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Hydration-optimized oriented phospholipid bilayer samples for solid-state NMR structural studies of membrane proteins

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Abstract

The preparation of oriented, hydration-optimized lipid bilayer samples, for NMR structure determination of membrane proteins, is described. The samples consist of planar phospholipid bilayers, containing membrane proteins, that are oriented on single pairs of glass slides, and are placed in the coil of the NMR probe with the bilayer plane perpendicular to the direction of the magnetic field. Lipid bilayers provide a medium that closely resembles the biological membrane, and sample orientation both preserves the intrinsic membrane-defined directional quality of membrane proteins, and provides the mechanism for resonance line narrowing. The hydration-optimized samples overcome some of the difficulties associated with multi-dimensional, high-resolution, solid-state NMR spectroscopy of membrane proteins. These samples have greater stability over the course of multi-dimensional NMR experiments, they have lower sample conductance for greater rf power efficiency, and enable greater rf coil filling factors to be obtained for improved experimental sensitivity. Sample preparation is illustrated for the membrane protein CHIF (channel inducing factor), a member of the FXYD family of ion transport regulators.

Keywords: Membrane protein; Structure; Solid-state NMR; Oriented lipid bilayers; Hydration-optimized sample

1. Introduction

Solid-state NMR spectroscopy of oriented samples can be used to determine the structures of membrane proteins [1]. The samples consist of proteins embedded in planar phospholipid bilayer membranes, that are oriented on glass slides, and placed in the rf (radio frequency) coil of the NMR probe so that the membrane plane is perpendicular to the direction of the applied magnetic field. The use of lipid bilayers provides a medium that closely resembles the biological environment, and sample orientation serves as a mechanism for resonance line narrowing, and also preserves the membrane-defined protein topology. The structures of gramicidin, the M2 transmembrane segment from the acetylcholine receptor, the transmembrane segment of the M2 H^+ channel protein from influenza virus, and the membrane bound fd coat protein (PDB files 1MAG, 1CEK, 1MP6, 1MZT), have been determined from solid-state NMR orientational restraints using this approach [2–5].

The method relies on the direct relationship that exists between the spectra of oriented proteins and their molecular structure and membrane topology. When highly oriented samples are prepared, multi-dimensional NMR experiments, that are based on the $^1H/^{15}N$ PISEMA pulse sequence [6,7], yield high-resolution spectra [8,9] where the frequencies measured for each resonance depend on the orientation of the corresponding molecular site with respect to the direction of the applied magnetic field, and thus, serve as orientational restraints for three-dimensional structure determination. For membrane proteins in oriented bilayers, the sample orientation is fixed, and each frequency reflects the orientation of a specific site in the protein with respect to the membrane.

The major technical challenges of this technique are associated with the preparation and physical properties of the samples. Once isotopically labeled membrane proteins are expressed, purified, and reconstituted in

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oriented lipid bilayers for NMR structural studies, the physical characteristics of the samples complicate the performance of multi-dimensional NMR experiments. In particular, planar lipid bilayer samples oriented on glass slides have low mechanical stability and high conductance, are susceptible to dehydration during the course of NMR experiments, and are accompanied by poor rf coil filling factors which result from the use of several glass slides per sample. In this report, we describe the preparation of hydration-optimized lipid bilayer samples, that can overcome these difficulties by utilizing a single pair of glass slides and by reducing the water content.

2. Results and discussion

2.1. Bilayers oriented on a single pair of glass slides

The first goal was to improve the signal to noise ratio by maximizing the rf coil filling factor. Typically, a sample for NMR structural studies contains 0.1–3.0 molecules of protein per 100 molecules of lipid, with 30–100 mg of lipid oriented on 15–40 glass slides of dimensions $11 \times 11 \times 0.07$ mm, and the lipids are DOPC (di-oleoyl-phosphocholine) and/or DOPG (di-oleoyl-phosphoglycerol). Multiple glass slides are required to improve the handling properties of these slippery and mechanically fragile samples, and are perceived to be necessary for obtaining a high degree of alignment. The stacked sample fills a square rf coil that is typically 11.5 mm wide, 14 mm long and 2–5 mm thick, however, more than half of the coil volume is occupied by glass. In order to test the degree of alignment that can be obtained with a single pair of glass slides, 30 mg of DOPC in chloroform solution, were deposited on the surface of a single glass slide. After removing the solvent under vacuum, a second glass slide was placed on top of the lipid, and the sample was equilibrated for 12 h at 93% RH (relative humidity).

The ^{31}P chemical shift NMR spectrum obtained for this sample at 22 °C is shown in Fig. 1b. The single resonance frequency at 27 ppm, its narrow line width (<1 ppm), and the lack of substantial intensity at −18 ppm, are all indicative of high (90%) uniaxial orientation of the lipid bilayers perpendicular to the magnetic field. The ability to prepare samples on a single pair of glass slides more than doubles the rf coil filling factor, improving the experimental sensitivity by a factor of at least 1.4, and also enables smaller coils to be utilized, for improved rf power efficiency and additional sensitivity. The spectrum obtained from unoriented lipid bilayers dispersed in water at 22 °C is shown in Fig. 1a, for comparison. This motionally averaged powder pattern is characteristic of phospholipids in liquid crystalline bilayers [10–12], as expected for DOPC well above its chain melting transition temperature of −18 °C [13,14]. In this spectrum, the 45 ppm chemical shift anisotropy reflects an average orientation of the phosphocholine headgroups nearly parallel to the bilayer surface [15].

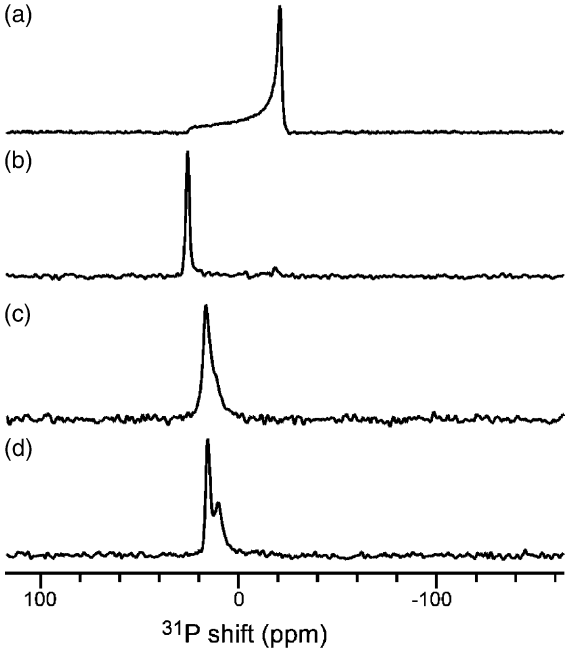

Fig. 1. ^{31}P chemical shift NMR spectra of DOPC in (a) fully hydrated, excess water, unoriented lipid bilayers at 22 °C, (b) fully hydrated, $n_w \approx 10$, oriented bilayers at 22 °C, (c) hydration-optimized, $n_w \approx 2$, oriented bilayers at 22 °C, and (d) hydration-optimized, $n_w \approx 10$, oriented bilayers at 50 °C. The percentage fractions of oriented lipids, estimated from the area under each single resonance from oriented lipid, and under the residual powder pattern from unoriented lipid, are (b) 90%, (c) 100%, and (d) 100%. For the unoriented sample 30 mg of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) (Avanti, AL) were vortex-mixed in water, and transferred to a cylindrical glass tube for NMR studies. The oriented lipid bilayer sample was prepared by depositing 30 mg of DOPC, dissolved in chloroform, on the surface of a single $11 \times 11 \times 0.07$ mm glass slide (Marienfeld, Germany). After removing the chloroform under high vacuum for 2 h, the sample was covered with a second identical glass slide and placed in a sealed chamber, where a constant RH of 93% was maintained with a saturated solution of ammonium phosphate, and monitored with a hygrometer. Oriented bilayers formed after equilibration in this chamber, at 40 °C for 12 h. For hydration-optimization, the oriented lipid bilayer sample was further equilibrated at 40 °C, in a sealed chamber containing drierite, which provides an atmosphere of 5% RH. The water concentration was estimated by weighing each sample before and after each equilibration step. The water concentration was $n_w \approx 10$ after 12 h at 93% RH, $n_w \approx 4$ after 24 h at 5% RH, and $n_w \approx 2$ after 96 h at 5% RH. The sample was wrapped and sealed in a thin plastic film prior to insertion into the rf coil of the NMR probe. ^{31}P NMR spectra were obtained with an echo pulse sequence and continuous ^{1}H irradiation during acquisition, on a Chemagnetics-Otsuka Electronics CMX 400 spectrometer. The temperature was maintained with heated nitrogen gas. Chemical shifts are referenced for phosphoric acid at 0 ppm.

The phosphocholine headgroup conformation is extremely sensitive to the level hydration, at water concentrations below 10 water molecules per lipid

($n_w \approx 10$), but is not affected by additional water above this number [11,16,17]. Therefore, the observation of identical frequencies (27 ppm) for the single resonance in Fig. 1b, and the high-frequency discontinuity of the residual powder pattern in Fig. 1a, demonstrates that the headgroup conformation in glass-oriented bilayers is similar to that in multilamellar vesicles in excess water. This is also consistent with our water content weight estimate of $n_w \approx 10$ in the oriented sample.

2.2. Hydration optimization

The second goal was to improve the low mechanical stability and high conductive properties of the sample, and to alleviate its susceptibility to dehydration during the course of NMR experiments. These detrimental properties result from high water content, but can be corrected by reducing the extent of lipid hydration, therefore, we equilibrated the oriented DOPC sample at 5% RH for 96 h, in order to reduce the water concentration from $n_w \approx 10$ to $n_w \approx 2$. The lipid chain melting phase transition temperature is hydration dependent, and for DOPC it increases from −18 °C, the limiting value obtained at $n_w = 9$, to 4 °C, the value observed at $n_w \approx 1$ [18]. At room temperature and $n_w \approx 2$, DOPC lipid bilayers are in the liquid crystalline phase, and two water molecules are sufficient to maintain the anisotropic rotations of the phosphocholine headgroup, and to produce axially symmetric ^{31}P NMR powder spectra [11]. Indeed, the ^{31}P NMR spectrum we obtained at 22 °C after dehydration (Fig. 1c), is similar to the spectrum obtained after full sample hydration (Fig. 1b), in that it displays a single characteristic resonance frequency. The complete lack of intensity at −18 ppm reflects a very high degree of orientation (100%), better than that observed for fully hydrated bilayers, a feature that has been previously noted for oriented bilayers with low water content [19]. Sample equilibration in a low RH atmosphere, after full hydration, as opposed to initial partial hydration, ensures the preparation of homogeneous highly oriented bilayer samples, which we refer to as hydration-optimized lipid bilayers.

Two features distinguish the spectrum in Fig. 1c ($n_w \approx 2$) from that in Fig. 1b ($n_w \approx 10$): the resonance frequency is shifted from 27 to 17 ppm, and the resonance line width is broader (≈2.5 ppm). The frequency shift is consistent with a hydration-dependent conformational change of the phosphocholine headgroup [11,15,16]. At water concentrations below $n_w \approx 10$, the conformation of phosphocholine is extremely sensitive to the number of waters in its hydration shell, and with progressive dehydration the headgroup tilts towards the lipid bilayer plane causing a reduction in the ^{31}P chemical shift anisotropy of the phosphate group. The broadening observed in the spectrum from hydration-optimized bilayers is not a result of increased mosaic spread, but rather of non-homogeneous hydration of the lipids by the small number of water molecules remaining in the sample, and a similar effect has been observed in the spectra of unoriented lipid bilayer dispersions [20]. Indeed, heating the sample to 50 °C enables two resonances to be resolved (Fig. 1d), and each resonance is interpreted to reflect a distinct headgroup conformation, induced by a greater (17 ppm), or smaller (12 ppm), number of bound water molecules.

2.3. Membrane proteins in hydration-optimized lipid bilayer samples

Since the ultimate goal is to develop samples that are useful for NMR structure determination of membrane proteins, an essential requirement is that they maintain protein structural integrity under hydration-optimized conditions. This was tested by preparing a sample of the uniformly ^{15}N-labeled membrane protein CHIF (channel inducing factor) in oriented lipid bilayers, and recording its ^{15}N chemical shift spectrum both before and after hydration-optimization. CHIF is a 67 amino acid, single-pass membrane protein, that is upregulated by aldosterone and corticosteroids in mammalian kidney and intestinal tracks, where it associates with the Na, K-ATPase to regulate Na^+ and K^+ homeostasis [21,22]. It is a member of the FXYD family proteins that are emerging as auxiliary, tissue-specific and physiological state-specific, subunits of the Na, K-ATPase [23]. We recently described the expression, purification and characterization of recombinant CHIF and other FXYD proteins [24]. The ability to produce these proteins in milligram quantities, enables structural as well as functional studies to be carried out.

The CHIF sample was prepared by mixing 1 mg of uniformly ^{15}N-labeled CHIF dissolved in trifluoroethanol, with 100 mg of lipids dissolved in chloroform, and depositing this mixture on the surface of 30 glass slides. The solvents were evaporated under vacuum, and the sample equilibrated for 12 h at 93% RH, 40 °C, to obtain a hydration level of $n_w \approx 10$. Both the lipids and protein in this sample are highly oriented, as evidenced by the single resonance in the ^{31}P NMR spectrum of the lipids (Fig. 2a), and the presence of several resolved resonances in the ^{15}N NMR spectrum of the protein (Fig. 2d).

The spectrum of CHIF in oriented bilayers (Fig. 2d) has several identifiable peaks at frequencies throughout the range of the ^{15}N amide chemical shift, and gives the first view of the structural organization of this protein in a membrane. Resonances around 200 ppm are from backbone amide sites in the single transmembrane helix that have their NH bonds nearly perpendicular to the plane of the membrane, and the rather narrow dispersion of ^{15}N resonances centered around 200 ppm suggests that the CHIF transmembrane helix crosses the

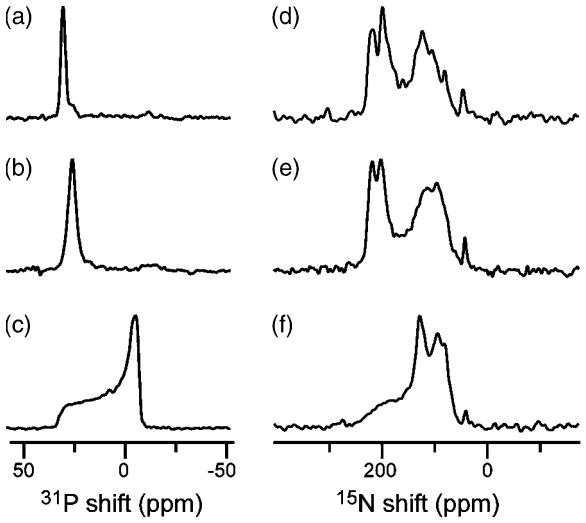

Fig. 2. ^{31}P and ^{15}N chemical shift NMR spectra of uniformly ^{15}N-labeled CHIF in (a, d) fully hydrated, $n_w \approx 10$, oriented lipid bilayers at 22 °C, (b, e) hydration-optimized, $n_w \approx 4$, oriented bilayers at 22 °C, and (c, f) fully hydrated, excess water, unoriented lipid bilayers at 22 °C. The ^{15}N NMR spectra from oriented CHIF in (d, e) were each obtained with 6,000 acquisitions. In the oriented samples, the percentage fractions of oriented lipids are (a) 90% and (b) 95%. Recombinant ^{15}N-labeled CHIF was cloned and expressed in *Escherichia coli*, grown in media containing ^{15}N-enriched ammonium sulfate (Cambridge Isotope Laboratories, MA) [24]. The oriented lipid bilayer sample was prepared by mixing 1 mg of CHIF dissolved in 0.5 mL of trifluoroethanol and 50 μL of β-mercaptoethanol, with 100 mg of lipid (DOPC/DOPG, 4/1, mol/mol) dissolved in chloroform, and depositing this mixture on the surface of thirty $11 \times 11 \times 0.07$ mm glass slides (Marienfeld, Germany). The solvents were evaporated under vacuum and the sample equilibrated for 12 h at 93% RH, 40 °C, to attain a hydration level of $n_w \approx 10$. For hydration-optimization the sample was additionally equilibrated for 48 h at 5% RH, 40 °C, to attain a hydration level of $n_w \approx 4$. The sample was wrapped and sealed in a thin plastic film prior to insertion into the rf coil of the NMR probe. For the unoriented sample, hydrated lipid bilayers containing CHIF were dispersed in a cylindrical glass tube. NMR spectra were acquired on a Chemagnetics-Otsuka Electronics CMX 400 spectrometer. ^{31}P NMR spectra were obtained with an echo pulse sequence and continuous ^{1}H irradiation during acquisition. ^{15}N NMR spectra were obtained with cross-polarization and continuous ^{1}H irradiation during acquisition. The temperature was maintained with heated nitrogen gas. Chemical shifts are referenced for liquid ammonia and phosphoric acid at 0 ppm.

lipid bilayer membrane with only a modest tilt angle [25,26]. Resonances around 80 ppm are from sites in the N- and C-terminal helices, with NH bonds nearly parallel to the membrane surface, and the peak near 35 ppm results from the amino groups of the lysine sidechains and the N-terminus. A preliminary analysis of these solid-state NMR data is possible since both CD and NMR spectroscopy in micelles show that the overall secondary structure of these proteins is α-helical [24].

The spectra obtained for this sample after equilibration for 48 h at 5% RH, and 40 °C, are shown in Figs. 2b and 2e. The single resonance in the ^{31}P spectrum moves to lower frequency and broadens, as expected for a sample with lower water content, but the sample retains its high degree of orientation. Likewise, the overall features of the ^{15}N spectrum are retained, with resonances from the transmembrane helix still clearly clustered around 200 ppm, resonances from the terminal helices around 80 ppm, and, importantly, no reduction in the signal to noise ratio, as should be expected with loss of orientation.

The ^{31}P and ^{15}N NMR spectra for a sample of CHIF in unoriented lipid bilayers are both powder patterns, and provide no resolution among resonances (Figs. 2c and 2f). The ^{31}P spectrum from the phospholipid headgroups is characteristic of liquid crystalline lipid bilayers, while in the protein, most of the backbone sites are structured and immobile on the time scale of the ^{15}N chemical shift interaction (10 kHz), and contribute to the characteristic amide powder pattern between about 220 and 60 ppm. Backbone sites that are mobile and unstructured, such as those near the N- and C-termini, give rise to the isotropic resonance band centered at 120 ppm. Therefore, while certain resonances near 120 ppm, in the spectrum of oriented CHIF, may reflect specific orientations of their corresponding sites, some others arise from mobile backbone sites.

3. Conclusions

It is possible to prepare samples of membrane proteins in oriented lipid bilayers that have significantly reduced water content, but preserve both a high degree of alignment, as well as the protein structural features. These hydration-optimized lipid bilayer samples offer several desirable advantages. In solid-state NMR structural studies of membrane proteins, multiple glass slides are required to improve the handling properties of slippery and mechanically fragile fully hydrated samples, albeit at the cost of greatly reduced rf coil filling factors and lower experimental sensitivity. In contrast, the lack of interstitial bulk water in the hydration-optimized samples greatly decreases their slippery quality, and renders them mechanically stable and easier to handle, thus enabling a greater number of lipids to be oriented on a smaller glass surface area (30 mg or more of lipid in a single pair of 11 mm^2 glass slides). This maximizes the rf coil filling factor, improving it by at least a factor of two, and leads to a significant increase in experiment sensitivity.

The reduced water content in the hydration-optimized samples limits both the conductance and/or dielectric losses in rf power, as well as the extent of rf heating, which typically result from the high conductances of solvated ionic (phosphoglycerol) and zwitterionic (phosphocholine) lipid headgroups. This enables high-resolution NMR experiments to be performed with rf power efficiencies approaching those attainable for crystalline samples.

Since the hydration-optimized bilayer samples are equilibrated at the lower limit of their water-binding capacity, prior to NMR spectroscopy, and since it is very difficult to remove the last tightly-bound water molecules from the headgroups of DOPC and DOPG, these samples are no longer susceptible to dehydration by rf heating, during the course of multi-dimensional solid-state NMR experiments, which can lead to conformational instability, as well as de-tuning of the NMR probe. The problems associated with rf heating can be further mitigated by performing the NMR experiments at low temperature. Indeed, even moderately low temperatures of 0–5 °C result in improved sample stability and reduced losses of rf power [27]. Our initial experiments with hydration-optimized samples at $n_w \approx 2$, indicate that lipid bilayer orientation is not lost upon lowering the temperature below the onset of headgroup rotations, suggesting that it might be possible to use these samples for experiments at very low temperature. Studies with hydration-optimized samples at low temperature, could be further aided by the addition of cryo-protectants, such as trehalose, which are also known to maintain the integrities of both lipid bilayer membranes, and protein structures, in anhydrobiosis [28].

With regard to membrane proteins, an important question is whether native structures are preserved in hydration-optimized samples. This can only be answered with structure determination, which is our ultimate goal, and it is possible that these samples will be well suited for structural studies of some, but not all, groups of membrane proteins. We anticipate that transmembrane helices will be stable in low-water lipid bilayers, since their tilts, rotations, and curvatures are inherent to the protein three-dimensional structure and supramolecular organization, and mediated by helix–helix contacts and amino acid sequences, while they are not very susceptible to changes in lipid bilayer thickness that accompany changes in hydration. For example, the transmembrane helix of the Influenza virus M2 H^+ channel adopts the same tilt in bilayers of DMPC (dimyristoyl-phosphocholine; 14C hydrocarbon chain) and DOPC (18C hydrocarbon chain), despite their considerable difference in thickness, suggesting that significant interactions occur between the helices in the tetrameric channel [29].

Finally, while the samples described in this report were prepared by organic solvent deposition of mixed lipids and protein on glass slides, oriented lipid bilayer samples for solid-state NMR structural studies can also be prepared by depositing aqueous suspensions of protein/lipid vesicles, obtained by detergent dialysis [9,27]. The method of choice for reconstitution is protein-dependent, and both methods yield highly oriented lipid bilayer samples that are ready for hydration optimization in reduced humidity atmosphere.

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