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A Resonance Assignment Method for Oriented-Sample Solid-State NMR of Proteins

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Oriented-sample (OS) solid-state NMR spectroscopy is capable of determining the three-dimensional structures of proteins in their native functional environments when they are immobilized and aligned in supramolecular assemblies,[1] such as virus particles or membranes. Examples include gramicidin,[2] the AchR M2 domain,[3] the M2 domain of the influenza A virus,[4] fd[5] and Pf1[6] phage coat proteins, phospholamban,[7] Vpu (from HIV-1),[8] and MerF.[9]

With improved sample preparation methods and the implementation of experiments that yield high-resolution separated local field (SLF) spectra, the principal roadblock to atomic-resolution structure determination is obtaining sequence-specific assignments of resolved resonances. While the "shotgun" approach[10] simultaneously assigns spectra and measures structural constraints, it relies on the preparation of multiple selectively isotopically labeled samples and is restricted to residues in regular secondary structures, whether α-helix or β-sheet. The goal is to utilize uniformly labeled samples for all steps of the structure determination process. The feasibility of using dilute spin exchange to identify signals from proximate sites has been demonstrated for both virus particles[11] and membrane proteins.[12] However, this is limited by the requirement of lengthy intervals (several seconds) for the spin exchange among the weakly coupled nuclei to occur. As an alternative to the recent cross-relaxation-driven method,[13] a proton-mediated dilute spin exchange experiment that has the potential to accelerate data acquisition has been implemented.[14] The latter method is based on the transfer of magnetization between the low-γ spins via the proton bath under mismatched Hartmann−Hahn (MMHH) conditions. ^{15}N−^{15}N correlations for distances of up to 6.7 Å can be identified.[14] In principle, the method is applicable to any dilute-spin system bridged by a strong proton dipolar network (e.g., amide backbone ^{15}N spins), thus providing a strategy for sequential assignment of resonances in OS solid-state NMR spectra of proteins. The cross-peaks are established within a few milliseconds, dramatically shortening the overall experiment time relative to conventional dilute spin exchange experiments[11,12] based on spin diffusion.[15] The MMHH method has been extended to the measurement of heteronuclear dipolar couplings[16] by inclusion of the SAMPI4 pulse sequence in the indirect dimension,[17] thus yielding a spin-exchanged high-resolution SLF spectrum.

Solid-state NMR spectra of the structural form of Pf1 coat protein in magnetically aligned bacteriophage particles and of the membrane-bound form reconstituted in magnetically aligned bicelles have previously been measured and assigned[6,18,19] and therefore can be used as a test system for the applicability of the MMHH method to biological samples. Figure 1A shows the PISEMA[20] spectrum of uniformly ^{15}N-labeled Pf1 bacteriophage. The PISEMA spectrum

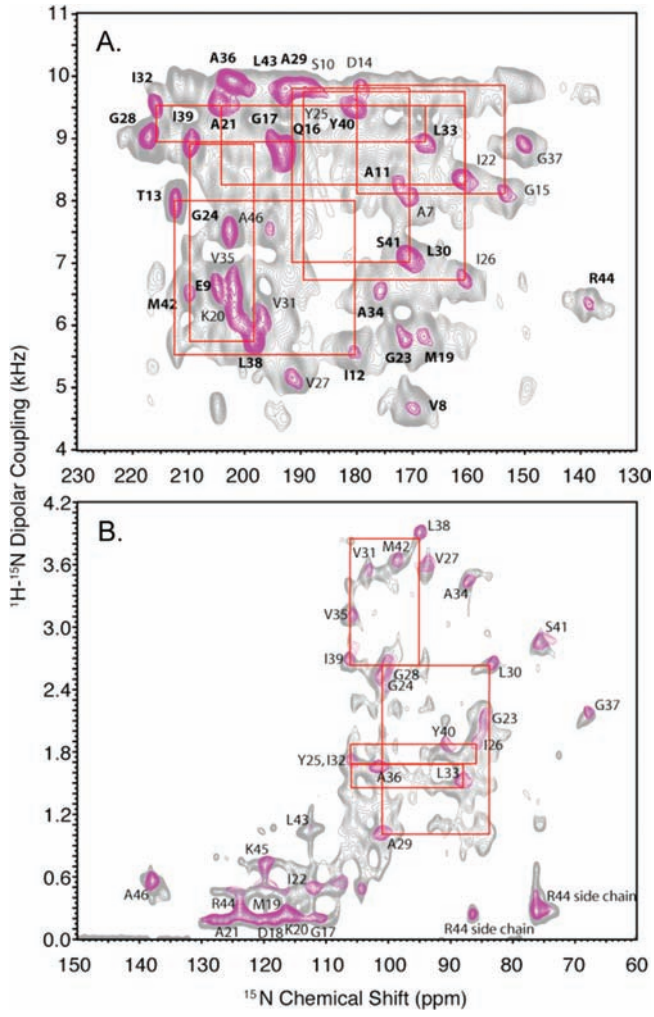

Figure 1. (A) PISEMA spectrum of Pf1 phage (magenta contours) overlaid with the spin-exchanged PISEMA spectrum (gray contours) acquired using the pulse sequence shown in Figure 2A. Data were acquired on a Bruker Avance II spectrometer operating at 500 MHz [temperature $T = -3$ °C, 64 scans, 50 kHz B_1 field, 80 t_1 points (magenta lines) and 1000 scans, 64 t_1 points, 4 ms contact time, 22% MMHH (gray lines)]. Only the helical part of the whole Pf1 spectrum is shown. Red boxes (depicted here for a number of representative residues) establish the connectivities between adjacent amide sites. (B) SAMPI4 spectrum of Pf1 coat protein reconstituted in magnetically aligned bicelles (magenta contours) overlaid with its spin-exchanged version acquired using the pulse sequence depicted in Figure 2B (gray lines). Data were acquired on a Bruker Avance III spectrometer operating at 700 MHz [32 scans, 50 kHz B_1 field, 82 linear t_1 points (magenta lines) and 1000 scans, 55 kHz ^{1}H B_1 field during the MMHH period and 50 kHz B_1 field everywhere else, 0.1 s Z filter, 82 linear t_1 points (gray lines)]. Representative connectivities for some of the residues (as in Figure 1A) are shown as illustrative examples.

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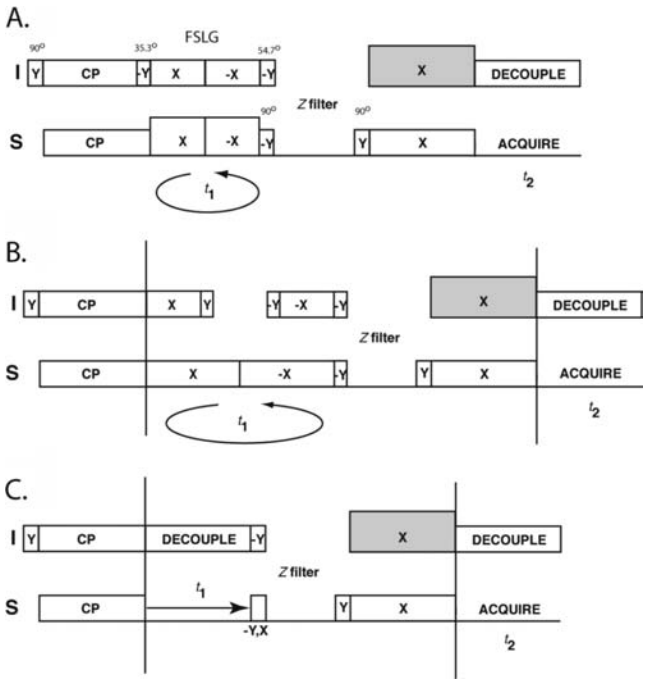

Figure 2. (A, B) Spin-exchanged SLF pulse sequences for spectroscopic assignment in solid-state NMR spectra of oriented samples. The t_1 dimension is evolved using either (A) the frequency-switched Lee−Goldburg scheme or (B) SAMPI4. This is followed by the *Z* filter and the proton-mediated spin exchange, during which the proton rf amplitude is set at 10−25% above the Hartmann−Hahn matching condition. (C) Homonuclear $^{15}N-^{15}N$ exchange employing mismatched Hartmann−Hahn magnetization transfer. The intermediate pulse before the *Z* filter selects either real or imaginary component of the t_1 evolution.

is overlaid with its spin-exchanged version acquired using the pulse sequence shown in Figure 2A. For the Pf1 bacteriophage spectra, PISEMA was used instead of SAMPI4 in order to measure larger (>5 kHz) dipolar couplings using the relatively low radiofrequency (rf) B_1 fields (<50 kHz) available on the spectrometer. For the spin-exchanged spectrum of Figure 1A, the proton-mismatch amplitude was set at 22% larger than the Hartmann−Hahn condition during the exchange period; the mixing time was set to 4 ms, and a 1 s *Z* filter was incorporated in order to eliminate residual proton magnetization and let the probe cool down before reapplication of a long period of continuous irradiation. The mismatch amplitude of ∼20% was found to be nearly optimal for the magnetization transfer among the backbone ^{15}N spins. In contrast, a 10% mismatch amplitude was previously found[16] to be optimal for the more distant ^{15}N spins in a NAL crystal (separated by as much as 6.7 Å as opposed to ∼2.8 Å for the ^{15}N spins in adjacent residues in an α-helix). Overlaying the spectra with and without spin exchange in Figure 1A allowed the cross-peaks to be distinguished from the main peaks and the sequential connectivity among the latter to be established. The red boxes depicted in Figure 1 connect two main peaks and two cross-peaks for each pair of interacting residues. For clarity, the results for residues I12−T13, D14−G15, A21−I22, A29−L30, Y25−I26, I32−L33, and L38-I39 are highlighted; many others are established by the displayed data. Notably, these assignments are in agreement with those obtained previously using a combination of selective isotopic labeling and the shotgun approach,[18] and the cross-peaks generally follow the $(i, i + 1)$ connectivity pattern. Additional weaker cross-peaks [e.g., $(i, i + 3)$] may arise from more distant $^{15}N-^{15}N$ interactions as a result of their proximity in helical structures; this merits additional investigation.

To demonstrate the feasibility of applying the MMHH method to membrane proteins, the membrane-bound form of the uniformly ^{15}N-labeled Pf1 coat protein was reconstituted into magnetically aligned bicelles as previously described.[19,21] Figure 1B shows the SAMPI4 spectrum of Pf1 coat protein in bicelles processed by the maximum entropy method (MEM)[22] (magenta contour lines). The SAMPI4 spectrum is overlaid with its exchanged version processed by MEM and acquired using the pulse sequence shown in Figure 2B (gray lines). Because of the perpendicular orientation of the normal relative to the magnetic field and the slightly reduced order parameter of the bicelle, the dipolar couplings were scaled by a factor of ∼0.4 relative to those of the magnetically aligned Pf1 phage. Therefore, the MMHH amplitude was reduced to ∼10% in order to retain the same average Hamiltonians[14] that result in the maximum magnetization transfer as in the case of magnetically

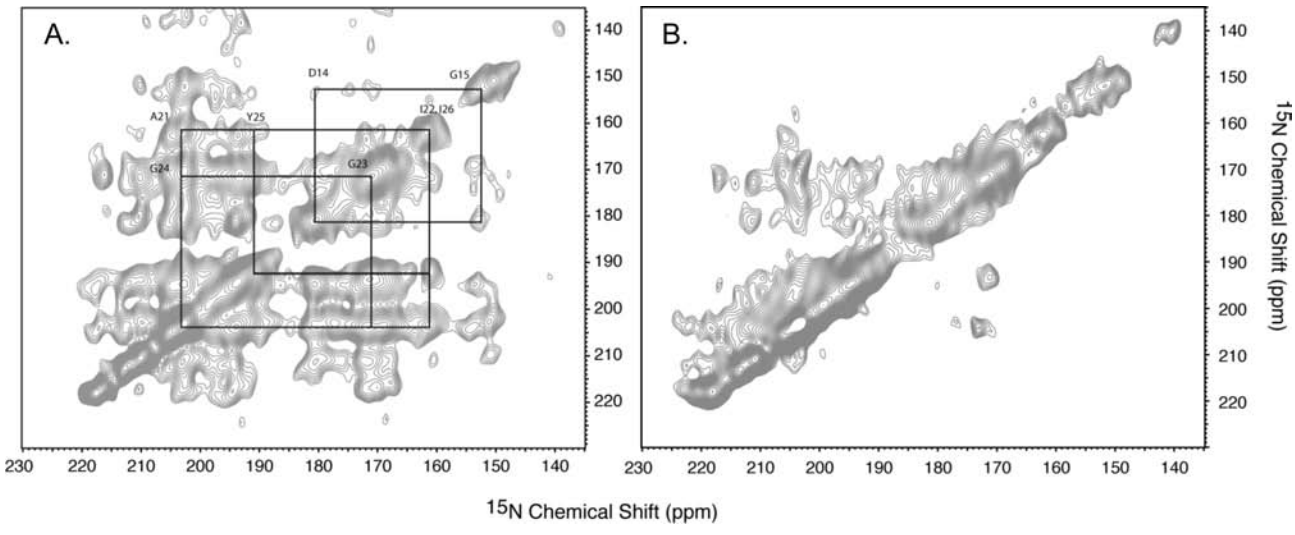

Figure 3. (A) $^{15}N-^{15}N$ exchange spectrum used to assist in the assignment process for magnetically aligned Pf1 phage (500 MHz ^1H field, 256 scans, 128 complex t_1 points, 1 s *Z* filter, 5 ms MMHH). Using the exchanged PISEMA spectra alone for spectral assignment may give rise to ambiguities when the main peaks have similar $^1H-^{15}N$ dipolar couplings, which causes the cross-peaks to be produced near the locations of the main peaks. As an illustrative example, the sequential assignments of Figure 1A for residues A21 through I26 were validated using the $^{15}N-^{15}N$ cross-peaks. (B) $^{15}N-^{15}N$ exchange spectrum acquired without using the MMHH block with 1 s exchange time (*Z* filter). Many of the cross-peaks in Figure 3A are missing from the spectrum.

aligned phage. Representative connectivities for some of the residues (as in Figure 1A) are shown; they are also consistent with earlier assignments.[19] This demonstrates that the MMHH spin exchange can be applied to assign spectra of membrane proteins in lipid bilayer environments.

In practice, the homonuclear $^{15}N-^{15}N$ spin exchange spectrum acquired using the pulse sequence shown in Figure 2C can provide independent validation of the assignment and aid in resolving ambiguities when the main peaks in the exchanged PISEMA spectrum have similar dipolar couplings. Figure 3A shows the $^{15}N-^{15}N$ exchange spectrum of magnetically aligned Pf1 phage, which provides the sequential assignment for residues A21 through I26 via cross-validation of the peaks in the corresponding exchanged PISEMA spectrum (Figure 1A). Other connectivities can also be established.

To further illustrate the efficiency of the MMHH transfer, a control experiment for Pf1 phage was performed without the MMHH block; the pulse sequence in this experiment essentially corresponds to that in the dilute spin exchange experiment[11,12] employing proton-driven spin diffusion. As can be seen from Figure 3B, only a very few cross-peaks are established after a mixing time of 1 s (corresponding to the 1 s *Z* filter in the MMHH experiment), and for such a short mixing time, the cross-peaks lack symmetry with respect to the main diagonal. More cross-peaks can be established when the mixing time is made longer than 3 s (results not shown); however, this considerably lengthens the overall time of the experiment.

The combination of MMHH spin exchange and high-resolution SLF spectroscopy accelerates the assignment and analysis of OS solid-state NMR spectra of uniformly labeled proteins. Moreover, inclusion of the additional dipolar coupling dimension helps eliminate ambiguities when the main peaks have overlapping chemical shifts. It has been shown that the method can be applied for various alignment media, including magnetically oriented bacteriophage and membrane proteins reconstituted in bicelles. Moreover, this purely spectroscopic technique is applicable to proteins of arbitrary topology. While selective labeling and the shotgun approach are still invaluable for the initial steps of assignment and elimination of potential ambiguities, this further development of the method improves the practicality of determining the three-dimensional structures of membrane proteins in their native phospholipid bilayer environments.

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