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Correlating lipid bilayer fluidity with sensitivity and resolution of polytopic membrane protein spectra by solid-state NMR spectroscopy ☆

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ABSTRACT

Solid-state NMR spectroscopy has emerged as an excellent tool to study the structure and dynamics of membrane proteins under native-like conditions in lipid bilayers. One of the key considerations in experimental design is the uniaxial rotational diffusion of the protein that can affect the NMR spectral observables. In this regard, temperature plays a fundamental role in modulating the phase properties of the lipids, which directly influences the rotational diffusion rate of the protein in the bilayer. In fact, it is well established that below the main phase transition temperature of the lipid bilayer the protein's motion is significantly slowed while above this critical temperature the rate is increased. In this article, we carried out a systematic comparison of the signal intensity and spectral resolution as a function of temperature using magic-angle-spinning (MAS) solid-state NMR spectroscopy. These observables were directly correlated with the relative fluidity of the lipid bilayer as inferred from differential scanning calorimetry (DSC). We applied our hybrid biophysical approach to two polytopic membrane proteins from the small multidrug resistance family (EmrE and SugE) reconstituted into model membrane lipid bilayers (DMPC-14:0 and DPPC-16:0). From these experiments, we conclude that the rotational diffusion giving optimal spectral resolution occurs at a bilayer fluidity of ~5%, which corresponds to the percentage of lipids in the fluid or liquid-crystalline fraction. At the temperature corresponding to this *critical value of fluidity*, there is sufficient mobility to reduce inhomogeneous line broadening that occurs at lower temperatures. A greater extent of fluidity leads to faster uniaxial rotational diffusion and a sigmoidal-type reduction in the NMR signal intensity, which stems from intermediate-exchange dynamics where the motion has a similar frequency as the NMR observables (i.e., dipolar couplings and chemical shift anisotropy). These experiments provide insight into the optimal temperature range and corresponding bilayer fluidity to study membrane proteins by solid-state NMR spectroscopy. This article is part of a Special Issue entitled, NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

Membrane protein structure determination is necessary for deciphering molecular-scale details of essential biological processes including ion homeostasis, cell signaling pathways, and transport of metabolites. Both NMR and X-ray crystallography have contributed to the knowledge database of the structures for these hydrophobic proteins. One method that has gained traction in recent years is solid-state NMR spectroscopy. In this technique, it is possible to study the proteins under native-like conditions such as phospholipid bilayers that are better mimics to cellular membranes than detergent micelles. Indeed, synthetic lipid membranes have been extensively studied and are often used for reconstituting membrane proteins for solid-state NMR and EPR spectroscopy [1–6]. The two major disciplines within solid-state NMR for characterizing membrane proteins are magic-angle-spinning (MAS) [7–10] and the oriented approach [11–13]. The latter has been extensively used to probe the tilt angles of membrane protein secondary structures with respect to the lipid bilayer as well as for backbone structure determination [1,14–22]. The MAS method is more commonly employed due to the ease of sample preparations that do not require the protein to be macroscopically aligned in the magnetic field. In addition, standard and afterglow [23,24] ^{13}C detection methods give increased sensitivity relative to ^{15}N-based experiments used in oriented solid-state NMR. For both methods, one of the intrinsic motional parameters that affects the ability to record high-quality structural data is the presence of uniaxial rotational motion about the membrane normal [25–28]. Indeed, when this diffusion rate is comparable to the frequency of the NMR observables, signal-to-noise and resolution suffer [29] and the spectra become difficult to interpret. Unlike microcrystalline proteins studied by MAS, these intrinsic motional properties also depend on the fluidity of the

Abbreviations: SSNMR, solid-state NMR; MAS, magic-angle-spinning; CP, cross-polarization; DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine

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membrane. In model phospholipid bilayers, the temperature relative to the main phase transition temperature (T_m) dictates the fluidity of the membrane [32]. Above T_m, the lipids are in the liquid-crystalline state, in which the hydrocarbon chains are disordered and fluid-like state [30]. Below T_m, there are two relevant phase regions that correspond to the gel and ripple phases [30,31]. The latter is also known as the pre-transition and coincides with the beginning of bilayer melting where some of the lipids are in a semi-ordered, gel-like phase interspersed with lipids in the more fluid and disordered liquid-crystalline phase [34,35]. Below the pre-transition, the bilayer is in a solid-like gel phase. It has been documented that below the main phase transition, the uniaxial rotational diffusion of membrane proteins is significantly slowed while above the T_m, the diffusional rate is increased [33]. The incorporation of membrane proteins into the lipid bilayer results in broadening of both pre- and main phase transitions such that at low lipid:protein ratios the former can be broad beyond detection [36–38].

In this work, we took a systematic approach to correlating the membrane fluidity with the solid-state NMR spectral quality from MAS (sensitivity and resolution) for polytopic membrane transporters from the small multidrug resistance (SMR) family. Our experiments involved measuring main phase transitions of proteoliposomes (SMR proteins in DMPC and DPPC) using differential scanning calorimetry (DSC) and acquisition of multidimensional MAS spectra under a wide range of temperatures. These findings serve as a guide for optimizing membrane protein studies by MAS and show that the optimal rotational dynamics for high quality NMR spectra of polytopic membrane proteins requires diffusional rates that are neither too fast nor too slow.

2. Methods

2.1. Growth and purification of EmrE and SugE

EmrE was expressed as a fusion protein with maltose binding protein (MBP) in BL21 (DE3) cells. To selectively incorporate [2-^{13}C,^{15}N] Leu, cells were grown in M9 minimal media containing 120 mg/L of [2-^{13}C,^{15}N] Leu (Sigma-Aldrich), 800 mg/L of natural abundance Ile and Val, and 300 mg/L of all other amino acids. For simultaneous incorporation of Leu and Val, the media contained 120 mg/L of [2-^{13}C,^{15}N] Leu, 120 mg/L of [$^{13}C_5$,^{15}N] Val (Sigma-Aldrich), 800 mg/L of natural abundance Ile, and 300 mg/L of all other amino acids. EmrE was purified as previously described [1,24]. Reverse-IL labeled SugE (U-[^{13}C,^{15}N] with natural abundance Ile and Leu) was grown and purified in the same manner as EmrE [24].

2.2. Reconstitution of EmrE into DPPC or DMPC liposomes

EmrE was purified in DDM detergent (buffer: 20 mM Na_2HPO_4 and 20 mM NaCl at pH 6.9) and reconstituted into 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (Avanti Polar Lipids) using Bio-Beads (Biorad) overnight at 4 °C (45:1 w:w of Bio-Beads to detergent). Proteoliposomes were pelleted via ultracentrifugation at 177,000 × *g* (max) for 1.5 h. For NMR samples, the buffer was exchanged to 20 mM HEPES (pH 6.9), 20 mM NaCl, 50 mM DTT, and 0.05% NaN_3 and packed into the 3.2 mm MAS rotor.

2.3. Differential scanning calorimetry

DSC experiments were performed with a nanoDSC (model 6300; TA Instruments). The samples were suspended in 20 mM Na_2HPO_4, 20 mM NaCl at pH 6.9. The apo EmrE experiments used a lipid:monomer ratio of 100:1 (mol:mol). The lipid concentration in the experiments was 9.2 mM. The temperature range for DSC was 4–55 °C with a scanning rate of 4.8 °C/h at a constant pressure of 3 atm and 600 s of equilibration time prior to scanning. A blank run was performed with buffer alone and subtracted from the sample curves to obtain ΔC_p. Data was analyzed using NanoAnalyze v2.4.1 (TA Instruments), Gnuplot v4.6, and Matlab vR2012a (MathWorks).

2.4. NMR spectroscopy

The pelleted proteoliposomes were partially dehydrated using lyophilization and then center-packed into 3.2 mm thin-walled rotors using sample spacers. All MAS experiments were performed at 14.1 T using a DD2 spectrometer (Agilent) with a bioMAS probe (Agilent) doubly tuned to ^{1}H and ^{13}C or triply tuned to ^{1}H, ^{13}C, and ^{15}N. The 90° pulses for ^{1}H, ^{13}C, and ^{15}N corresponded to frequency strengths $\omega/2\pi$ of 100 kHz, 45.5 kHz, and 45.5 kHz, respectively. MAS rates were 10 kHz or 12.5 kHz. The temperature titration 1D ^{1}H–^{13}C cross-polarization experiments used a contact time of 200 μs, an acquisition time of 25 ms, a ^{13}C spectral width of 100 kHz, and TPPM ^{1}H decoupling [39] at 100 kHz ($\omega/2\pi$). 2D NCA experiments were acquired on tetraphenylphosphonium (TPP^+) bound [2-^{13}C,^{15}N-Leu]-EmrE in DMPC. The ^{1}H–^{15}N contact time was 600 μs or 950 μs (shorter at warmer temperatures to compensate for ^{1}H $T_{1\rho}$ values). The transfer from ^{15}N to ^{13}CA used SPECIFIC-CP [40] and a contact time of 3.5 to 4.5 ms (shorter at warmer temperatures). The indirect dimension had a spectral width of 3125 Hz and 8 ms of evolution. The direct dimension had a spectral width of 100 kHz and 25 ms of acquisition time. The 2D spectra were acquired with 64 scans (−21 °C, −10 °C, −1 °C), 128 scans (9 °C), or 320 scans (14 °C) to partially account for the loss in signal-to-noise from increases in temperature. The variable temperature display and effect of spinning were calibrated using methanol [41]. The heating due to decoupling was determined with KBr inside a rotor containing DMPC hydrated with the buffer, to simulate a protein sample. Specifically, KBr (~16.5 mg) was placed between the top spacer and the rotor cap and did not mix with the lipid. Using the chemical shift difference of ^{79}Br [42] 20 ms of ^{1}H decoupling ($\omega/2\pi$ ~100 kHz, recycle delay of 2 s) increased the sample temperature by 1.5 °C, which is consistent with heating previously reported from the bioMAS scroll coil [43]. Data were processed with VnmrJ v3.1a (Agilent), NMRPipe [44], Gnuplot v4.6, and Sparky [45].

3. Results

3.1. Differential scanning calorimetry

To correlate the bilayer fluidity with the MAS solid-state NMR observables, we used membrane protein transporters from the SMR family [46,47] reconstituted into synthetic lipid bilayers. The primary sequence and predicted transmembrane (TM) domains of the model SMR protein EmrE are shown in Fig. 1A. For carrying out the differential scanning calorimetry (DSC) experiments, we reconstituted EmrE into DMPC (14:0) and DPPC (16:0) liposomes at a lipid:protein ratio of 100:1. The lipid only vesicles were reconstituted in an identical fashion through detergent to ensure similar liposome formation mediated by the polystyrene beads. The DSC thermogram data provides a plot of the heat capacity as a function of temperature with the peaks corresponding to phase transitions. In this work, we integrated the DSC data as a proxy for bilayer fluidity which is essentially the fraction of lipids in the liquid-crystalline phase [32]. All values of fluidity are reported as *fluid fraction* that resulted from cumulative integration of the main phase transition peak from the DSC thermogram data. In the gel phase, the bilayer is ordered and rigid (fluid fraction = 0), whereas in the liquid-crystalline phase, the bilayer is dynamic and disordered (fluid fraction = 1) [30,32,34]. Fig. 2A and D show thermogram data for DMPC and DPPC bilayers in the absence of protein where the pre-transition (gel to ripple phase transition) is seen as a small, broad peak at ~10 °C below the main phase transition. This phase transition is considered the start of bilayer melting, and is a period where the bilayer is dominated by rigid lipid acyl chains, periodically separated by more fluid lipid acyl chains, as well as marking the point where the fluid fraction begins to rise