Determination of Torsion Angles in Membrane Proteins

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Introduction

Bacteriorhodopsin (bR) harnesses light energy to transport protons across the cell membrane of *H. salinarium*. Absorption of a photon by the protonated retinal chromophore initiates a cycle in which the chromophore releases a proton to an aspartate on the extracellular side and reprotonates from an aspartic acid on the cytoplasmic side. Vectorial proton transport depends on a switch in accessibility of the chromophore Schiff base nitrogen from the extracellular to cytoplasmic side. Changes in the retinal conformation are expected to be particularly important for understanding the pumping mechanism. Twists about the chromophore polyene chain (Fig. 1) affect the proton affinity of the Schiff base nitrogen[1] as well as the relative orientation and

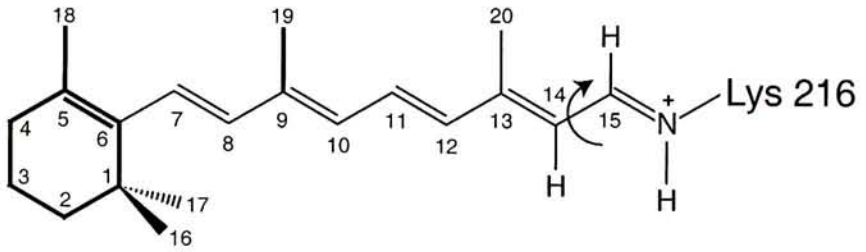

Figure 1: The protonated retinal Schiff base chromophore of bacteriorhodopsin. The measured H-C14-C15-H torsion angle, ϕ, is indicated by the curved arrow.

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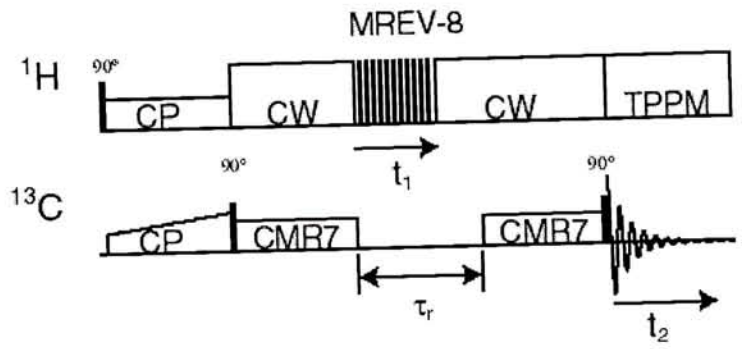

***Figure 2**: The HCCH pulse sequence, modified from the scheme of Feng et al[11] to incorporate the improved double quantum mixing sequence CMR7.[12] During the constant time of one rotor period, the coherence is allowed to evolve for a time t_1 under the heteronuclear ^1H-^{13}C couplings that are reintroduced by MREV-8[13,14] decoupling. CW irradiation is applied for the remainder of the constant time period to remove heteronuclear couplings.*

proximity of proton donor and acceptor groups. In this paper we briefly describe an experiment to measure the twist about various bonds in retinal bound to bR.

FTIR[2] and CD[3,4] measurements indicate distortion in the resting state of the protein, bR_{568}. Intense hydrogen out of plane bending modes indicate this distortion increases during the photocycle in the vicinity of the retinal C15.[2,5-7] Unfortunately, these methods do not provide precise measurements of the chromophore conformation. In contrast, X-ray structures[8-10] of bR_{568} suggest a planar conformation of the C14-C15 bond, with a dihedral angle of 174-180°. To resolve this ambiguity, we utilized solid-state NMR methods to measure the torsion about the C14-C15 bond.

Methods

Solid-state NMR methods permit quantitative measurements of torsion angles through correlation of anisotropic dipolar interactions. Measurement of the H-C-C-H dihedral angle can be accomplished with the experiment shown in Fig. 2.

Evolution of the double quantum signal (sum intensity) in the indirect dimension can be described by

$$a(t_1) = \left\langle \sin^2\left(\omega_{2Q}\tau_{2Q}\right)\prod_{\lambda}\cos\Psi_{\lambda}(t_1)\right\rangle \qquad (1)$$

where ω_{2Q} is the time- and orientation-dependent double quantum nutation rate from zeroth order average Hamiltonian theory,[12] τ_{2Q} is the double quantum excitation time, the index λ denotes a particular ^{1}H-^{13}C coupling, and the dephasing angle $\Psi_\lambda(t_1)$ is defined by the time average of the dipolar coupling,

$$\Psi_\lambda(t_1) = -\kappa\delta_\lambda \sum_{m=-2}^{2} D^{(2)}_{0,m}\left(\Omega^\lambda_{PR}\right) d^{(2)}_{m,0}\left(\beta_{RL}\right) \int_0^{t_1} e^{-im\omega_r t}\, dt, \qquad (2)$$

where the solid angle Ω_{PR} defines the rotation from the principal axis system of the dipolar tensor to the rotor-fixed frame, β_{RL} is the magic angle, $D^{(2)}_{0,m}$ is a Wigner rotation matrix, $d^{(2)}_{m,0}$ is a reduced Wigner rotation matrix, δ_λ is the magnitude of the static ^{1}H-^{13}C dipolar coupling, and κ is the scaling factor of the MREV-8 sequence. Isotropic chemical shift evolution is neglected by this treatment as placement of the carrier between the ^{13}C resonances results in the evolution of the DQ coherence under a sum chemical shift of zero. Chemical shift anisotropy terms commute with the heteronuclear couplings and are refocused over the constant time of one rotor period, τ_r.

Simulations: Information about the relative orientation (torsion and bond angles) between two couplings is contained in the difference between solid angles $\Omega^a_{PR} - \Omega^b_{PR}$ and can be extracted from experimental data through simulations utilizing the analytical expression in Eq. 1. Signal decay in t_1 due to insufficient ^{1}H-^{1}H decoupling during the MREV-8 sequence can be approximated as exponential decay when $T_2 >> \tau_r$. Bond angles were taken from the crystal structure of all-*trans* retinal. The value of the scaled heteronuclear dipolar coupling was set to the experimental value of 12.3 kHz measured in a DIPSHIFT[15] measurement of [2-^{13}C]leucine. The influence of the two-bond coupling to the Schiff base proton, the nearest neighbor to the H-C14-C15-H system, was found to be negligible for conformations of the C14-C15 and C15-N bonds that are close to *trans*. Protons even more distant are not expected to influence the signal evolution. Powder averaging was accomplished with 256 pairs of alpha and beta crystallite angles using the REPULSION method.[16]

Sample preparation: Synthesis of [14,15-$^{13}C_2$]retinal, enriched to 99%, was performed according to procedures described elsewhere.[17] Bacteriorhodopsin-containing purple membrane (PM) fragments were isolated from *H. salinarium* according to conventional procedures.[18] The labeled retinal was incorporated by bleaching the sample in 0.5 M hydroxylamine and subsequent regeneration of the apoprotein with the labeled retinal as described elsewhere.[19] Regenerated PM fragments were washed multiple times in 0.3 M guanidine at pH 10.0. Centrifugation of the suspended PM fragments produced a pellet that was packed into a 5 mm quartz rotor. The bR_{568} state was prepared in the custom-designed probe by illumination of the sample with the full visible spectrum of a 1000 W Xenon lamp at 0 °C for 2 hours.

Results and Discussion

The ^{13}C spectrum of the resting state, bR_{568}, of [14,15-$^{13}C_2$] retinal-labeled bR is presented in Fig. 3. Evolution of the double-quantum signal intensity (Fig. 4) is

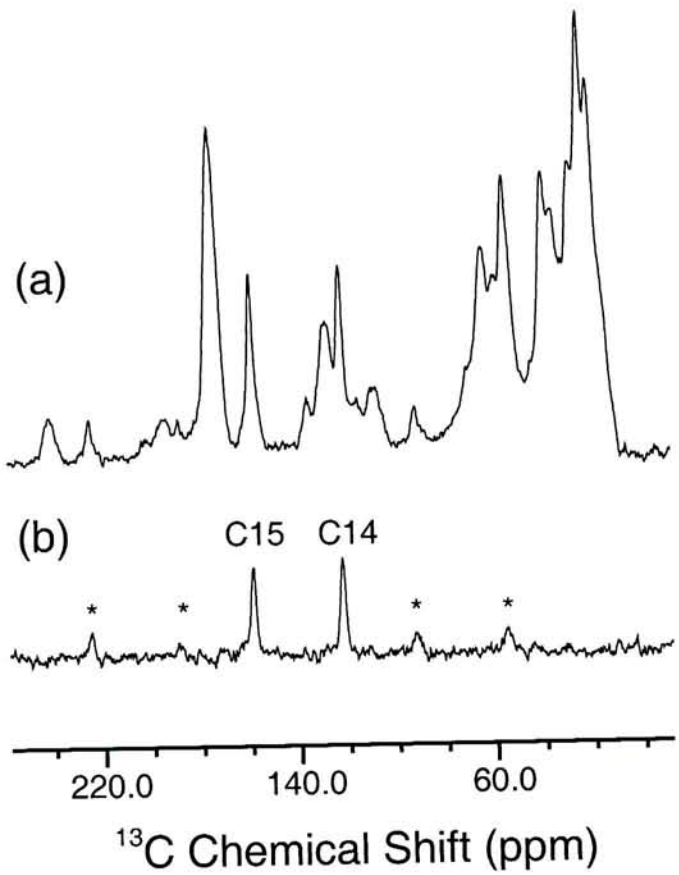

Figure 3: (a) Cross polarization and (b) double-quantum filtered ^{13}C spectra of light-adapted [14,15-$^{13}C_2$]retinal-labeled bR. Rotational sidebands are designated with asterisks. Notice the excellent suppression of the natural abundance background in the spectrum retaining only the coupled spins in the retinal with c.a. 50% efficiency. Spectra were collected on custom-designed spectrometer operating at a ^1H frequency of 317 MHz. The sample was maintained at –90 °C with a sample rotation rate of 5315±2 Hz. Proton fields of 50 kHz for cross polarization, 100 kHz for TPPM decoupling and 127.6 kHz for MREV-8 and CW decoupling were utilized. Carbon fields of 44.7 kHz for cross polarization and 37.2 kHz for CMR7 were applied. The durations of the cross polarization and CMR7 mixing periods were 2 ms and 752.64 ms, respectively. Each t_1 point is the average of 29,696 transients with a recycle delay of 2 s.

indicative of a torsion angle of ±164°. Reduced chi-square analysis indicates an error of ±4° at the 90% confidence level.

These results indicate considerable distortion of the bound chromophore from the planar conformation preferred by free retinal[20] and a protonated retinal Schiff base model compound.[21] That the C14-C15 bond should be twisted from the relaxed

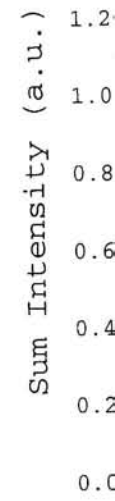

***Figure 4:** Modulo*
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Conclusions

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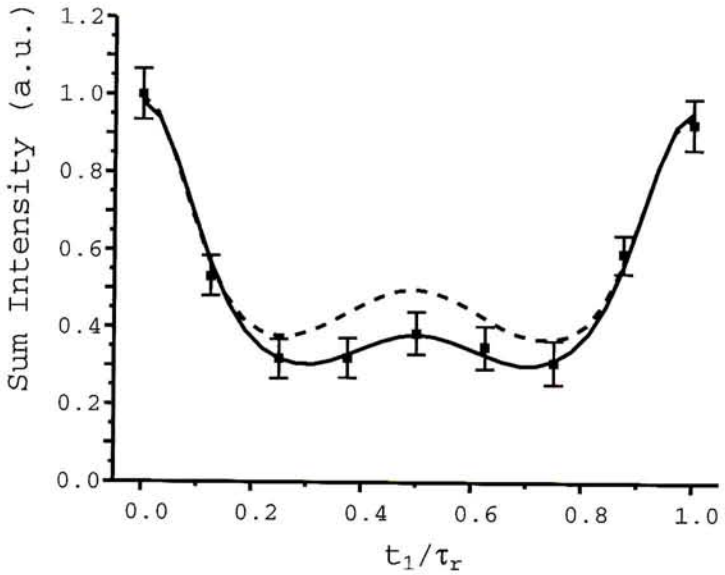

Figure 4: *Modulation of the sum polarization of light-adapted [14,15-$^{13}C_2$] retinal-labeled bR in the indirect dimension of the HCCH experiment. The solid line denotes the best fit to ±164°. The simulated curve for a planar trans conformation (dotted line) is shown for comparison.*

planar structure in the bR resting state by interactions with the binding pocket is surprising. Although nominally a single bond, *ab initio* calculations[1] indicate that the protonated Schiff base C14-C15 bond has a larger barrier of rotation than the neighboring nominal double bonds due to the influence of the conjugated π system. Clearly, it is not safe to assume that ligated retinal will behave in the same fashion as it would in the absence of the protein environment.

Conclusions

Knowledge of the conformation of the retinal in the ground and photointermediate states of bR is crucial to understanding the proton translocation mechanism of this membrane protein. We find that the C14-C15 bond is non-planar, with $|\phi| = 164 \pm 4°$. These results indicate the presence of local structural features unobserved in X-ray structures that have the potential to influence the protein function. More extensive characterization of the chromophore conformation in the bR resting state and upon photoexcitation is warranted to produce a detailed picture of the active site.

We have outlined an experiment based on double quantum excitation and reconversion to measure the ϕ angle about the H-C14-C15-H bond. The experiment cleanly separates the spectrum of the coupled spins from the natural abundance background. It should be equally applicable to other positions in the retinal molecule as well as to measurements of sidechain torsion angles in the protein itself.

Acknowledgements

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