Measurement of Dipolar Couplings in a Uniformly ^{13}C,^{15}N-Labeled Membrane Protein: Distances between the Schiff Base and Aspartic Acids in the Active Site of Bacteriorhodopsin

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In recent years, a number of magic-angle spinning (MAS) solid-state nuclear magnetic resonance (SSNMR) methods have been developed[1] for ^{13}C and ^{15}N resonance assignments in uniformly ^{13}C,^{15}N-labeled peptides and proteins.[2] The ^{13}CO, ^{13}Cα, ^{13}Cβ, and amide ^{15}N chemical shifts can be used to estimate the backbone torsion angles ϕ and ψ.[3] Additional constraints on the local structure in U-^{13}C,^{15}N-labeled systems can be obtained from measurements of the relative orientations of dipolar tensors.[4] Long-range dipolar couplings in peptides and proteins can provide valuable information about tertiary structure. However, the accurate determination of weak dipolar interactions in U-^{13}C,^{15}N-labeled molecules is complicated by the presence of strong couplings.[5,6] This problem can be circumvented by controlled "dilution" of the multiple spin system[7] or by the use of spectrally selective dipolar recoupling techniques.[8,9] To date, selective recoupling techniques have been applied to accurate measurements of multiple long-range ^{13}C−^{13}C[10] and ^{13}C−^{15}N[9] distances in small U-^{13}C,^{15}N-labeled peptides. In this Communication we demonstrate the application of frequency-selective REDOR (FSR)[9] to the measurement of two ^{13}C−^{15}N dipolar couplings in the active site of light-adapted [U-^{13}C,^{15}N]bacteriorhodopsin (bR) in its native purple membrane. The measured distances are in reasonable agreement with ones reported in recent diffraction structures of light-adapted bR, and the NMR methods described are directly applicable to bR photocycle intermediates, for which high-resolution diffraction structures are more difficult to obtain. The experiments presented here are the first example of long-range MAS NMR distance measurements in a U-^{13}C,^{15}N-labeled macromolecule.

Bacteriorhodopsin (bR) is a 26 kDa integral membrane protein produced by *Halobacterium salinarum*. The single polypeptide chain forms a bundle of seven transmembrane helices enveloping a chromophore formed by a Schiff base (SB) between retinal and Lys216. Dark-adapted bR comprises two species: bR_{555} and bR_{568}, with different retinal conformations. Light adaptation of bR, by irradiation with white light, converts bR_{555} to bR_{568}, which is the starting point of the proton pumping photocycle (see ref 15 for a review on bR).

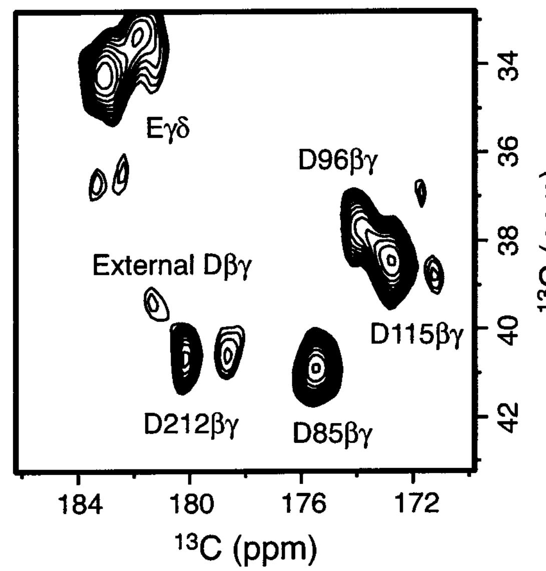

Figure 1. Two-dimensional RFDR ^{13}C−^{13}C chemical shift correlation spectrum of dark-adapted [U-^{13}C,^{15}N]bR displaying Asp Cβ−Cγ and Glu Cγ−Cδ cross-peaks. Spectra were recorded at 11.7 T, 12.5 kHz MAS, and −80 °C. To eliminate Asn Cγ and Gln Cδ signals in t_2, the 2D RFDR sequence[11] was followed by a REDOR[12] ^{13}C−^{15}N dipolar filter: CP−t_1−$(\pi/2)_\psi$−RFDR−$(\pi/2)$−REDOR filter−t_2. RFDR and REDOR filter lengths were 0.96 and 1.44 ms, respectively. Hypercomplex data were acquired by shifting phase ψ according to Ruben and co-workers.[13] The data were acquired as (16, 512) complex points with dwell times (320, 20) μs. Each FID was 512 scans, with a 4.0-s recycle delay, resulting in a total measurement time of ∼18 h. ^{13}C chemical shifts are indirectly referenced to the methyl ^{1}H resonance of DSS.[14]

In the one-dimensional MAS spectrum of bR_{568}, the Schiff base nitrogen resonates ∼135 ppm downfield from the ζ-NH_3^+ groups of Lys residues and ∼50 ppm downfield from the amide backbone peak.[16] This enables the selective inversion of the SB nitrogen and FSR distance measurements[9] to ^{13}C nuclei in the active site. Recent diffraction structures of bR_{568} (from 1.55 to 2.9 Å resolution)[17−19] report distances to the SB nitrogen in the 4.3−5.0 Å range for Asp85 Cγ and in the 4.0−4.4 Å range for Asp212 Cγ (see Supporting Information).

Figure 1 shows a region of a 2D RFDR[11] ^{13}C−^{13}C chemical shift correlation spectrum of dark-adapted [U-^{13}C,^{15}N]bR displaying Asp and Glu side chain methylene to carboxyl cross-peaks.

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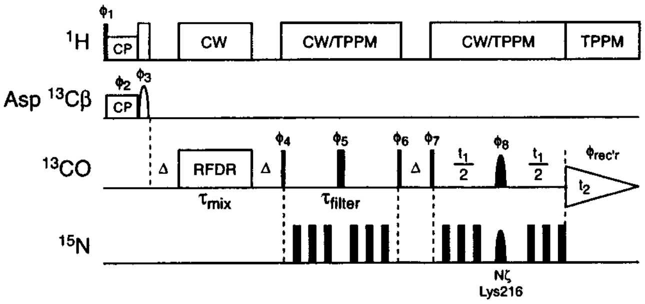

Figure 2. Two-dimensional experiment for the measurement of dipolar couplings between the retinal SB nitrogen and Asp Cγ in [U-^{13}C,^{15}N]-bR. Spectra were recorded at 7.4 T, 6494 Hz MAS, and -30 °C. Light-adapted bR was prepared as described in the Supporting Information. Narrow and wide rectangles represent $\pi/2$ and π pulses, respectively. The ^{13}C Gaussian $3\pi/2$ pulse applied in the Asp Cβ spectral region following CP was 1.232 ms long. RFDR and REDOR filter times were both 1.232 ms. REDOR and FSR ^{15}N π pulse lengths were 13 μs (XY-4 phase cycling). During FSR (t_1) Gaussian π pulse lengths were 0.308 and 4.004 ms for Asp ^{13}CO and Lys216 ^{15}Nζ, respectively. Gaussian pulses were divided into 64 steps and truncated at 1%. The z-filters were $\Delta = 50$ μs. Proton decoupling was 125 and 100 kHz during mixing and acquisition, respectively (see ref 9). Phase cycle: $\phi_1 = 1$, $\phi_2 = 1$, $\phi_3 = 24$, $\phi_4 = 1$, $\phi_5 = 2$, $\phi_6 =$ 11111111 11111111 11111111 11111111 33333333 33333333 33333333 33333333, $\phi_7 =$ 11111111 22222222 33333333 44444444, $\phi_8 =$ 11223344, $\phi_{\mathrm{rec'r}} =$ 42244224 31133113 24422442 13311331 24422442 13311331 42244224 31133113, where $1 = x$, $2 = y$, $3 = -x$, $4 = -y$.

The resonance assignments are based on previous studies of selectively [4-^{13}C]Asp-labeled bR.[20] Asp85 and Asp212 Cβ-Cγ cross-peaks are well-resolved, and, in principle, the distances to the SB can be measured via a 3D FSR experiment (where the decay rate of cross-peaks in the FSR dimension is determined by the dipolar coupling to SB ^{15}N). However, with the current technology, a 3D experiment for a membrane protein, such as bR, is subject to signal-to-noise and time limitations. Therefore, we have reduced the experiment to 2D (see Figure 2) by implementing a filter prior to FSR, designed to retain only Asp Cγ resonances in the CO spectral region, while eliminating all other signals (although in practice some residual Glu Cδ intensity remains). The filter consists of a selective storage pulse applied to Asp Cβ, followed by coherence transfer to Cγ and Cα via RFDR.[11] The subsequent REDOR filter eliminates signals in the CO spectral region, which originate from nuclei directly bonded to a ^{15}N nucleus (Asn Cγ, Gln Cδ, and C′). We have confirmed that the resulting 1D spectrum is equivalent to a weighted projection through the Asp C$\beta-$Cγ and Glu C$\gamma-$Cδ region in the 2D correlation spectrum (see Supporting Information).

FSR distance measurements from Lys216 Nζ to Asp85 Cγ and Asp212 Cγ in light-adapted bR are presented in Figure 3. Spectra in Figure 3a,b clearly show that Asp85 and Asp212 Cγ are selectively dephased by the retinal SB ^{15}N, while the other Asp signals remain unchanged. FSR dephasing curves for several ^{13}C$-$^{15}N distances in the 4.0$-$5.5 Å range were calculated using the SIMPSON NMR simulation software[21] (see Supporting Information). Experimental S/S_0 curves for Asp85 and Asp212 are compared with 4.0, 4.5, 5.0, and 5.5 Å simulations in Figure 3c,d. Asp85 and Asp212 distances were found to be 4.7 ± 0.3 and 4.9 ± 0.5 Å, respectively. The Asp85 distance is in agreement with recent diffraction distances (4.3$-$5.0 Å), and the Asp212 distance appears to be somewhat longer than the diffraction values (4.0$-$4.4 Å).[17-19]

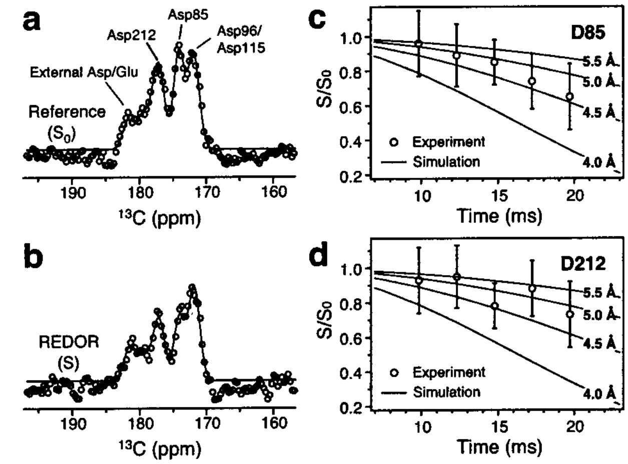

Figure 3. Distance measurements from the Schiff base (Lys216 Nζ) to Asp85 Cγ and Asp212 Cγ in light-adapted [U-^{13}C,^{15}N]bR. (a) Reference (S_0) and (b) REDOR (S) spectra recorded using the pulse sequence in Figure 2 with $t_1 = 19.712$ ms. Experimental spectra (○) and fits with Gaussian line shapes (lines) are juxtaposed. The deviation of the baseline from zero in (a) and (b) is within the experimental noise level as determined from the entire spectrum. (c) Asp85 Cγ and (d) Asp212 Cγ REDOR S/S_0 curves. Experimental data (○) were obtained with 32,768 and 65,536 transients for $t_1 < 15$ ms and $t_1 > 15$ ms, respectively (interleaving S_0 and S spectra for each t_1 point in blocks of 256 transients). The error bars in the experimental S/S_0 points were calculated from the uncertainties in the Gaussian line shape fits. Using a 2.0-s recycle delay, the total duration of the experiment was ~10 days. Simulations (lines) for 4.0, 4.5, 5.0, and 5.5 Å carbon$-$nitrogen distances were generated using SIMPSON.[21] The best-fit distances are 4.7 ± 0.3 Å for Asp85 and 4.9 ± 0.5 Å for Asp212.

In summary, we have applied frequency-selective REDOR to distance measurements between the Schiff base and aspartic acids in the active site of uniformly ^{13}C,^{15}N-labeled bacteriorhodopsin. The measured distances are in reasonable agreement with recent diffraction studies and complement previous structural investigations of the bR active site by SSNMR.[22] The methods described can be applied to the characterization of bR photocycle intermediates.

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Supporting Information Available: Table of diffraction distances, bR spectra, [U-^{13}C,^{15}N]bR preparation, and simulation details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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