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Probing membrane protein ground and conformationally excited states using dipolar- and J-coupling mediated MAS solid state NMR experiments

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Abstract

The intrinsic conformational plasticity of membrane proteins directly influences the magnitude of the orientational-dependent NMR interactions such as dipolar couplings (DC) and chemical shift anisotropy (CSA). As a result, the conventional cross-polarization (CP)-based techniques mainly capture the more rigid regions of membrane proteins, while the most dynamic regions are essentially invisible. Nonetheless, dynamic regions can be detected using experiments in which polarization transfer takes place via J-coupling interactions. Here, we review our recent efforts to develop single and dual acquisition pulse sequences with either ¹H or ¹³C detection that utilize both DC and J-coupling mediated transfer to detect both rigid and mobile regions of membrane proteins in native-like lipid environments. We show the application of these new methods for studying the conformational equilibrium of a single-pass membrane protein, phospholamban, which regulates the calcium transport across the sarcoplasmic reticulum (SR) membrane by interacting with the SR Ca²⁺-ATPase. We anticipate that these methods will be ideal to portray the complex dynamics of membrane proteins in their native environments.

Keywords

Solid-State NMR; ¹H detection; membrane proteins; magic angle spinning; conformationally excited states; phospholamban; RI-TOBSY; RI-HSQC; rINEPT; SERI-HSQC; INEPT-HETCOR

INTRODUCTION

As with all biopolymers, membrane proteins exist in their native environment as an ensemble of conformational states that are in equilibrium^{1–4}. Changes in the environment, e.g., lipid compositions, mutations, and ligand binding shift the conformational equilibrium

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toward active or inactive states. Solid-state NMR (ssNMR) is the method of choice for probing structure and dynamics of membrane proteins at atomic resolution^{5–7}. However, sensitivity and resolution of multidimensional NMR spectra remain problematic, hampering the detection of sparsely populated, high-energy conformational states. Moreover, the high redundancy of the primary sequences of the transmembrane (TM) segments and their intrinsic dynamics significantly broaden the NMR resonances in the homo- and hetero-correlated spectra^{8,9}.

Several reviews have illustrated the most significant achievements in ssNMR of membrane proteins, which involve the use of high-field magnet technology, advances in probe design, isotopic-labeling schemes, improved sample preparation, and new pulse sequence design^{10–13}. Recently, several outstanding examples on the application of these new techniques have been reported^{14–17}. A parallel development in ssNMR has been the use of dynamic nuclear polarization (DNP) techniques¹⁸ which would boost the NMR signals of membrane proteins up to 90 fold¹⁹. Although there are a few examples of its application to membrane proteins, the intrinsic challenges encountered when setting up DNP experiments is limiting their routine use. In addition, the sensitivity of membrane proteins to the composition of the membrane environment warns against doping the samples with large concentrations of spin labels necessary for the DNP transfer. Therefore, the characterization of sparsely populated states represents an ongoing challenge for the membrane protein field, and yet they are often central to proteins' biological function^{20–22}.

Depending on the exchange rates between the ground and the conformationally excited states, solution NMR provides several techniques allowing for their atomic resolution structure determination. These include rotating frame relaxation experiments ($R_{1\rho}$), Carr-Purcell-Meiboom-Gill (CPMG), chemical exchange saturation transfer (CEST) and zz -exchange experiments (R_{1zz})⁴. Only if the exchange between well-defined conformational states is slow compared to the frequency difference of their associated chemical shifts, is it possible to directly identify discrete populations of resonances and characterize these states structurally.

In these past years, our group has been redesigning MAS ssNMR experiments to boost both sensitivity and resolution of membrane protein spectra. Our approach, Polarization Optimized Experiments or POE, utilizes orphan spin operators to recover nuclear spin polarization for obtaining multiple spectra from one experiment using only one receiver^{23–25}. The basis for POE methodology is simultaneous cross polarization (CP) and generation of long-lived ¹⁵N polarization of isotopically labeled proteins. Recently, we extended POE to detect sparsely populated states of membrane proteins^{26,27}. This was possible thanks to ¹H detection, which has drastically enhanced POE sensitivity. While ssNMR experiments rely predominantly on the use of CP-based experiments, dynamic regions of membrane proteins and protein fibril preparations can be mapped using J -coupling mediated experiments such as INEPT^{27–31}. In fact, mobile domains of membrane proteins that are solvent exposed may undergo fast motions that average out dipolar interactions, rendering these domains essentially 'invisible' to CP-based NMR detection. However, the increase of the transverse relaxation time (T_2) of these mobile residues facilitates their detection via through-bond polarization transfer (i.e., J -coupling mediated

polarization transfer). Similarly, ^{13}C detected rINEPT and CP experiments have been used for studying mobile and rigid domains of semi-solid materials^{32–35}.

Here, we review the principles and the use of CP- and INEPT-based 1D and 2D pulse sequences that were successfully used to study the conformational dynamics of the single-pass membrane protein phospholamban (PLN), whose conformational equilibrium is essential for regulating calcium transport in cardiomyocytes (Figure 1A), and thereby cardiac muscle contractility^{2,36}. We also describe new POE pulse sequences that combine CP- and INEPT-based experiments into a single pulse sequence to map both rigid and dynamic domains simultaneously²⁶.

DESCRIPTION OF THE PULSE SEQUENCES

Figures 1B and 1C depict the pulse sequences for CP and rINEPT experiments that are used for mapping the amide spectra of rigid and dynamic domains, respectively^{37,38}. CP-based experiments utilize strong homo- and hetero-nuclear DC networks to transfer polarization from a ^1H spin bath to ^{15}N or ^{13}C nuclei through a collective spin phenomenon that occurs with typical transfer periods ranging from 0.3 to 1 ms depending on the $R_{1\rho}$ relaxation mechanism. In contrast, ^{15}N rINEPT transfer exploits a $1/J_{\text{NH}}$ evolution period (typically ~10 ms) that selects the polarization from mobile residues with long T_2 , while dephasing signals of shorter T_2 such as membrane-bound residues³⁹. The rate of polarization transfer in the rINEPT experiment is an order of magnitude slower than the corresponding CP experiment, and the combination of these two methods enables one to probe membrane protein dynamics in a wide range of time scales.

In principle, implementation ^{15}N -detected 1D experiments into a 2D ^{15}N - ^1H chemical shift correlated experiment is feasible. However, the low sensitivity of ^{15}N nuclei represents a significant challenge for heteronuclear-detected multidimensional experiments. We have overcome this problem by using ^1H -detected pulse sequences. Figures 1D and 1E show the basic ^1H -detected 2D pulse sequences for CPHSQC and RI-HSQC^{27,40}. The ^1H -detected RI-HSQC pulse sequence consists of a water suppression element (τ_1) and a t_1 evolution period that are sandwiched between two ^1H - ^{15}N refocused INEPT periods²⁹. On the other hand, in the CP-HSQC pulse sequence (Figure 1D) the water suppression and t_1 evolution periods are sandwiched between two ^1H - ^{15}N CP elements followed by ^1H detection under a windowed PMLG sequence, which significantly improves ^1H linewidths by suppressing homonuclear DC^{40,41}.

In the HNH CP-based experiments under moderate MAS conditions, larger ^1H line widths of fully protonated membrane bound residues pose another significant challenge for obtaining residue-specific resolution. In this case, ^{13}C -detection for residue specific resonances achieves reasonable spectral sensitivity and resolution. In particular, ^{13}C - ^{13}C correlated spectra can provide numerous through-bond and through-space correlations from backbone carbons to side chain carbons for resonance assignments. Figures 1F and 1G show the pulse sequences for the 2D ^{13}C - ^{13}C correlation experiments CP-DARR and RITOBYSY. These experiments are crucial for mapping the ^{13}C fingerprints of immobile and dynamic regions of membrane proteins. The initial polarization for CP-DARR and RI-TOBSY experiments is

obtained from CP and rINEPT pulse sequence blocks, respectively, followed by a t_1 evolution period. During the ^{13}C - ^{13}C homonuclear mixing period, the DARR experiment uses dipolar-assisted polarization transfer⁴², whereas the polarization transfer during the TOBSY experiments occurs via through-bond homonuclear transfer by a $\text{P}9^1_6$ mixing sequence⁴³. During the acquisition period (t_2), the RI-TOBSY utilizes a WALTZ-16 heteronuclear J -decoupling sequence⁴⁴. On the other hand, in the CP-DARR, a SPINAL-64 sequence suppresses heteronuclear DC⁴⁵. Note that CP-DARR is capable of detecting long-range inter-residue distances (5–7 Å) when the mixing time is set to a range of 300–500 ms^{16,46}. In contrast, the TOBSY mixing time (typically 6 to 12 ms) can only detect intra-residue correlations^{28,43}. In recent years, we have developed POE methods to concatenate multiple CP-based 2D and 3D experiments⁴⁷. As an example, Figure 1H shows the extension of POE methodology for ^1H detected hybrid pulse sequence that combines both dipolar and J -coupling based experiments into a single experiment²⁶. The sensitivity enhanced (SE) RIHSQC is acquired during the first acquisition period followed by a 90° pulse on ^{13}C that creates ^{13}CO direct polarization used for recording CP-HSQC in the second acquisition period. Note that the sensitivity of RI-HSQC (or SERI-HSQC) obtained from single and dual acquisition methods is almost identical (Figures 1B and 1H); whereas the sensitivity of the CP-HSQC obtained from hybrid pulse sequence (Figure 1H) is about 40 to 45% lower with respect to single acquisition pulse sequence (Figure 1D). Nevertheless, almost no additional time and no repetition of the recycle delay is required for the acquisition of the CP-HSQC spectrum together with the RI-HSQC experiment.

Experimental considerations and details

Solid-state MAS NMR experiments on proteins are carried out at either moderate spinning speeds with 3.2 or 4 mm rotors, or fast spinning speeds with 1.3 mm diameter rotors. Due to the small sample volume, the 1.3 mm probes and rotors require high concentrations of proteins such as micro-crystalline preparations in which the hydration levels are low. In the case of 3.2 mm rotors, the larger sample volume constitutes a significant advantage for membrane protein preparations, which require high lipid-to-protein ratios and sufficient hydration to mimic physiological conditions. The experiments outlined in this work are mainly optimized for MAS spinning rates ranging from 10–15 kHz that preserve the function of enzymes such as Ca^{2+} -ATPase, which is regulated by PLN. The description of the procedures for protein reconstitution in lipid membranes and MAS sample preparations are reported in our previously published protocols². Note that our membrane protein preparations contain at least 100 lipids per protein to maintain native like conditions and avoid adventitious protein aggregation. Typically, the reconstituted samples contain 2–3 mg of protein with a hydration level of approximately 120% (W/W). In our experiments, amide signals in 1D ^{15}N CP and rINEPT experiments were used to quantify the population of the conformationally excited state of PLN relative to the ground state. Also, the comparison of 2D CP and INEPT-based experiments was used to probe residue-specific structural changes upon perturbation of the conformational equilibrium.

All solid-state NMR experiments were acquired at the Minnesota NMR Center using Bruker or Agilent spectrometers operating at a ^1H Larmor frequency of 600 or 700 MHz equipped with 3.2 mm MAS probes with reduced RF heating technology. The recycle delay was set to

3 s and the t_2 spectral width was set to 100 kHz. Note that the acquisition and evolution times of CP-based experiments are generally shorter than the corresponding INEPT-based sequences. The t_2 acquisition time was set to 30 and 20 ms for INEPT and CP-based experiments, respectively. The ^{15}N and ^{13}C CP contact times were set to 1 ms, whereas ^{15}N and ^{13}C rINEPT periods (2τ) were set to 10.4 and 6.2 ms respectively. For the ^1H -detected experiments, water suppression was achieved by spin-lock pulses with phases x and y with RF amplitude of 30 kHz and τ_1 set to 200 to 300 ms⁴⁸. The number of t_1 increments for RI-HSQC and CP-HSQC was set to 80 and 40, respectively, with a dwell time of 200 μs resulting in a maximum t_1 evolution period of 16 and 8 ms. For ^{13}C -detected RI-TOBSY and CP-DARR experiments, the transmitter offset was positioned at 40 and 100 ppm, respectively. For the TOBSY experiment, the dwell time was set to 60 μs and a total of 60 t_1 increments were used. The DARR experiment was acquired with 256 t_1 increments with 30 μs dwell time. For the INEPT-based experiments, heteronuclear decoupling for ^1H , ^{13}C , or ^{15}N was obtained using a WALTZ-16 sequence with 10 kHz RF amplitude. For the CP-based experiments, a 100-kHz SPINAL decoupling on ^1H was used during t_1 and t_2 periods. During the t_2 ^1H acquisition of CP-HSQC experiment, a window-PMLG (phase modulated Lee-Goldberg) was applied on ^1H channel⁴¹. The window acquisition parameters were optimized using a U- ^{13}C , ^{15}N NAVL (N-acetyl-Valine-Leucine) sample as a standard, with ^1H linewidths of 120–150 Hz. In each cycle of window-PMLG, a detection period of 1.8 μs was inserted between 1.3 and 0.8 μs delay periods that account for receiver and probe ringing, followed by m5m PMLG sequence with RF amplitude of 100 kHz and length 14 μs .

RESULTS AND DISCUSSION

In this section, we summarize our recent findings on PLN conformational dynamics using the pulse sequences shown in Figure 1^{26–28,37,42,43}. In lipid membranes, PLN undergoes a conformational equilibrium between an ordered T state (ground state) and a dynamic R state (conformationally excited state). In the T state, the transmembrane domain crosses the membrane at an angle of ~ 20 degrees and the cytoplasmic domain is adsorbed onto the surface of the membrane forming an amphipathic α -helix⁴⁹. PLN anchors and inhibits the function of Ca^{2+} -ATPase via intramembrane interactions, docking in a groove formed by helices TM4 and TM9 of the ATPase^{2,50}. The dynamic cytoplasmic domain of PLN constitutes the regulatory region, which harbors the phosphorylation sites for protein kinase A (Ser16) and CaMKII (Thr17). We found that it is possible to increase Ca^{2+} transport by the Ca^{2+} -ATPase by shifting the population of the conformational ensemble toward the excited state via single site mutations^{51,52}. Other PLN variants support these findings, showing that the population of these high energy states can be shifted by phosphorylation, genetic or engineered mutations (R14 deletion, and S16E, T17E), or lipid composition. Although it is difficult to determine the absolute R-state population, we utilized the relative integrated intensities from the rINEPT and CP experiments to obtain a semi-quantitative estimate of the R-state population^{27,53}. Figure 2 shows the comparison of the 1D ^{15}N signal intensities for CP and rINEPT spectra, which display significant changes in the conformational equilibrium among different PLN variants. The rINEPT experiment detects the resonances of the cytoplasmic domain of R-state exclusively, and enables us to estimate the relative change of signal intensity among different PLN variants with respect to

corresponding CP signal. When reconstituted in zwitterionic DMPC lipids, the R9C mutant shows the lowest R-state population (0.3%), whereas the R14del and R25C mutants display 20 and 25% R state population, respectively. The R state population of PLN, which is about 3% in DMPC, can be enhanced up to 6 and 25% by incorporating the positively charged lipids, ePOPC, which interacts with the positively charged Lys and Arg residues of the cytoplasmic region of PLN^{27,53}.

The 2D ¹H detected CP-HSQC (Figure 3) features very broad line widths due to the strong ¹H-¹H DC anisotropic interactions, whereas the fast dynamics of the conformationally excited R-state quenches the anisotropic interactions, giving rise to narrow line widths as shown in the RI-HSQC spectra. In these experiments, we observed a significant variation in the relative peak intensities as well as line widths between 45 to 105 Hz, indicating a high degree of conformational heterogeneity dynamics. In particular, serine and threonine residues (Ser10, Ser16 and Thr17) located between 112 to 117 ppm in the ¹⁵N dimension are extremely sensitive to conformational dynamics. The serine and threonine peaks are very intense in the R25C sample, which has higher population of the R state, whereas these peaks are either undetectable or broadened beyond detection in other PLN mutants, displaying a lower population of R-state. In fact, serine and threonine residues are located in the hinge of cytoplasmic region that connects the structural regions and are very sensitive to a shift of the conformational equilibrium.

To further investigate the conformational changes occurring upon mutations, we acquired ¹³C-¹³C correlated spectra using RI-TOBSY and CP-DARR experiments. Figures 4 and 5 show residue-specific changes for PLN^{AFA}, R9C and R25C samples reflecting the T-to-R state equilibrium⁵³. The CA-CB assignments of the R-state in the TOBSY spectra (shown in red) for residues 1–20 were transferred from the solution NMR data of PLN in isotropic bicelles^{29,53}. Whereas the assignments of T-state residues of CPDARR spectra were previously carried out by our group using MAS ssNMR experiments^{2,54}. It is apparent that the cytoplasmic Ala residues (Ala11 and Ala15) are in slow conformational exchange with two distinct peaks at 57.5 and 53.0 ppm assigned to T and R state, respectively. These two populations are apparent for PLN^{AFA} and R25C; however, they are missing or undetectably broad in the TOBSY spectrum of the R9C mutant. For the R25C mutant, the signals arising from serine and threonine residues located in the hinge connecting domains Ia and Ib are essentially missing in the DARR spectrum, but are detectable in the TOBSY spectrum due to their high mobility. Note that resonances corresponding to these residues are also present in the spectra of PLN^{AFA} and R9C, albeit with significantly lower intensities. Taken together, these observations demonstrate that the loop region is more mobile for R25C than the PLN^{AFA} and R9C mutants. Finally, Figure 6 shows further evidence of cytoplasmic region dynamics. In this experiment, we mapped the ¹⁵N-¹H backbone resonances of dynamic and immobile residues simultaneously by acquiring RI-HSQC and CP-HSQC in a single experiment at various temperatures (Figure 1H)²⁶. While the peak positions of the CP-HSQC detected residues remains the same, the R-state cytoplasmic domain peaks shift toward an unfolded state as function of the temperature. Note that the chemical shifts of the resonances in the cytoplasmic domain represent a weighted average of a range of conformations including T and R states^{51,54,55}. A complete unfolding of the cytoplasmic

domain occurs only by truncating the transmembrane domain and analyzing only the peptide corresponding to residues 1–23 of PLN⁵⁶.

Recent progress on solid-state NMR of membrane proteins have demonstrated the importance of using both dipolar- and J-coupling mediated pulse sequences for complete structural mapping 2,28,30,39,57. Phospholamban is a typical example for the application of these methods to demonstrate the change in conformational equilibrium using 1D and 2D experiments^{26,27,53,58}. Similar pulse sequences using both ¹³C- and ¹⁵N-detected CP- and rINEPT-based experiments have also been applied to various single- and multi-pass membrane proteins including sarcolipin, α -synuclein, bacterial chemotaxis receptor, GPCR, and BamA complex^{16,28,59–63}. However, the lower sensitivity of these experiments dramatically increases the total experimental time. In fact, the 2D ¹⁵N-detected HSQC experiment was not feasible for the PLN samples due to the low population (less than 5%) of the dynamic R state⁵³. To overcome this problem, we developed ¹H-detected RI-HSQC and SERI-HSQC methods that hasten the 2D acquisition of INEPT-based experiments up to 100 times compared to the corresponding ¹⁵N detection experiments²⁷. While ¹H detection can also be applied for CP-based experiments via window-PMLG acquisition, fewer window acquisition points limit the sensitivity of this experiment⁶⁴. In general, ¹H detection for CP-based experiments has higher performance at fast MAS rates using either fully protonated or perdeuterated protein samples^{65–69}.

In conclusion, we have shown that ssNMR is a unique technique for probing immobile and mobile regions of membrane proteins. A combination of CP and INEPT-based experiments has been successfully employed to demonstrate the relative change of the populations of the T and R states of PLN variants reconstituted in hydrated proteoliposomes. The combination of ¹H and ¹³C-detected CP and INEPT-based experiments made it possible to map the relative structural changes of ground and excited states of different PLN mutants. We anticipate that these methods will be routinely used to probe the motions and conformationally excited states of membrane proteins of higher complexity than single-pass membrane proteins.

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Highlights

CP-based experiments probe rigid regions of membrane proteins

INEPT-base experiments image dynamic regions of membrane proteins

Dual acquisition enables the simultaneous detection of rigid and dynamics regions Solid-state NMR detects ground and excited states of membrane proteins

Figure 1:

(A) Schematic of the conformational landscape of PLN, which is sensitive to polarization transfer via dipolar and J -couplings under moderate MAS (10 to 15 kHz) conditions, adapted from reference²⁶ with permission. One-dimensional ^{15}N -detected pulse sequences CP (B) and rINEPT (C), that respectively probe immobile and dynamic regions of membrane proteins^{37,38}. (D, E) ^1H detected 2D CP-(H)NH and RI-HSQC pulse sequences that acquire N- H^{N} correlation spectra^{27,40}. (F, G) ^{13}C -detected CP-DARR and RI-TOBSY experiments for the acquisition of ^{13}C - ^{13}C correlation spectra via dipolar and J -couplings respectively^{42,43}. (H) ^1H -detected pulse sequence for N-H correlation spectra of dynamic

and immobile residues by acquiring SERI-HSQC and CP-HSQC in first and second acquisitions respectively²⁶. TOBSY pulse sequence was implemented with 10 kHz MAS rate using rotor synchronized P¹₆ mixing⁴³. Whereas all the remaining pulse sequences were implemented at 12 kHz MAS rate.

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Figure 2:

A relative estimation of the conformationally excited state of various PLN samples using backbone ^{15}N spectra obtained from CP and rINEPT pulse sequences (Figures 1B and 1C) under identical conditions. The CP experiment detects T-state and transmembrane (TM) domain of R-state, whereas rINEPT exclusively detects cytoplasmic domain of R-state. (A) CP and rINEPT spectra of PLN samples where the integrated intensity of rINEPT was normalized with respect to CP and drawn in (B), demonstrating a systematic change in R-state population. Reproduced from ref. s^{26,27,53} with permission.

Figure 3:

(A) CP-HSQC spectrum of PLN^{AFA} obtained from the pulse sequence of Figure 1D. (B) 2D RIHSQC spectra of various PLN samples obtained from the pulse sequence of Figure 1E. Reproduced from Ref. s^{26,27,53} with permission.

(A) CP-DARR:

(B) RI-TOBSY:

Figure 4:

Comparison of ^{13}C - ^{13}C correlation spectra (A) CP-DARR and (B) RI-TOBSY that respectively map immobile and dynamic residues of PLN^{AFA} , PLN^{R25C} , and PLN^{R9C} samples. Reproduced from Ref.⁵³ with permission.

Figure 6: Simultaneous acquisition of RI-HSQC and CP-HSQC spectra of U- ^{13}C , ^{15}N PLN using the pulse sequence of Figure 1H. Reproduced from Ref.²⁶ with permission.