Internuclear Distance Measurement in a Reaction Intermediate: Solid-State ^{13}C NMR Rotational Resonance Determination of the Schiff Base Configuration in the M Photointermediate of Bacteriorhodopsin

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Rotational resonance (R^2) is a technique for selectively recoupling homonuclear dipolar interactions in MAS NMR spectroscopy.[1-4] Since the strength of the dipolar interaction is directly related to the internuclear distance, R^2 provides an approach for structure determination in polycrystalline or amorphous solids. The method has been used to obtain ^{13}C–^{13}C and ^{31}P–^{31}P distances for a number of proteins in the ground state.[5-8] Clearly, the technique is also applicable to structural studies of reaction intermediates if they can be trapped for extended periods. Here we present the first such application.

Bacteriorhodopsin (bR), the sole protein in the purple patches of the plasma membrane of *Halobacterium halobium*, comprises a single polypeptide chain, folded into seven transmembrane helices, and a retinal chromophore, which is situated in the center of the helix bundle and linked to lysine$_{216}$ by formation of a Schiff base (SB). During the photocycle of the light-adapted form of the protein, a proton is released on the extracellular side of the membrane and replaced from the intracellular side. The mechanism for this light-driven proton transport involves the photoisomerization of the retinal chromophore from all-*trans* to 13-*cis*, a transient drop in the pK of the SB (which leads to deprotonation and reprotonation of the nitrogen in the formation and decay of the M intermediate), and conformational changes that alter the connectivity of the proton transport pathway to prevent proton backflow. Of particular interest is the configuration of the C=N bond of the SB in the M intermediate, as this determines the orientation of the lone pair of the nitrogen at this key step. Previous solid-state NMR studies have used the chemical shifts of the 14-retinal and the ϵ-lysine$_{216}$ carbons to monitor the SB configuration during the photocycle.[9,10] Model compound studies show that these carbons are shifted upfield by *anti* to *syn* isomerization of the intervening C=N bond, due to the steric interactions between their protons in the latter case (see Figure 1). However, since chemical shifts are also subject to other influences, including for these particular carbons the protonation state of the nitrogen, the interpretation of chemical shifts is always somewhat tentative.

Here we make an unambiguous determination of the configuration about the SB linkage in M by directly measuring the distance between the 14-retinal and the ϵ-lys$_{216}$ carbons in a sample of [14-^{13}C]retinal,[ϵ-^{13}C]lys bR[11] thermally trapped in the M

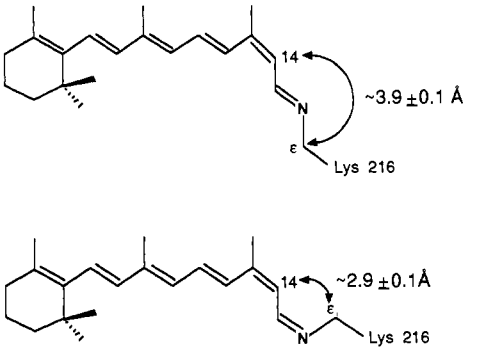

Figure 1. Comparison of the *anti* and *syn* C=N configurations for a 13-*cis*-deprotonated retinal Schiff base.

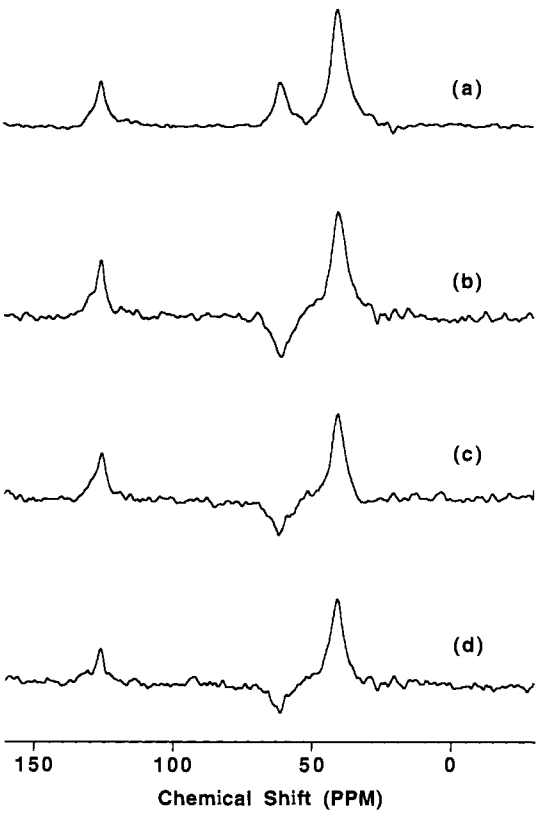

Figure 2. Solid-state ^{13}C NMR MAS difference spectra obtained by subtraction of a spectrum of natural abundance bR from spectra of [ϵ-^{13}C]Lys,[14-^{13}C]retinal bR in the M state. CPMAS difference spectrum (a) and $n = 1$ R^2 magnetization exchange difference spectra for mixing times of 0.2 (b), 5.0 (c), and 20.0 (d) ms.

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(11) [14-^{13}C]retinal,[ϵ-^{13}C]lys bR was prepared as described previously.[10]

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state.[12] A CPMAS difference spectrum[13] of [14-^{13}C]retinal,[ϵ-^{13}C]lys bR in the M state (Figure 2a) shows the 14-retinal and ϵ-lys$_{216}$ resonances at 125 and 60 ppm, respectively, with the resonances of the six free lysines at 40 ppm. The complete conversion of the sample is indicated by the absence of peaks at 122 and 53 ppm and at 110 and 48 ppm, corresponding respectively to the C=N *anti* and C=N *syn* protonated retinal Schiff bases found in dark-adapted bR.[10] The distance measurement is performed by following the rate of rotor-driven magnetization transfer that occurs when the magic angle spinning speed is set to satisfy the $n = 1$ R^2 condition.[1] At a speed of 5.15 kHz, we selectively induce magnetization exchange between resonances 65 ppm apart. The ϵ-lys$_{216}$ magnetization is first inverted, using a weak 180° inversion pulse, in order to establish a non-equilibrium initial condition. After a variable mixing time, a nonselective 90° pulse is applied to measure the distribution of magnetization between the two signals of interest (Figures 2b–d). Figure 3 shows the observed and simulated evolution[3] of the normalized difference magnetization. We see that the data are consistent with a distance of 3.9 ± 0.1 Å, corresponding to an *anti* C=N configuration (and inconsistent with a 2.9 Å distance for a *syn* C=N configuration[7]). The same configuration has also been deduced from resonance Raman spectra for the M state at room temperature.[14] The present results support the earlier interpretation of ^{13}C chemical shifts.[9,10] They also establish the feasibility of using solid state rotational resonance NMR to measure distances in a reaction intermediate.

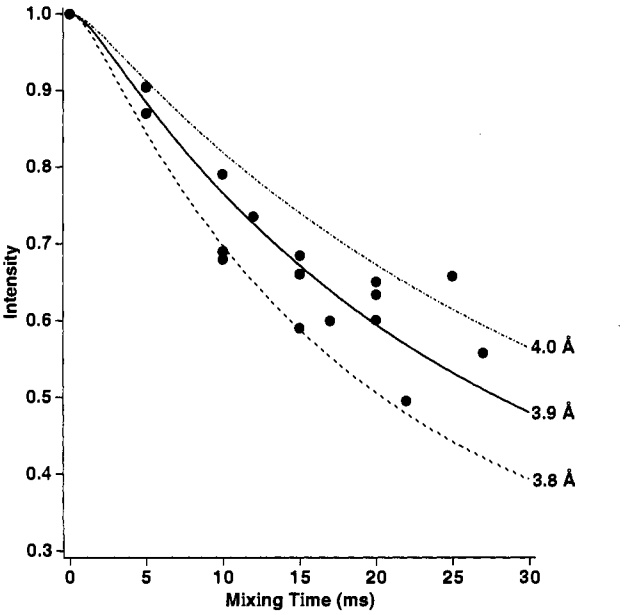

Figure 3. Observed and simulated magnetization exchange between the ϵ-lysine$_{216}$ and 14-retinal carbons of bR in the M state under the $n = 1$ R^2 condition.

(12) Trapping the M photointermediate: [ϵ-^{13}C]Lys,[14-^{13}C]retinal bR was washed with a solution of 0.3 M guanidine–HCl at pH 10.0, conditions which slow the decay of the M intermediate in the photocycle but preserve proton transport.[15] The resultant pellet was transferred to a 7-mm cylindrical single-crystal sapphire rotor (Doty Scientific, Columbia, SC). The sample was light-adapted at 0 °C for 1 h using a 500-W projector lamp for illumination. The M photointermediate was then trapped by illuminating the light adapted sample for a few hours at −50 °C with light of wavelengths >540 nm. A dry nitrogen gas atmosphere was used to cool the sample during illumination. Under these conditions, the sample is completely yellow, which is consistent with the presence of the M intermediate. Upon warming, the sample regains its purple color, with an NMR spectrum characteristic initially of the light-adapted state and ultimately of the dark-adapted state. The methods described here are slight variations of earlier techniques.[8-10]

(13) Solid-state NMR spectroscopy: magic angle spinning (MAS) NMR spectra were obtained on a home-built spectrometer at a field of 7.4 T (^{13}C and ^{1}H frequencies of 79.9 and 317.6 MHz, respectively), using a standard cross-polarization pulse sequence[16] with a mixing time of 2 ms, sample rotation rates of 4.0 to 5.15 kHz, and continuous proton decoupling during data acquisition. The proton 90° pulse length was typically 4.2 μs. The sample spinning speed was controlled to within ±10 Hz during the 4–10 h of data acquisition,[17] and the sample temperature was maintained at −60 °C to stabilize the M photointermediate. Acquisition time ranged from 10 to 37 ms, and typically 6000 transients were accumulated per spectrum. The recycle delay was 3 s, which was adequate to allow for full relaxation (>5 × ^{1}H T_1s).

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