Communication

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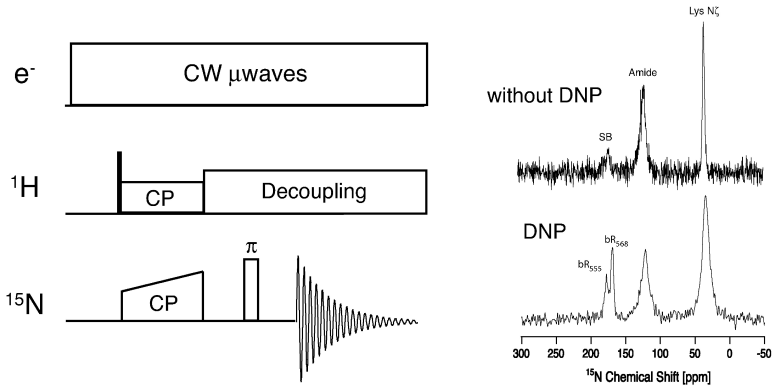

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High-Frequency Dynamic Nuclear Polarization in MAS Spectra of Membrane and Soluble Proteins

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One of the principal promises of magic angle spinning (MAS) solid-state NMR (SSNMR) experiments is the possibility of determining the structures of molecules in states that are not accessible via X-ray or solution NMR experiments—e.g., membrane or amyloid proteins. Recently, the first examples of this potential were realized with papers describing the complete structures of a small peptide, N-*f*-MLF-OH,[1] an 11-mer peptide in a amyloid fibrils derived from the protein transthyretin,[2] and a 62-amino acid SH3 domain derived from α-spectrin.[3] The success of these experiments is due largely to the ability to record multidimensional MAS NMR spectra that provide ^{13}C and ^{15}N spectral assignments and precise measurements of internuclear distances and torsion angles. These results validate many aspects of the methodology developed for structural studies but concurrently highlight the relatively low signal-to-noise (S/N) of the experiments.

To address this sensitivity problem, we have recently developed high-field dynamic nuclear polarization (DNP) experiments where we transfer the large spin polarization of an exogenous electron spin reservoir to the nuclear spin system via microwave irradiation at the electron resonance frequency.[4] This requires doping the sample with a paramagnet, such as 4-amino-TEMPO (4AT),[5] and the use of a 140 GHz[6,7] gyrotron microwave source that can generate a few watts of CW power for extended periods. In this communication, we report the successful application of MAS DNP to samples of cryoprotected bacteriorhodopsin (bR) and α-lytic protease (α-LP), where we have observed DNP enhancements of up to 50 in ^{15}N MAS spectra. These experiments represent dramatic improvements over our previously published results.[8−10] The spectra were recorded at ∼90 K where MAS is experimentally straightforward. Thus, similar DNP experiments could significantly expand the applicability of MAS NMR experiments to structural studies of a number of important biological systems.

Membrane proteins are important targets for SSNMR structural investigations since they are often insoluble and/or crystallize only under conditions that perturb function. The archetypical membrane protein is bR, a 26.6 kD molecule which forms purple patches in the plasma membrane of halophilic archaea. The highly hydrophobic purple membrane is rich in methyl groups, and thus, even at the moderately low temperatures employed in these experiments, the ^1H T_1 is short (∼1 s) due to the 3-fold hops of the $-CH_3$'s. Nevertheless, the results in Figure 1 demonstrate that it is possible to observe enhancements of 50 in a sample of bR that has been generally labeled via $^{15}NH_4Cl$. In the MAS NMR spectrum (Figure 1b) the amide and η-arginine ^{15}N's are not visible until the vertical gain is increased by a factor of 10. In contrast the DNP-enhanced spectrum (Figure 1a**)** was obtained by irradiating the sample with 1.4 W of CW microwave power at 91 K and demonstrates excellent S/N with a much shorter acquisition time. The DNP enhancement is 50.

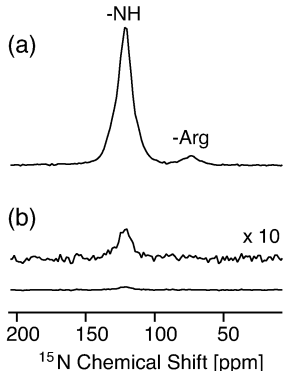

Figure 1. ^{15}N CP MAS spectra of γ-^{15}N bR pellet (a) with and (b) without DNP, illustrating an enhancement of 50. The sample consisted of an aqueous solution containing γ-^{15}N bR dispersed in 15% glycerol, 40 mM 4AT, and 0.1 NaCl. The centrifuge pellet was packed into a 4 mm sapphire rotor with 57 μL sample volume. The prominent line in the spectra at 121 ppm is from the peptide backbone, while the line at 73 ppm arises from the 14 η-^{15}N's of the seven Arg's in the sample. The spectra were recorded at 5 T (211 MHz ^1H) with cross-polarization (CP) from ^1H to ^{15}N and a refocusing echo on ^{15}N. The signal enhancement process involves transfer of polarization from electrons to ^1H's near 4AT, and subsequently $^1H-^1H$ spin diffusion distributes the enhanced spin polarization throughout the protein. Both experiments were recorded at $T = 91$ K, $\omega_r/2\pi = 4.0$ kHz, and with a 2 s. recycle delay. (a) Ninety-six transients with 1.4 W of CW 140 GHz microwave power at the top of the probe; (b) 256 transients without microwave power.

Another example of the utility of DNP is shown in Figure 2 for ζ-^{15}N-Lys-bR. These spectra illustrate that the large signal enhancements extend to the Schiff base ^{15}N site that is buried deep inside the membrane. The S/N in the DNP-enhanced spectrum (Figure 2b) is dramatically improved over that in the conventional spectrum (Figure 2a) even though B_0 is lower by a factor of 1.5, the sample is smaller by a factor of ∼3, and the signal averaging time is shorter by a factor of ∼14. We did not measure the enhancement because the signal was weak without microwaves, but we expect it to be similar to that measured in other samples. However, with only an hour of acquisition time, the DNP-enhanced spectrum shows excellent S/N and clear resolution of the Schiff base resonances arising from bR_{555} and bR_{568}. Most significantly, the dramatic improvement in S/N will facilitate important measurements in the resting state and photointermediates of bR, including torsion angles

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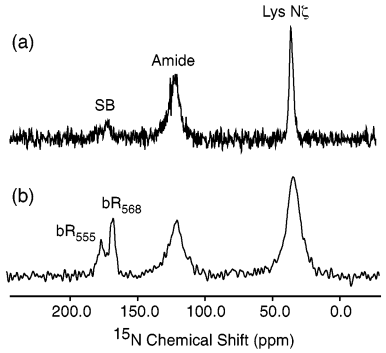

Figure 2. ^{15}N MAS spectra of ζ-^{15}N-Lys-bR. The lines in the spectra arise from the Schiff base ^{15}N in the 13-cis (bR_{555}) and all-trans (bR_{568}) conformations of the chromophore present in dark-adapted (DA) bR (178 and 169 ppm), the natural abundance amide backbone (121 ppm), and the six free Lys (33 ppm) present in the protein. (a) The trace was recorded without microwave irradiation on a spectrometer operating at 317 MHz for ^{1}H using a 5 mm rotor with a nominal 100 μL volume, $T = 190$ K, $\omega_r/2\pi = 5.00$ kHz and required 25,856 transients or 14.4 h of acquisition. The data were processed with a baseline correction and no line broadening. (b) Spectrum (211 MHz) recorded on a DNP spectrometer using a 4 mm sapphire rotor, a nominal 57 μL sample volume, $T = 90$ K, $\omega_r/2\pi = 4.2$ kHz, with 0.9 W of 140 GHz microwave power at 50% duty cycle. The DNP-enhanced data were processed in a manner identical to that for (a), and required 1280 scans or 64 min of acquisition. The membrane samples were prepared from a 40 mM 4AT/15% glycerol solution. The bR is partially light-adapted since the bR_{555}:bR_{568} signal ratio is ∼1:2, which is reversed from the normal ∼2:1 in DA-bR.[11]

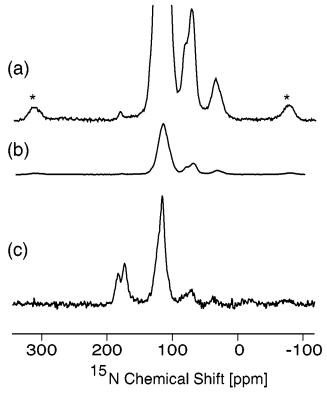

Figure 3. DNP-enhanced ^{15}N MAS spectra of (a,b) uniformly ^{13}C−^{15}N labeled α-LP (120 mg/ml) in water/glycerol (50%) with 40 mM 4AT at pH = 10. Trace (b) shows the entire spectrum acquired in 1536 scans, and (a) is a vertical expansion illustrating that a signal from the single protonated His imidazole ^{15}N in the protein is observed (183 ppm). Signals from the amide backbone (120 ppm), the 12 Arg's (82 and 73 ppm), and the Lys's (35 ppm) are also observed with excellent S/N. The experiment was performed with a 10 s recycle delay at 93 K, $\omega_r/2\pi = 4.0$ kHz, and with 0.7 W of microwave power at 50% duty cycle. (c) Spectrum obtained from a sample of (U-^{13}C,^{15}N-His)-αLP (150 mg/mL) in water/glycerol with 40 mM 4AT at pH = 4.8. The two His imidazole ^{15}N's are protonated and their signals resolved. The spectrum was recorded with 2560 scans, a recycle delay of 10 s, microwave power of 0.7 W (50% duty cycle), $T = 94$ K, $\omega_r/2\pi = 4.2$ kHz, using 4 mm sapphire rotors.

and distances that are essential to elucidating the ion-pumping mechanism of bR.

In a final demonstration of the utility of DNP, Figure 3 presents spectra of the soluble protein α-LP that show the ability to observe the single His residue in this 18 kD protein. The His is part of the distinctive catalytic triad found in serine proteases, and a knowledge of its pK_a, tautomeric state, and H-bonding interactions is crucial to understanding the mechanism of this important class of enzymes.[12] The traces in (a) and (b) were obtained in ∼1500 scans from a U-^{13}C,^{15}N-α-LP sample in frozen water/glycerol/4AT. In addition to the resonance from an individual protonated nitrogen atom ($N_{\delta 1}$) in the His, we also observe signals from the amide backbone N−H as well as the 12 Arg's and the two Lys's with excellent signal-to-noise as is clear from the expanded vertical trace shown in (a). Figure 3c shows a spectrum of U-^{13}C,^{15}N-His-α-LP recorded at pH = 4.8. In this case both His imidazole nitrogens, $N_{\delta 1}$ and $N_{\epsilon 2}$, are protonated and are observed at 184 and 174 ppm, respectively, very close to the values observed for these resonances in aqueous solutions at room temperature.[13] DNP signal enhancements of up to 45 were measured on samples of α-LP. As was the case with bR, the spectral quality and S/N enabled by DNP will permit structural studies that are currently not feasible. Experiments are already underway to measure the ^{15}N−^{1}H distances in the catalytic triad.

Two final points are worth mentioning. First, the enhancement factors reported here (∼50 at 90 K) are very encouraging and should stimulate a variety of new applications. Nevertheless, they were observed at a 5-T field which is modest for contemporary NMR. Efforts are underway to extend DNP experiments to higher fields, and initial results at 9 T (250 GHz/380 MHz) are reported elsewhere.[7] In addition, we anticipate that the enhancements reported here (∼50) can be increased by a factor of ∼3−8 when equipment to perform MAS at 10−20 K becomes available. These predictions are based on experiments in which we have observed signal enhancements up to ∼400 at ∼10 K in static samples.[14] For these reasons we anticipate that DNP experiments such as described here could have a significant impact on MAS experiments on proteins as well as applications in other areas.

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Supporting Information Available: Additional ^{15}N MAS spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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