach, even though it takes longer.

Low RH (16%) in a 37 °C incubator	Dehydrate to the point where there is only a small opaque region remaining at the center of each glass slide (~20 min) (Fig. 3, step 3)
96% RH chamber at 20–22 °C	Dehydrate so that each slide is almost completely transparent (within 24 h). Just before stacking the slides, add 2 μl of water to the center of each slide

▲ **CRITICAL STEP** For both of these dehydration options, it is important that the proteoliposome film not be completely dehydrated before the slides are stacked. In the 16% RH environment, it is important that the slides be stacked before the sample has become completely transparent, i.e., there is a small region in the center of each slide that is still opaque. In a 96% RH environment, there is less danger of excessive dehydration, although leaving the samples in this RH for a much longer period of time than we recommend may lead to poor orientation of the samples. We surmise that excessive dehydration of the proteoliposome surface may lead to protein denaturation.

? **TROUBLESHOOTING**

iv. Stack the glass slides as shown in Figure 3 and incubate them for 4–5 d in the 96% RH chamber at 37 °C. During this time, the stack becomes compacted as the hydrated bilayers on top of each slide interact with the slide above and the remaining bulk water is removed. In doing so, the height of stack becomes compatible with the height of the sample cell, and the stack can be inserted into the sample cell as shown in Figure 3, step 5. Typically, 35 slides fIll the sample cell for this preparation protocol.

! **CAUTION** The hygroscopic behavior of lipids and proteins varies, and consequently, the timing and temperature for the dehydration and rehydration steps may need to be adjusted depending on the lipids and proteins being used.

! **CAUTION** Alignment of the sample is only partially determined by the parallel layering of the glass slides. The magnetic field interaction with the diamagnetic helices of a transmembrane helix in membrane proteins enhances the alignment (with a mosaic spread of <1°). Small variations in the orientation of the stacked slides can be ignored.

v. Sample clarity starts from the center of the cell to the corners upon rehydration. Once the stack of slides becomes completely clear, seal the sample cell with a cap and screw, as well as with bee wax and Parafilm, as shown in Figure 3. Weigh the final sample now so that you have a reference weight to compare with the sample weight after the NMR experiments to confirm that no hydration has been lost from the sample.

B. Mas ssNMR sample packing in Mas rotor ● TIMING ~20 min

i. Prepare a proteoliposome pellet using a protein-to-lipid ratio of 1:30 (~15–20 mg of lipid) by either reconstitution method described above.

ii. Transfer the pellet into a 3.2-mm MAS rotor as described in Box 2 and Figure 4, or by using another effective transfer method. In our experience, the 3.2-mm thin-wall rotors are optimal for the spectroscopy of these proteoliposome samples.

! **CAUTION** These MAS rotors are fragile and expensive. Handle rotors and caps with care, especially when centrifuging the rotor.

▲ **CRITICAL STEP** Longer centrifugation of the proteoliposome solution is recommended to prepare the MAS pellet to reduce the size of the pellet. Larger (i.e., softer) pellets make it difficult to transfer 100% of the sample into the MAS rotor.

■ **PAUSE POINT** As noted for the OS sample preparation, the proteoliposomes are stable provided that the detergent has been completely removed. As a result, the centrifuge pellet can be stored either above or below the phase-transition temperature of the lipids until spectrometer time is available. We recommend transferring the sample to the MAS rotor before this point in order to minimize desiccation of the centrifuge pellet.

iii. After the complete transfer of the pellet, seal the MAS rotor with a cap (Fig. 4, step 9). The sample is ready for MAS ssNMR experiments.

Acquisition of NMR spectra ● TIMING ~1 h-7 d

3| Acquire 1D and 2D NMR spectra from the protein/lipid samples prepared using option A for oriented samples and option B for MAS samples. These NMR experiments are essential for validating the sample preparation.

A. OS sNMR studies

i. Acquire a 1D ^{31}P spectrum. 1D ^{31}P spectroscopy is exemplified here with spectra of gramicidin A, a polypeptide test sample (supplementary Methods) that we frequently use (Fig. 6a,b). These spectra of the phospholipids show how well the lipid bilayers are aligned. The resonances from a single-lipid type generate a single strong resonance at ~30 p.p.m. when obtained above the gel-to-liquid crystalline-phase transition temperature. Mixtures of lipids may give rise to multiple resonances in the same vicinity in a well-aligned sample.

ii. Obtain ^{1}H-^{15}N cross-polarization spectra for a test sample such as gramicidin. Make sure that the spectrometer is appropriately set up for the protein samples. As shown in Figure 6c,d, the resonances of a uniformly ^{15}N-labeled protein will be severely overlapped, and consequently it is difficult to assess the quality of alignment from such samples.

iii. Specific amino acid labeling and 2D PISEMA or SAMPI4 experiments obtained above the phase-transition temperature of gel to liquid crystalline demonstrate unequivocally the uniform alignment of the samples (Fig. 7). Record these spectra using the acquisition conditions noted in Table 3 (Fig. 6).

? **TROUBLESHOOTING**

iv. Weigh the sample and note any loss in hydration.

! **CAUTION** After an experiment, look for moisture droplets that may have accumulated on the inside of the sample cell. Sample heating should be minimized in all experiments. In our laboratory, we use low-electric-field probes (low E probe) made at the NHMFL[71]. Various commercial vendors now sell such probes. To further reduce sample heating from the ^{1}H radio frequency (RF) duty cycle, lengthen the recycle delay or reduce the acquisition time.

B. (B) MAS ssNMR studies

i. Record 2D ^{13}C-^{13}C DARR spectra of uniformly ^{13}C-labeled samples with a short mixing time (Fig. 8). Here we use 30 ms so that minimal-to-moderate or no long-range cross-correlations are observed. Spectra were recorded using the acquisition conditions noted in Table 4.

ii. The quality of the sample preparations is given by the linewidths of individual resonances. Such resonances are relatively rare in transmembrane helical proteins, but observations in both of these samples show that single-site resonances have linewidths between 0.5 and 1.0 p.p.m.

? **TROUBLESHOOTING**

? **TROUBLESHOOTING**

step 1A(viii), reconstitution using MβcD

If the final proteoliposome sample is not transparent, consider adding more MβCD powder and repeating Step 1A(vi–viii).

Step 1B(vi), reconstitution by dialysis

Poor reconstitutions during dialysis are recognized by the formation of cloudy precipitate rather than translucent liposomes (Fig. 5d). The precipitation of the protein from a failed reconstitution should be evident by the second day of dialysis. However, more dialysis time may be needed for larger proteins and detergents with low CMC values. Make sure that you dialyze above the phase-transition temperature for a given lipid combination. Other lipid combinations and detergents can be screened to improve reconstitution efficiency.

Step 2A(ii), sample loading on glass slides

For OS ssNMR sample preparation, we recommend loading 1.5 mg of sample (lipid + protein) per slide to prepare an optimal (considering sensitivity and resonance linewidths) OS spectrum. If the NMR data are of poor quality and the liposome sample looks good, load more or less liposome samples per slide to achieve more signal or better alignment, respectively.

Step 2A(iii), OS sample hydration

Sample hydration mentioned in the protocol is an important step; good samples contain ~40% (wt/wt) water. If the sample is too dry or overhydrated, equilibrate the sample for a longer period of time in the hydration chamber.

Step 3A(iii), OS ssNMR spectroscopy

If the OS ssNMR spectra provide evidence of poorly aligned samples, open the sample and equilibrate it for a longer period of time in the hydration chamber, as mentioned before. Some samples need a longer equilibration time than the standard 4–5 d.

Step 3B(ii), Mas ssNMR spectroscopy

If the spectral resonances are broad, vary temperature, screen for different lipids—some membrane proteins require negatively charged lipids and so on—or increase the molar fraction of lipid in the samples[70].

TIMING

Step 1, reconstitution of purified membrane protein into liposomes: 1–4 d

Step 2, solid-state NMR sample preparation: ~4–5 d

Step 3, acquisition of NMR spectra: ~1 h–7 d

Box 1, expressing membrane proteins and purifying them in detergent micelles: ~2 d

Box 2, MAS ssNMR sample packing in an MAS rotor: 20 min

ANTICIPATED RESULTS

To illustrate the procedures presented here, we show the results from CrgA and Rv1861 membrane proteins. Figure 5a–c shows 12% (wt/vol) SDS-PAGE gels for good and poor reconstitutions of membrane proteins in lipid bilayers. Figure 5a shows the aggregation of CrgA owing to incomplete reconstitution. Lanes E1 and E2 show CrgA elutions in DPC detergent where the protein did not aggregate, whereas in lanes A1 and A2 oligomeric states of CrgA are observed in POPC/POPG liposomes after reconstitution. This is because of partial incorporation of CrgA into POPC/POPG liposomes followed by protein aggregation. The result of the incomplete reconstitution (lanes A1 and A2) is broad resonances in the MAS NMR spectra and powder pattern intensity in the OS NMR spectra (data not shown). Figure 5b,c shows good reconstitution for CrgA (POPC/POPG) and Rv1861(DMPC/DMPG) into liposomes, respectively. The aggregation of protein in the final pellet (lane P2) is absent, and the detergent during reconstitution was effectively removed by MβCD leading to a good reconstituted CrgA sample (Fig. 5b). For Rv1861, all protein is incorporated into the liposomes, and a monomeric band is observed in the lane P of the gel (Fig. 5c). Furthermore, no protein is unincorporated into the liposomes as there is no protein in the supernatant after centrifugation. Reconstitution failure is easily observed. In Figure 5d, the loss of transparency and formation of precipitate are signs of unincorporated protein in liposomes.

The preparation of glass slide samples is evaluated using 1D NMR spectroscopy. Lipid alignment for oriented samples is quickly evaluated using ^{31}P NMR. Figure 6a,b shows examples of aligned and unaligned DMPC lipid bilayers for the gramicidin A peptide. The ^{31}P spectrum of uniformly oriented sample (Fig. 6b) shows a single peak near 30 p.p.m., whereas the partially aligned bilayer (Fig. 6a) has multiple peaks. The ^{15}N NMR spectra require more spectrometer time but directly evaluate protein alignment in oriented samples. Figure 6c,d shows the spectra of uniformly ^{15}N-labeled CrgA proteoliposomes collected at 13 °C and the uniformly ^{15}N-labeled Rv1861 collected at 37 °C where both samples were above the phase transition temperature of the lipids. In the CrgA spectra, the resonance intensity near 200 p.p.m. is dominated by the backbone amide sites of the transmembrane helices, where the N–H bonds are roughly parallel to the bilayer normal. The frequency of these resonances and their dispersion in this region are dependent on the tilt of the transmembrane helices. For CrgA, the helices have a small tilt angle to the bilayer normal, and the transmembrane residues have resonances near 200 p.p.m. For helices with a larger tilt angle as in Rv1861, the transmembrane helical resonances are centered about 170 p.p.m. The isotropic frequency for the amides is near 120 p.p.m. The intensities in the vicinity of 60–80 p.p.m. are from the sites where the amide N–H bond is almost parallel to the bilayer surface, such as those in an amphipathic helix on the surface of a bilayer. Often the ^{15}N resonances present in the termini and the loop regions of the protein are parallel to the bilayer surface or near the isotropic frequency rather than being parallel to the bilayer normal, but these resonances can be distributed throughout the spectrum.

2D NMR experiments on specifically labeled amino acid samples provide more detailed information for OS samples. Figure 7a,b shows the 2D PISEMA spectra of ^{15}N alanine (black)–,^{15}N valine (green)labeled CrgA and 2D SAMPI4 spectra of ^{15}N leucine

(green)–, ^{15}N valine (red)labeled Rv1861, respectively. For the CrgA spectra (Fig. 7a), the transmembrane helix resonances are consistent with PISA wheels characteristic of small helical tilts (15° ± 1), whereas the resonances for Rv1861 (Fig. 7b) are consistent with a PISA wheel characteristic having a tilt angle of 38° or more with respect to the bilayer normal. Resolved resonances in these spectra are indicative of a high degree of alignment in the sample representing a mosaic spread in orientation of <1° and an order parameter near 1.0. The high degree of alignment in the sample directly leads to the linewidths observed in the spectra, and with even a mosaic spread of a couple of degrees, the spectral resolution shown in Figure 7a,b would be lost[72].

MAS samples are assessed using both ^{13}C and ^{15}N NMR. Figure 8 a,b shows 2D DARR-MAS spectra of [U-^{13}C] CrgA and Rv1861 in liposomes at 243 K using a 30-ms DARR-mixing time. Characteristic helical and nonhelical isotropic resonance frequencies can be observed for the Val/Leu/Ile resonances in CrgA and Rv1861 spectra. As the samples are helical membrane proteins, we can interpret nonhelical resonance frequencies as arising from the loops and extramembranous domains, which typically have broader resonance linewidths and decreased intensity in the dipolar-based DARR spectra compared with the less-dynamic transmembrane helical resonances. With partial assignment of the unique resonances in the helices, it is possible to achieve sparse through space distance restraints.

Outlook

The combined use of MAS and OS spectra for obtaining orientational and distance restraints to characterize a protein structure has been demonstrated using the conductance domain of the M2 protein from Influenza A[17]. Here we demonstrate that high-quality samples can routinely be prepared for both OS and MAS NMR spectroscopy for two helical membrane proteins, but our success extends to six other proteins than those we have worked with in our laboratory. We are convinced that this is a robust protocol that could be easily adopted by many other labs. The combination of these experiments permits the structural characterization of membrane proteins in a native-like environment that appears to be frequently required for the characterization of native-like structures, especially of small helical membrane proteins[3].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 | Expressing membrane proteins and purifying them in detergent micelles ● TIMING ~2 d

Examples of ^{15}N-, ^{13}C-labeled membrane protein expression and purification are presented in the Supplementary Methods. This protocol follows the methodology developed over decades of work in many laboratories with some modifications, mainly in the detergent choices[2773]. Because membrane protein purification in high yield is always a bottleneck for structural biology, here we mention briefly some considerations relating to the six main steps that are involved in this process.

Considerations

- **i.** Express the His-tagged protein in *E. coli* cells: for expression, choose an optimal temperature. Some proteins express very well at 37 °C for ~45 min following induction, whereas other proteins express better at low temperature, such as 20–26 °C for 16–18 h after induction.
- **ii.** Lyse the cells by mechanical or chemical lysis: during lysis, urea and guanidinium chloride reagents along with detergents in the lysis buffer are used to maximize the extraction of overexpressed membrane proteins from the cellular membrane or inclusion bodies.
- **iii.** Solubilize the proteins in the lysate using detergent: here the choice of detergent for solubilization is important. A poor detergent choice can cause aggregation of the overexpressed membrane proteins and is difficult to reverse during chromatography-mediated protein purification.
- **iv.** Purify the overexpressed membrane protein of interest using metal affinity column chromatography: choice of detergent for the final elution step is critical and protein dependent leading to either of the two detergent removal processes mentioned in the reconstitution step.
- **v.** Analyze the membrane protein purity by 12% (wt/vol) SDS-PAGE (Supplementary Fig. 1).
- **vi.** The purified proteins can be stored in the elution buffer at an appropriate pH at 4 °C for as long as 4 months, but the use of fresh preparations for reconstitution is recommended.

It is worth noting that the storage conditions and shelf-life are protein dependent. Some proteins may not have such stability in detergent solutions.

Box 2 | MAS ssNMR sample packing in an MAS rotor ● TIMING ~20 min

1. Place the centrifuge tube with the final proteoliposome pellet upside-down into a 15-ml centrifuge tube, which is in turn inserted into a 1-ml Eppendorf tube as shown in Figure 4 (steps 2 and 3). Join this construct by wrapping it in Parafilm.
2. Place this construct inside a 50-ml Falcon tube (Fig. 4, step 4).
3. Spin the 50-ml tube at 3,000*g* for 5 min at room temperature. The pellet will be transferred completely into the Eppendorf tube (Fig. 4, step 5).
4. Cut the Eppendorf tube above the pellet and fix it upside-down inside a 200-μl pipette tip. Place the pipette tip mouth into a 3.2-mm MAS rotor (Fig. 4, steps 6 and 7). Wrap the joints with Parafilm.
5. Place this construct with the MAS rotor into a 15-ml centrifuge tube, and then secure its position (Fig. 4, step 8).
6. Centrifuge it at 3,000*g* for 5–7 min at room temperature. The pellet will be completely transferred to the MAS rotor, which should then be capped.
7. The sample is ready for 1D cross-polarization (CP) MAS- and DARR-type solid-state MAS NMR experiments.

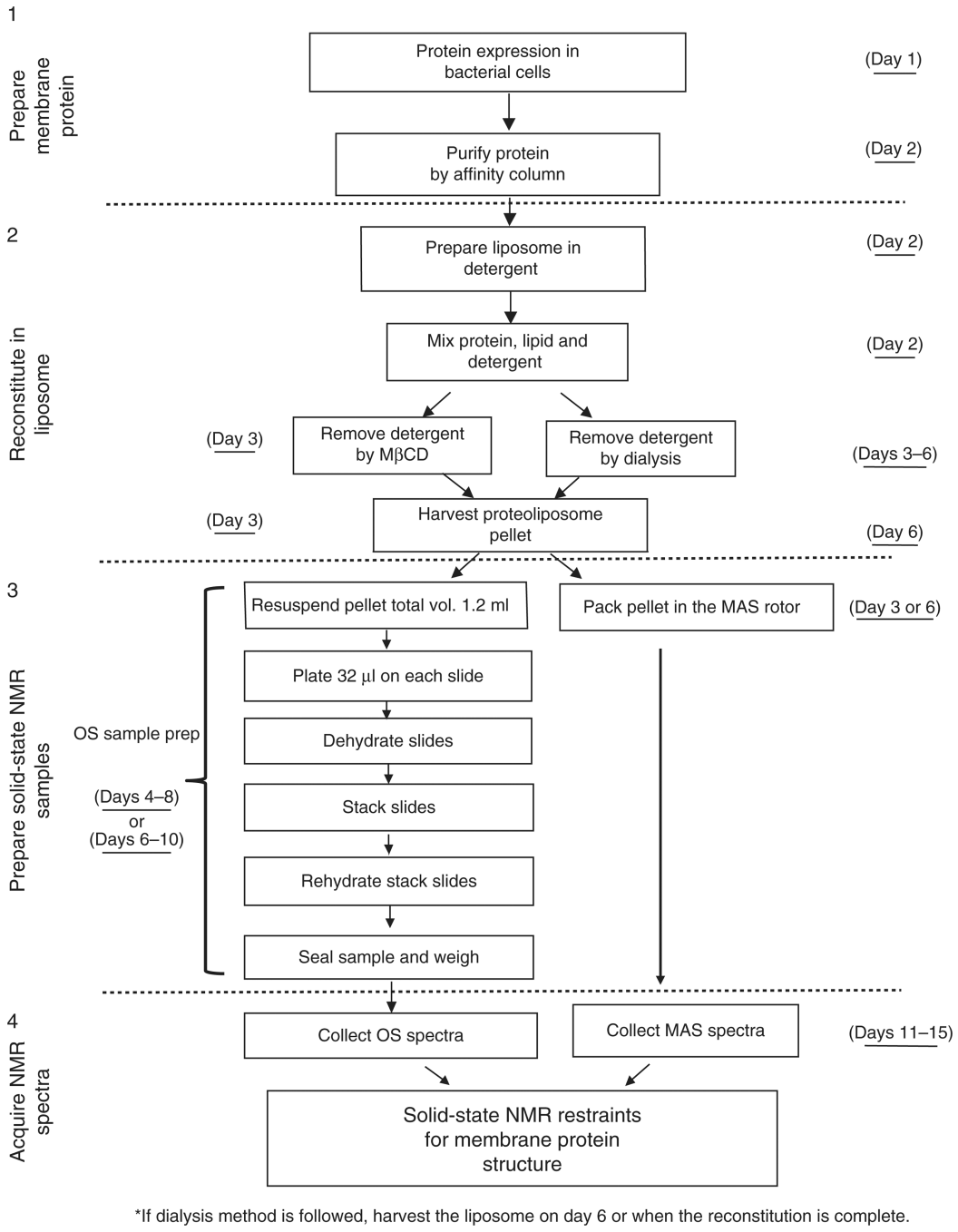

Figure 1.

Schematic of the experimental procedure with expected timeline from membrane protein expression to NMR spectroscopy. The process is divided into four main segments including membrane protein expression and purification, protein reconstitution into liposomes, ssNMR sample preparation and preliminary ssNMR spectroscopy for membrane protein structure determination. The focus in this manuscript is on steps 2 and 3 in this flowchart. It takes ~10 d from cell culture to sealing the NMR samples. Preliminary ssNMR spectra can be obtained in a few days.

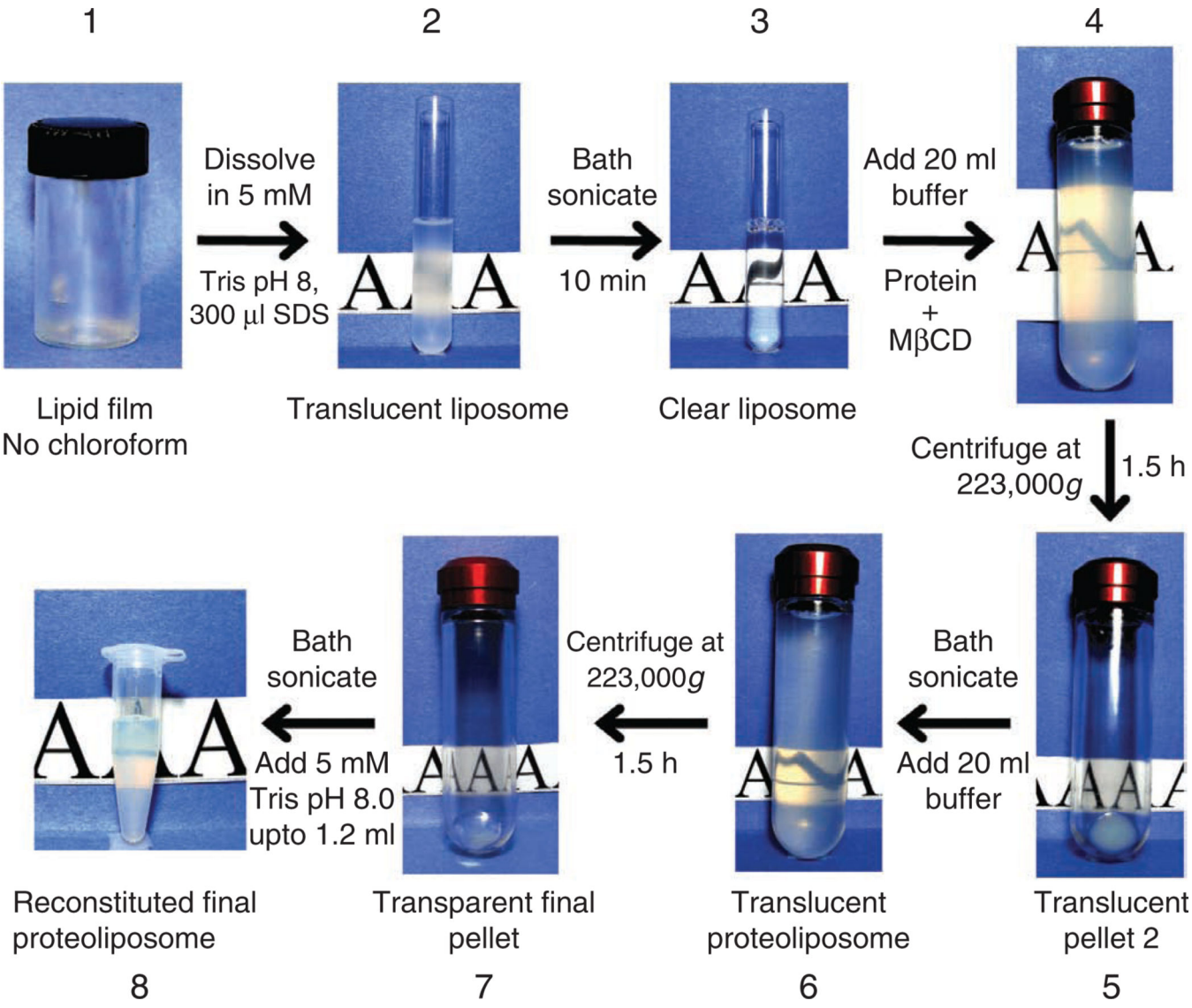

Figure 2.

Reconstitution of membrane proteins by MβCD. Images of (1) lipid film preparation by evaporation of chloroform under N_2; (2) lipid-detergent-mixed micelle preparation, resulting in translucent liposomes; (3) clear liposome preparation after bath sonication; (4) purified membrane protein in detergent mixed with the clear liposomes, followed by MβCD addition (this step turns the proteoliposome sample translucent again); (5) collection of the proteoliposomes by centrifugation, forming pellet 1; (6) resuspension of pellet 1 in 5 mM Tris buffer (pH 8.0) for the removal of any residual detergent-MβCD complex; (7) collection of the final proteoliposome pellet by centrifugation; and (8) suspension of the pellet by bath sonication in 1.2–1.3 ml of 5 mM Tris buffer (pH 8.0).

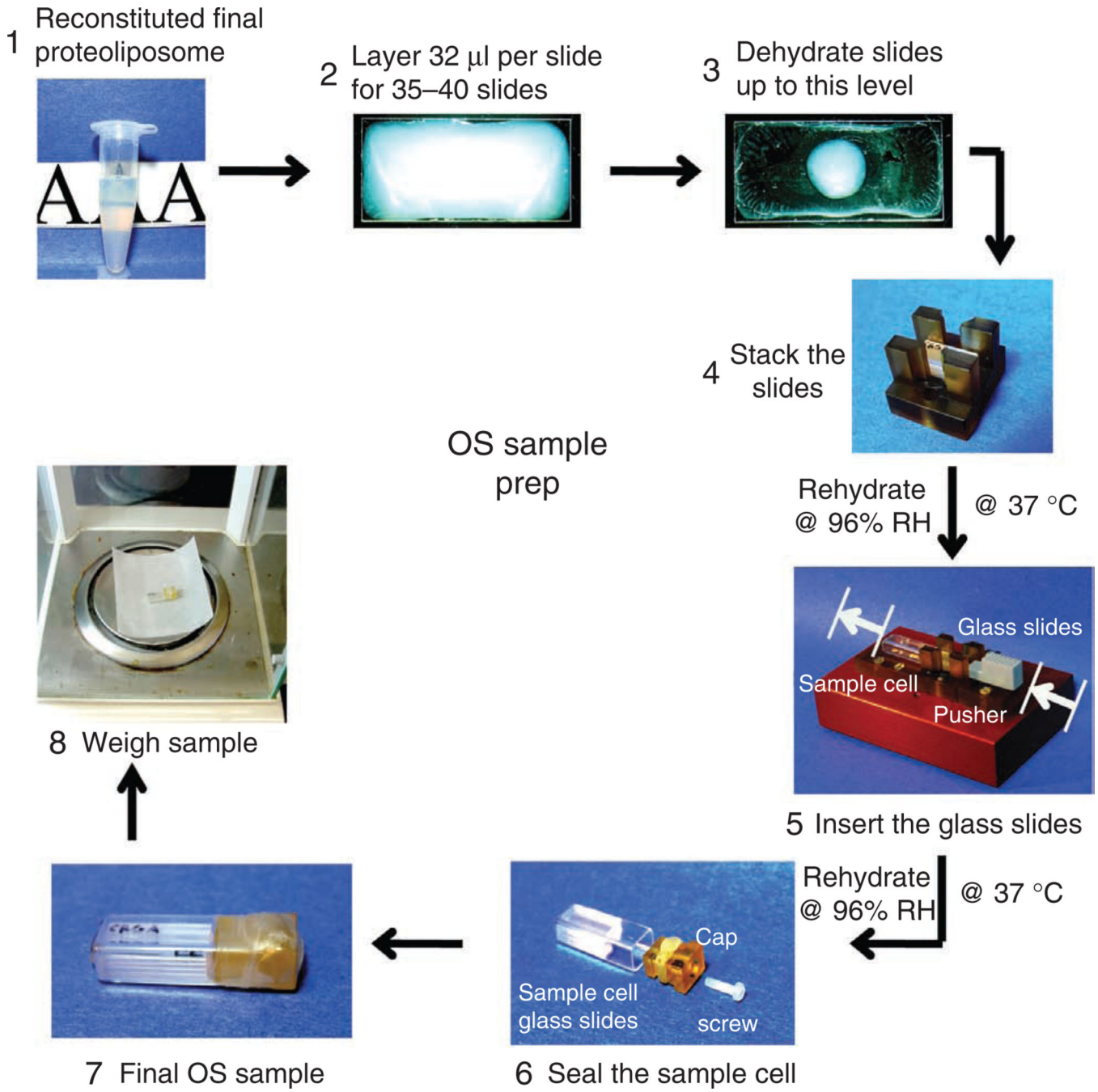

Figure 3.

Detailed procedure for mechanically aligned glass slide– supported OS ssNMR sample preparation of membrane proteins. Images illustrate (1) final reconstituted proteoliposome solution, (2) layering of 32 µl of the solution on each slide (total 35–40 slides), (3) partial dehydration of the slides in a 16% RH and 37 °C (shown) or 96% RH and 22 °C (not shown), (4) stacking of the slides in the slide holder and incubation of the stacked slides at 96% RH and at 37 °C, (5) insertion of the stacked glass slides into an NMR rectangular sample cell, (6) continuing rehydration of the sample for a total of 3–4 d in 96% RH

chamber at 37 °C until the sample turns completely transparent and then sealing, (7) the final OS sample ready for NMR spectroscopy, (8) weighing the sample before and after NMR spectroscopy to monitor potential hydration loss.

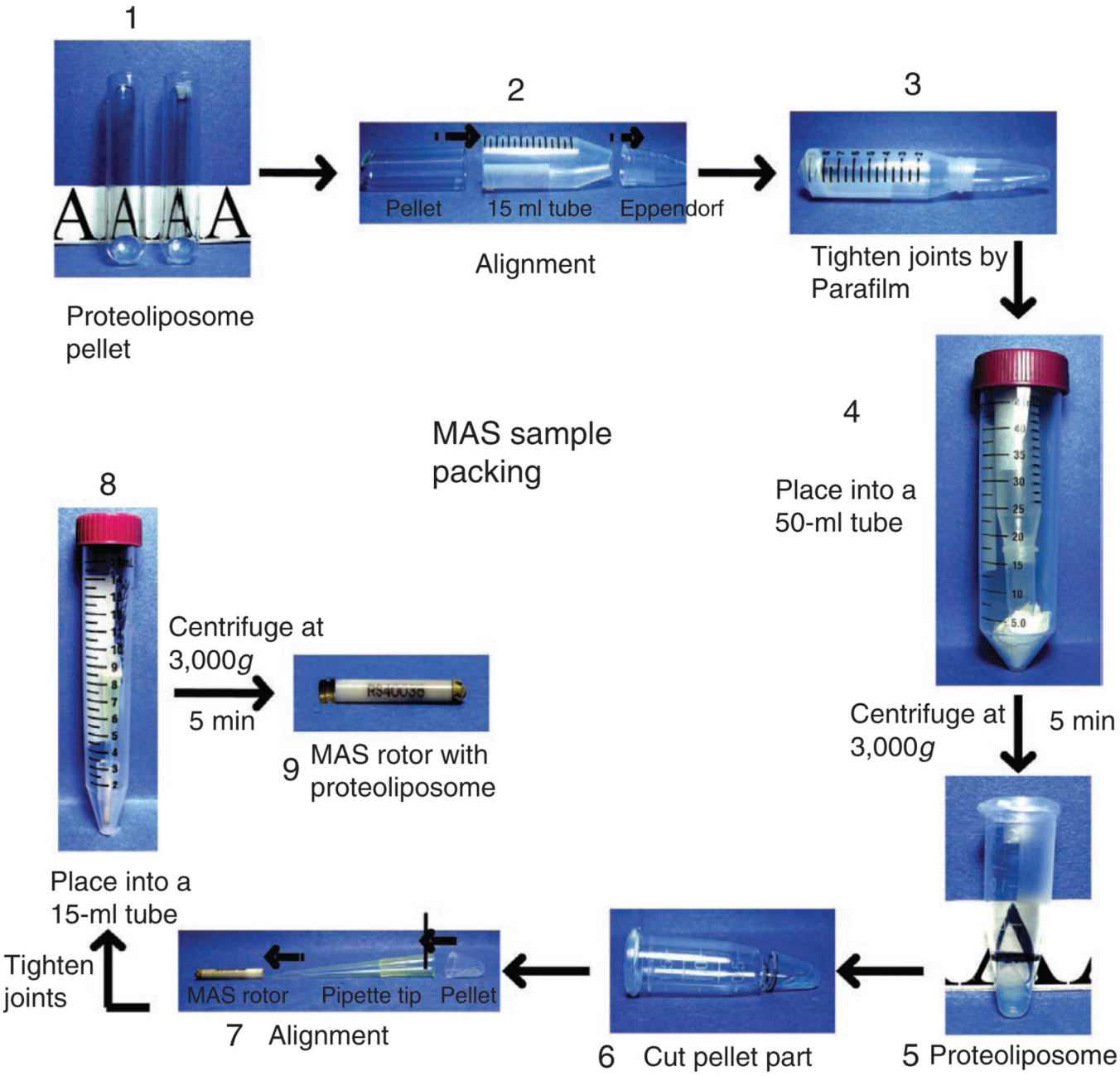

Figure 4.

Step-by-step procedure for MAS sample transfer to a thin-walled 3.2-mm MAS rotor. This process helps to obtain ~100% sample transfer for MAS ssNMR experiments. Images show (1) appearance of the final proteoliposome pellet; (2) cutting a 15-ml tube and aligning it with the pellet and a 1-ml Eppendorf tube as shown above; (3) sealing the joints with Parafilm; (4) inserting the construct into a 50-ml tube, capping it tightly and spinning the 50-ml tube at 3,000*g* for 5 min at room temperature; (5) collection of the MAS pellet in the Eppendorf tube; (6) cutting the Eppendorf tube above the pellet; (7) connecting the Eppendorf tip with pellet to a 200-μl pipette tip as shown in the figure above, inserting the pipette tip into the MAS 3.2-mm rotor, and then sealing both joints with Parafilm; (8)

inserting the MAS rotor portion of this new construct into a 0.5-ml Eppendorf tube without cap, sliding the whole construct into a 15-ml centrifuge tube, capping it securely, and then spinning at 3,000*g* for 5 min; and, finally, (9) sealing the sample in the MAS rotor with its cap for MAS ssNMR experiments.

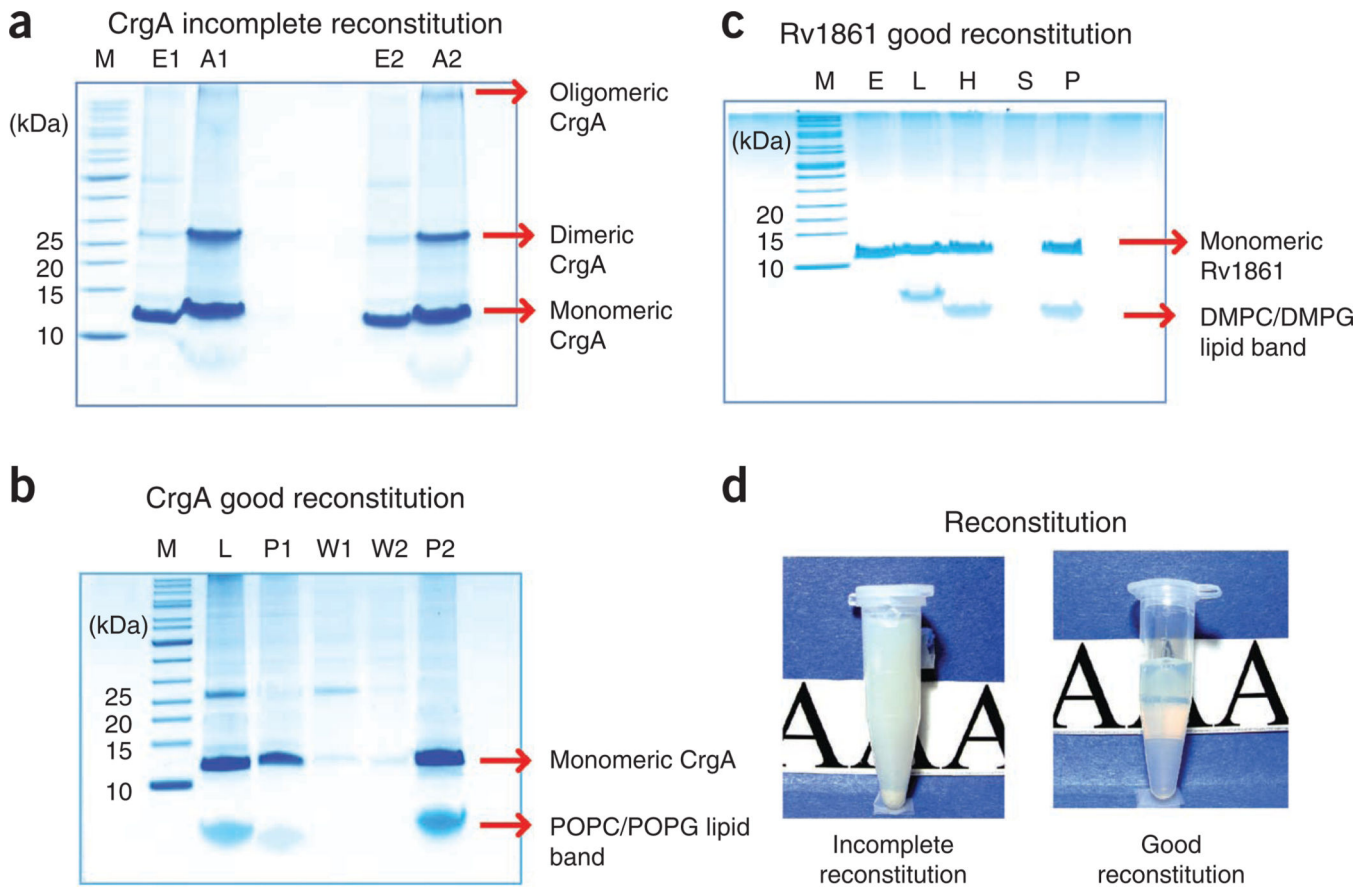

Figure 5.

SDS-PAGE (12%) gels illustrating incomplete and good reconstitution. (**a**) Lanes E1 and E2 show the purified CrgA membrane protein elution in Tris buffer (pH 8.0) and 0.4% (wt/vol) DPC detergent. Lanes A1 and A2 show the oligomerization of the protein after incomplete reconstitution in POPC/POPG liposomes (4:1 molar ratio). (**b**) Good reconstitution of CrgA by the MβCD protocol. Lane L shows the CrgA, lipid, detergent and MβCD mixture. Lane P1 is the pellet after the first centrifugation (Step 1A(vii)), and lane W1 is the supernatant of that step. Lane W2 is the supernatant of wash 2 (Step 1A(viii)) and lane P1 is the pellet for the final proteoliposome. (**c**) The various steps of the good reconstitution of Rv1861 in DMPC/DMPG (4:1) liposomes by dialysis protocol. Lane E is the elution step of Rv1861 in HEPES buffer (pH 7.5), 1.1% (wt/vol) DDGly detergent; lane L is Rv1861, lipid and detergent mixture (Step 1B(iv)); lane H is the dialysis solution on day 3 (Step 1B(vi)); lane S is the ultracentrifuge supernatant and lane P shows the final proteoliposome pellet solution (Step 1B(vi)). (**d**) Visual determination of incomplete and good reconstitution of membrane proteins in liposomes. In the incomplete reconstitution, precipitated protein is clearly observed, whereas in the good reconstitution sample, a translucent proteoliposome solution is observed.

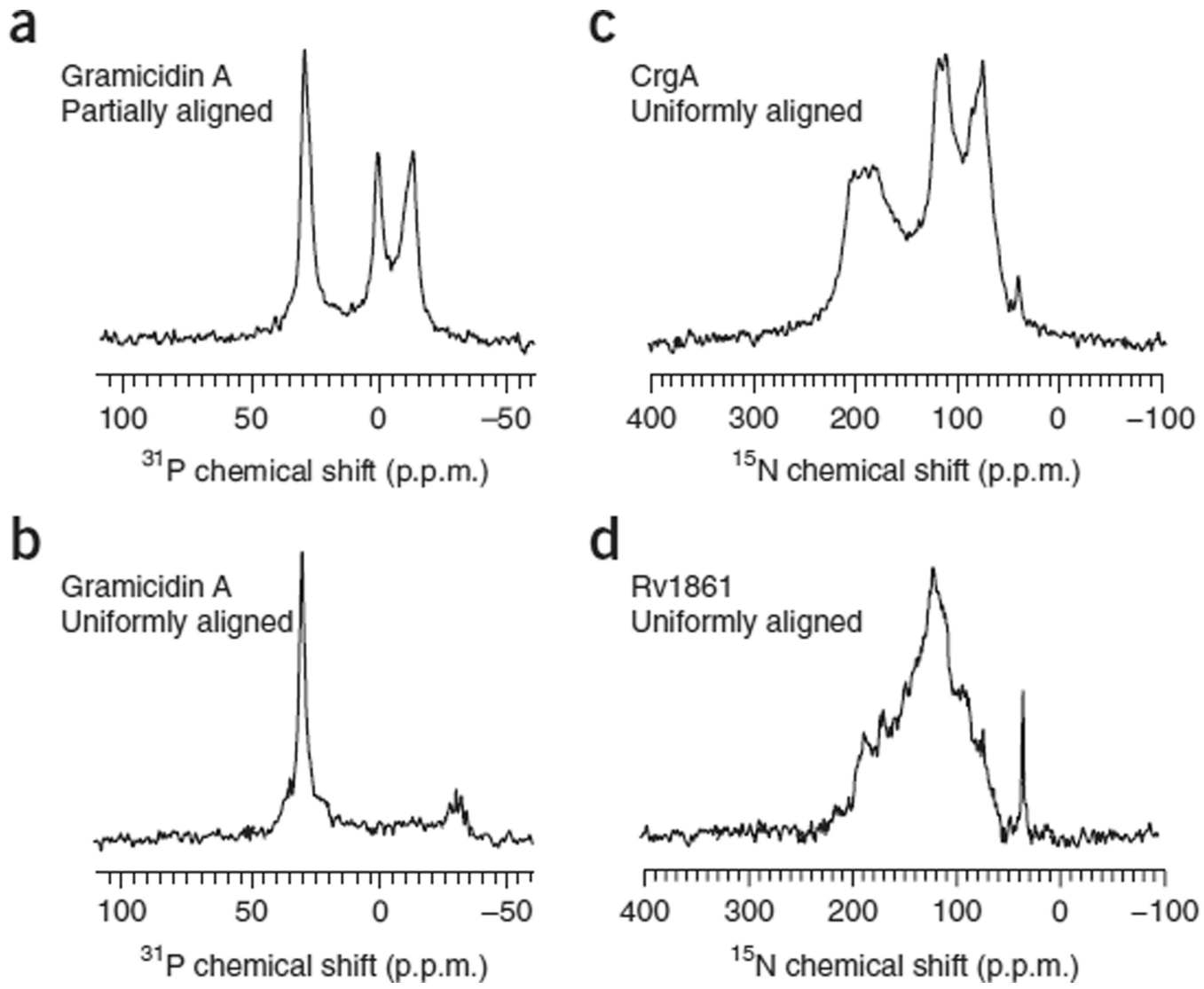

Figure 6.

ssNMR ^{31}P and ^{15}N 1D spectra of uniformly ^{15}N-labeled gramicidin A, CrgA and Rv1861 in lipid bilayers. (**a**) ^{31}P spectra of gramicidin A in DMPC partially aligned lipid bilayers at 42 °C showing multiple resonances. (**b**) ^{31}P spectra of gramicidin A in DMPC uniformly oriented lipid bilayers at 42 °C show single resonance at 30 p.p.m. (**c,d**) ^{15}N chemical shift spectra of uniformly ^{15}N-labeled CrgA and Rv1861 in uniformly oriented POPC/POPG (4:1 mol/mol) and DMPC/DMPG (4:1 mol/mol) bilayers. The CrgA spectrum was collected at 13 °C, whereas Rv1861 spectra was collected at 37 °C, both above the gel-to-liquid crystalline phase-transition temperature. Resonances near 200 p.p.m. are from amino acid residues in CrgA transmembrane helices, whereas resonances near 170 p.p.m. in Rv1861 are from more highly tilted transmembrane helices.

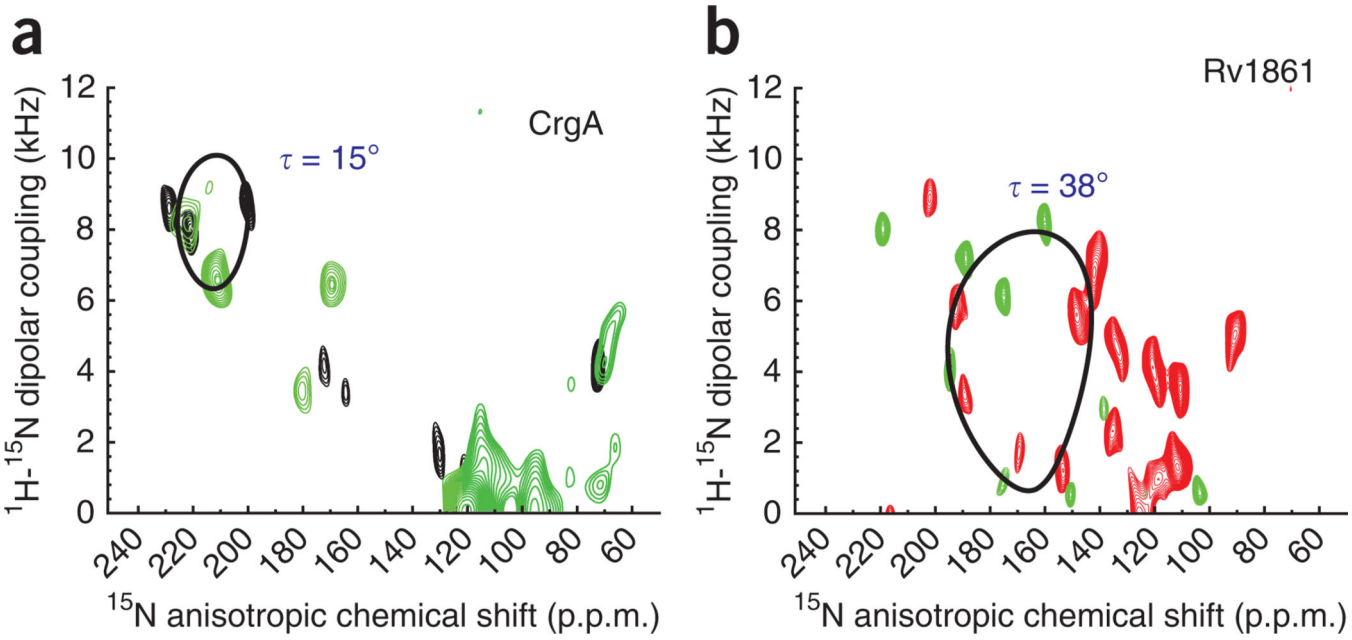

Figure 7.

Two-dimensional separated local field (SLF) ssNMR spectra of CrgA and Rv1861 in oriented lipid bilayers. (**a**) PISEMA spectra of CrgA ^{15}N-labeled alanine (black) and ^{15}N valine (green) are superimposed. Calculated PISA wheel with ~15° helix tilt angle is superimposed on the spectra. (**b**) SAMPI4 spectra of Rv1861 ^{15}N-labeled leucine (red) and ^{15}N valine (green) are superimposed and a PISA wheel for one of the transmembrane helices with tilt angle of ~38° is shown. The tilt angle of the transmembrane helix dictates the spectral dispersion and the center of mass for the resonances of the helix. For CrgA, both transmembrane helices (Val present only in transmembrane helix 1 and Ala present only in transmembrane helix 2) have similar tilt angles, whereas the Rv1861 3 transmembrane helices have tilt angles that vary between 38° and 45°. Multiple ^{15}N amino acid–specific labeling can result in 100% residue-specific ^{15}N backbone assignments and high-resolution structures for the backbones of these helices. All spectra were collected with 32-t1 increments with 4,000 scans in either a 600-MHz or 900-MHz magnet.

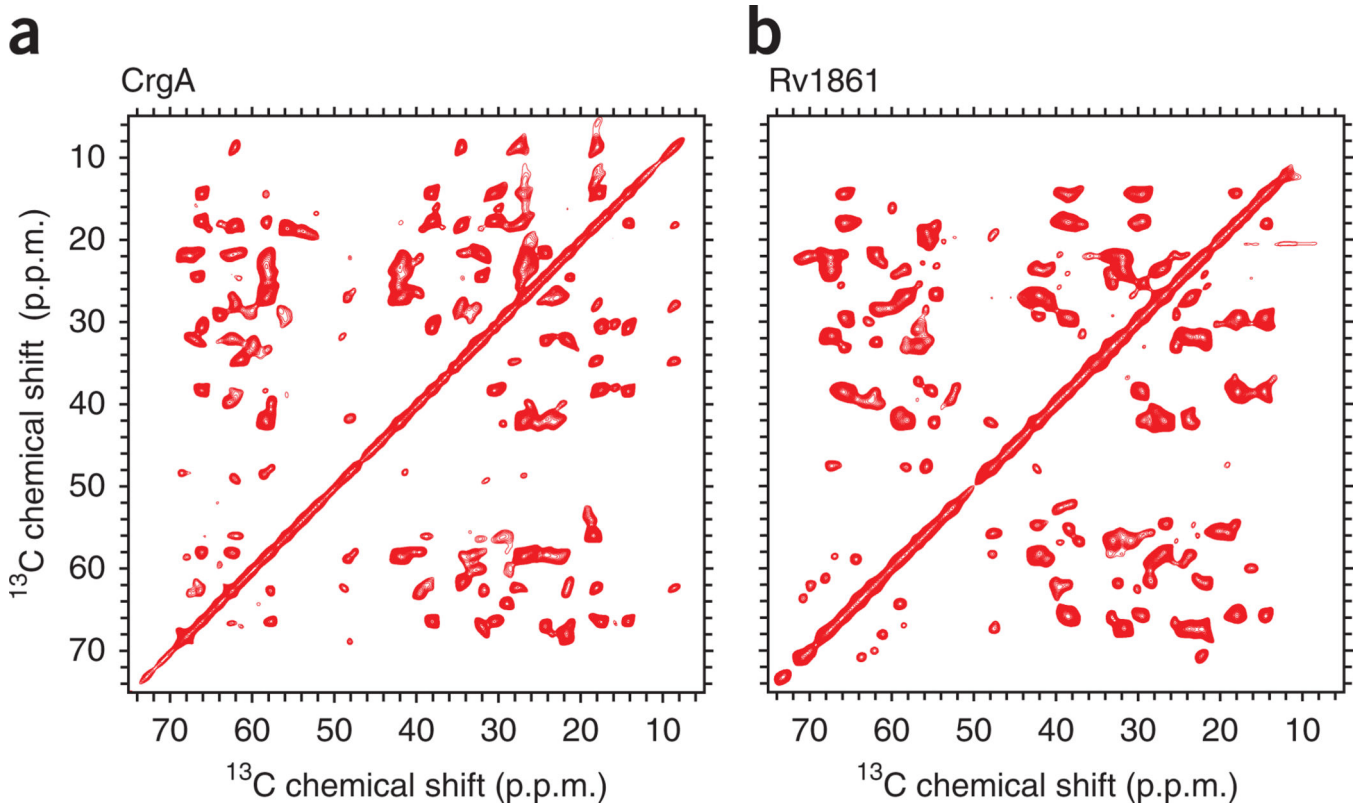

Figure 8.

Two-dimensional ^{13}C-^{13}C DARR-MAS spectra of CrgA and Rv1861 in liposomal preparations at 243 K with a 10-kHz spinning rate and 30-ms mixing time. Both spectra were obtained at 600 MHz. (**a**) Uniformly ^{13}C-labeled CrgA in POPC/POPG liposomes. (**b**) Uniformly ^{13}C-labeled Rv1861 in DMPC/DMPG liposomes. Spectral overlap is partially resolved in these 2D spectra, but there is still severe overlap between all of the hydrohobic transmembrane residues of a given type, making unique resonance assignments very challenging.

TABLE 1

Summary[a] of cloning and expression of *M. tuberculosis* target genes in *E. coli* bacterial cells.

Gene name	Organism	Molecular weight (kDa)	Position of His tag	Vector used	Expression host system[b]	Yield (mg per liter)
CrgA (Rv0011c)	*M. tuberculosis*	11.6	C-terminal	pET29b	BL21(DE3)RP$^+$	30
Rv1861	*M. tuberculosis*	11.4	N-terminal	pET16b	BL21(DE3)RP$^+$	30

[a] Detailed cloning, membrane protein expression and purifcation information has been previously published[5,20,27,71].

[b] BL21(DE3)RP$^+$*E. coli* cells with arginine (R) and proline (P) codon-plus cells. The expression of all membrane proteins was higher in codon-plus cells than in BL21(DE3) cells.

TABLE 2

Detailed description of choices of lipids for different membrane proteins for reconstitution process.

Lipid name	Abbreviation	Type of lipids	Membrane protein	Lipid molar ratio	P/L molar ratio[a]
1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine	POPC	Bilayer-forming	CrgA	PC:PG 4: 1	1:30, 1:80, 1:100
1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol)	POPG	Non-bilayer-forming			
1,2-Dimyristoyl-sn-glycero-3-phosphocholine	DMPC	Bilayer- forming	Rv1861	PC:PG 4:1	1:30, 1:80, 1:100
1,2-Dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol)	DMPG	Non-bilayer-forming			
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	DOPC	Bilayer-forming	M2 (22–62) and full length[b]	PC:PE 4:1	1:30, 1:100
1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine	DOPE	Non-bilayer-forming			

[a] P/L molar ratio: Protein-to-lipid molar ratio.

[b] OS and MAS sample preparation information has been previously published[14,17].

TABLE 3

Experimental conditions for OS ssNMR spectroscopy.

	1D CP Expt.	2D PISEMA/SAMPI4 Expt.
Spectrometers	Bruker Avance 600 and 900 MHz	Bruker Avance 600 and 900 MHz
Probes	Low-E probe	Low-E probe
CP contact time	800 μs–1 ms	1 ms
D1 (recycle delay)	4 s	4 s
^{1}H RF field	50 or 62.5 kHz	50 or 62.5 kHz
^{15}N RF field	50 or 62.5 kHz	50 or 62.5 kHz
^{1}H decoupling RF field	62.5 kHz	62.5 kHz
NS (no. of scans)	1,000–2,000	3,000–5,000
TD (complex data points)	512	512/32 (t2/t1)
Experimental time	~1–2 h	~4–7 d

TABLE 4

Experimental conditions for MAS ssNMR spectroscopy.

	2D ^{13}C-^{13}C DARR Expt.
Spectrometer	Bruker Avance 600 and 900 MHz
Probe	Low–E 3.2-mm MAS
Sample spinning	10 kHz
Sample temperature	243 K, 273 K, 286 K
CP contact time	1 ms
^{15}N, ^{13}C, ^{1}H 90° pulse lengths	5 μs (^{13}C) and 2.5 μs (^{1}H)
^{1}H decoupling RF field	100 kHz
^{13}C/^{15}N-^{1}H CP RF field 90–110% linear ramp on ^{1}H	50 kHz
D1 (recycle delay)	1–2 s
NS (no. of scans)	128–256
DARR mixing time	30 ms
TD (complex data points)	2,048/324 (t2/t1)
Experimental time	~1–2 d