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ARTICLE

Three-dimensional experiment for solid-state NMR of aligned protein samples in high field magnets

Alexander A. Nevzorov · Sang Ho Park · Stanley J. Opella

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Abstract A pulse sequence that yields three-dimensional ^{1}H chemical shift / ^{1}H-^{15}N heteronuclear dipolar coupling / ^{15}N chemical shift solid-state NMR spectra is demonstrated on a uniformly ^{15}N labeled membrane protein in magnetically aligned phospholipid bilayers. Based on SAMPI4, the pulse sequence yields high resolution in all three dimensions at a ^{1}H resonance frequency of 900 MHz with the relatively low rf field strength (33 kHz) available for a lossy aqueous sample with a commercial spectrometer and probe. The ^{1}H chemical shift frequency dimension is shown to select among amide resonances, which will be useful in studies of larger polytopic membrane proteins where the resonances overlap in two-dimensional spectra. Moreover, the ^{1}H chemical shift, which can be measured from these spectra, provides an additional orientationally dependent frequency as input for structure calculations.

Keywords Bicelle · Membrane protein · Aligned sample · Protein structure

Two-dimensional experiments generally provide adequate resolution for NMR studies of proteins with fewer than about 100 residues; higher dimensional experiments are required to disentangle overlapping resonances in spectra of larger proteins, especially those with helical secondary structure. Three-dimensional experiments are standard practice in studies of isotropic samples by solution NMR (Cavanagh et al. 1996) and powder samples by magic angle spinning (MAS) solid-state NMR (Heise et al. 2005). The routine implementation of three-dimensional experiments is the next essential step in the development of solid-state NMR of aligned samples, since it is feasible to obtain spectra with similarly narrow resonance line widths from membrane proteins with between 37 and 350 residues in magnetically aligned phospholipid bilayers (De Angelis et al. 2004, 2006; Park et al. 2006a). In this Communication, we describe a pulse sequence that yields three-dimensional ^{1}H chemical shift/^{1}H-^{15}N heteronuclear dipolar coupling/^{15}N chemical shift spectra, and demonstrate its application to a uniformly ^{15}N labeled membrane protein in magnetically aligned phospholipid bilayers. This experiment, which is based on the newly developed SAMPI4 pulse sequence (Nevzorov and Opella 2006), is sufficiently robust that the resonances have narrow line widths in all three frequency dimensions in spectra obtained at 900 MHz with a commercial spectrometer and probe on a lossy aqueous sample.

The three-dimensional structures of membrane proteins with one and two trans-membrane helices in magnetically aligned phospholipid bilayers have been determined by solid-state NMR of aligned samples (De Angelis et al. 2006; Park et al. 2006b). In these initial examples, the principal sources of structural constraints are the orientationally dependent frequencies associated with the amide resonances in two-dimensional separated local field (SLF) spectra of ^{15}N labeled proteins, namely the ^{15}N chemical shift and the

Both Alexander A. Nevzorov and Sang Ho Park contributed equally to this work.

A. A. Nevzorov · S. H. Park · S. J. Opella (✉)
Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, San Diego, CA 92093-0307, USA
e-mail: sopella@ucsd.edu

^{1}H-^{15}N heteronuclear dipolar coupling frequencies. It is likely that it will be possible to determine the structures of membrane proteins with up to three or possibly four trans-membrane helices relying primarily on two-dimensional experiments. However, studies of larger membrane proteins, such as those with seven trans-membrane helices (Park et al. 2006a), will almost undoubtedly require the routine use of three-dimensional experiments on high field spectrometers.

The initial three-dimensional ^{1}H/^{15}N double-resonance experiments designed for aligned samples (Kumar and Opella 1991, 1993) were demonstrated using single crystals of peptides because of the limited resolution achievable with the first generation of SLF (Hester et al. 1976; Waugh 1976) and heteronuclear correlation (HETCOR) experiments. Polarization inversion spin exchange at the magic angle (PISEMA) (Wu et al. 1994) yields SLF spectra with narrower line widths in the heteronuclear dipolar coupling frequency dimension, and when incorporated into three-dimensional experiments (Ramamoorthy et al. 1995), yields well-resolved spectra of aligned samples of uniformly ^{15}N labeled proteins (Jelinek et al. 1995; Marassi et al. 1997). However, PISEMA has a limited bandwidth due to its reliance on precisely chosen off-resonance frequencies for the ^{1}H irradiations that effect homonuclear decoupling (Lee and Goldburg 1965), and this can be a limiting factor when the experiments are performed in high magnetic fields where there is a substantial spread of ^{1}H chemical shift frequencies. Indeed, even though the dipolar coupling and chemical shift frequency ranges for proteins in magnetically aligned phospholipid bilayers (bicelles) aligned with their normals perpendicular to the direction of the magnetic field are about 40% of those of the full values observed in crystal samples (De Angelis et al. 2004; Nevzorov et al. 2005), it is still challenging to obtain undistorted spectra over the full frequency spans on lossy aqueous samples with limited rf field strengths at high fields. The SAMMY pulse sequence (Nevzorov and Opella 2003), which addresses this limitation, has now been further developed (Nevzorov and Opella 2006); SAMPI4 not only yields narrower line widths, but also is less sensitive to frequency offsets when used to achieve selective spin exchange between the ''dilute'' (e.g., ^{15}N or ^{13}C) and ''abundant'' (i.e., ^{1}H) spins under conditions of homonuclear (^{1}H/^{1}H) decoupling.

Figure 1 is the timing diagram for a three-dimensional pulse sequence that incorporates HETCOR (t_1) and SAMPI4-based SLF (t_2 and t_3) experiments. Following cross-polarization and the subsequent spin-temperature inversion, during the t_2 interval, spin exchange occurs under conditions of homonuclear

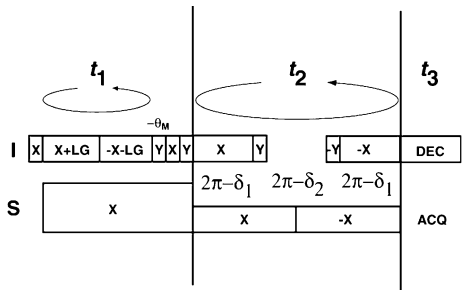

Fig. 1 Timing diagram for a pulse sequence that yields three-dimensional spectra of ^{15}N labeled proteins by evolution of the ^{1}H chemical shift during t_1, ^{1}H-^{15}N heteronuclear dipolar coupling during t_2, and ^{15}N chemical shift spectra during t_3

decoupling. The cancellation of the homonuclear terms over the total dwell is achieved using the first-order average Hamiltonians similar to Magic-Echo experiments (Rhim et al. 1971; Takegoshi and McDowell 1985). Choosing $\delta_1 = \pi/(4\omega_1)$ and $\delta_2 = \pi/(2\omega_1)$, where ω_1 is the B_1 rf field strength, compensates for the finite 90Y pulses in the middle of the pulse sequence. An integral number of SAMPI4 transfers are incremented as the t_2 dimension, with additional 180° phase cycling for the even dwells relative to the odd dwells to compensate for offsets due to chemical shifts and higher order imperfections. Prior to evolution during t_2, in order to transfer both the real and imaginary components of the ^{1}H chemical shift, σ_H, an intermediate 90Y pulse is applied. After the magic-angle pulse $-\theta_M$ that is followed by a 90X pulse, the density matrix can be approximated (assuming complete homonuclear decoupling) as:

$$\rho(t_1) = I_z \cos\left(\frac{\sigma_H}{\sqrt{3}} t_1\right) - I_x \sin\left(\frac{\sigma_H}{\sqrt{3}} t_1\right)$$

The component of the magnetization along the Z-axis is not transferred by SAMPI4; as a result, when the third 90Y pulse is included, it selects the cosine term by placing the I_z term along the X-axis, and when it is absent, only the sine term is selected.

The experimental three-dimensional spectrum presented as a cube in Fig. 2 was obtained by applying the pulse sequence diagramed in Fig. 1 to a sample of uniformly ^{15}N labeled Pf1 coat protein in magnetically aligned phospholipid bilayers. Uniformly ^{15}N labeled bacteriophage was prepared as described previously (Thiriot et al. 2004), the coat protein was purified by HPLC, and the sample was prepared using 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-di-O-hexyl-*sn*-glycero-3-phosphocholine (6-O-PC) from Avanti Polar Lipids, Inc. (http://www.avantilipids.com), also as described previously (De Angelis

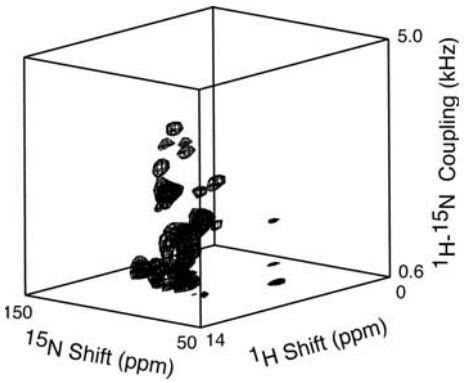

Fig. 2 Three-dimensional ^{1}H chemical shift / ^{1}H-^{15}N heteronuclear dipolar coupling / ^{15}N chemical shift spectrum of the membrane-bound form of uniformly ^{15}N labeled Pf1 coat protein in magnetically aligned phospholipid bilayers. The ^{1}H resonance frequency was 900 MHz and the rf field strength was 32.9 kHz. During ^{1}H chemical shift evolution the amplitude of the ^{15}N irradiation power was arranged to be approximately 50% greater than that used for the Hartmann-Hahn match. The experimental three-dimensional data set consisted of 20 complex t_1 points and 40 real t_2 points. 32 free induction decays (t_3) were co-added for each data point. The data were acquired on a spectrometer configured with a standard-bore Magnex 900/52 magnet (http://www.Magnex.com), a Bruker Avance console (http://www.Bruker-biospin.com), and a Bruker triple-resonance probe with a 5 mm diameter horizontal solenoid coil (PH BIOPE900SB N/C(P)/H). The data were zero filled and yielded a $1024 \times 256 \times 128$ real matrix. The data were processed using the program FELIX (http://www.accelrys.com). The 0.577 scaling factor was applied in the ^{1}H shift dimension. The sample contained 5 mg of protein in a 160μl solution at pH 6.8. The molar ratio between long-chain lipids and short-chain lipids (q) was 3.2 and the lipid concentration was 26% (w/v)

et al. 2004). The resulting sample had a high dielectric constant, which limited the rf field strength that could be reliably generated by the probe (32.9 kHz, corresponding to a 90° pulse length of 7.6 μs). The narrow linewidths and consequently high resolution of the spectrum reflect adequate decoupling in all three dimensions. This demonstrates that the unfavorable combination of the low rf field strength and high static magnetic field strength is well compensated by the efficacy of the SAMPI4 homonuclear decoupling during t_2 and SPINAL 16 heteronuclear decoupling (Fung et al. 2000; Sinha et al. 2005) during t_3. In general, we find that the frequency-switched Lee-Goldburg irradiation is less sensitive to frequency offsets when it is used to provide homonuclear decoupling during ^{1}H chemical shift evolution during t_1, than is the case during spin-exchange in PISEMA.

Resonances from the N-terminal 17 residues of the 46-residue Pf1 coat protein are not detected in solid-state NMR spectra obtained on the membrane-bound form of the protein in magnetically aligned bilayer samples due to the effects of motional averaging. As a result, the spectrum in Fig. 2 has 29 backbone amide resonances, most of which contribute to the wheel-like pattern characteristic of a tilted trans-membrane hydrophobic helix (Marassi and Opella 2000; Wang et al. 2000). As a membrane protein with a single trans-membrane helix, these resonances have frequencies typical of those encountered in the spectra of larger polytopic membrane proteins (De Angelis et al. 2006). The properties of these resonances are shown more clearly as two-dimensional spectral planes in Fig. 3; the line widths are 300 Hz in the dipolar coupling dimension, 1 ppm in the ^{1}H chemical shift dimension, and 2 ppm in the ^{15}N chemical shift dimension. Since only a limited number of increments were obtained in the indirect dimensions, the line widths observed in this spectrum can be further narrowed by acquisition of additional points in t_1 and t_2 or by the use of non-linear sampling and maximum entropy reconstruction (Jones and Opella 2006).

In summary, the SAMPI4 decoupling scheme is demonstrated to effect broadband magnetization transfer between the covalently bonded ^{15}N and ^{1}H spins under the conditions of homonuclear decoupling over the entire ^{1}H chemical shift range present in this sample. This enables three-dimensional NMR spectra to be obtained on a lossy membrane protein sample at high magnetic fields with relatively low rf fields using a commercial spectrometer and probe. The limited number of resonances in each of the spectral planes

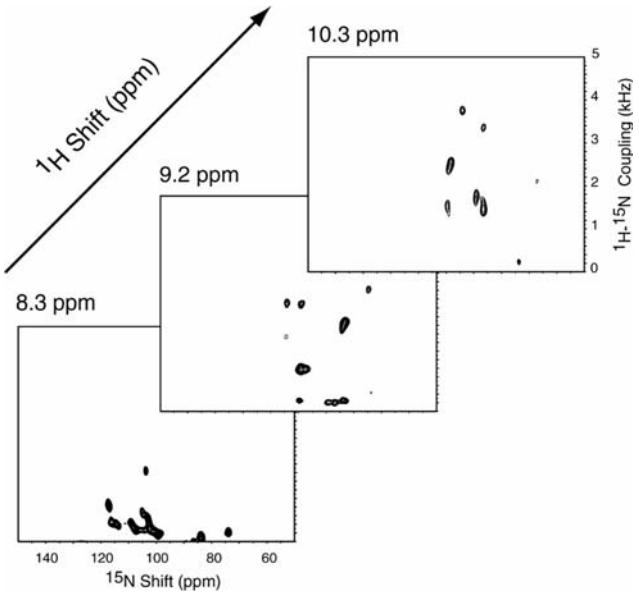

Fig. 3 Two-dimensional ^{1}H–^{15}N dipolar coupling / ^{15}N chemical shift planes taken from the three-dimensional spectrum shown in Fig. 2. The ^{1}H chemical shift frequencies are designated for each plane

indicates that the ^{1}H chemical shift dispersion available at 900 MHz will provide an effective mechanism for resolving among resonances that are overlapped in two-dimensional spectra. In addition, the anisotropic amide ^{1}H chemical shift interaction provides an angular constraint for protein structure determination.

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