

Double-Quantum ^{13}C Nuclear Magnetic Resonance of Bathorhodopsin, the First Photointermediate in Mammalian Vision

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Dim-light vision in mammals is initiated when a photon is absorbed by the retinylidene prosthetic group of the seven-helix transmembrane protein rhodopsin, a G-protein coupled receptor (GPCR) which is abundant in the rod cells of the retina at the back of the eye. The photon absorption leads to an ultrafast (<100 fs) and highly selective isomerization of the retinylidene conjugated chain from the initial 11-*Z* configuration to a distorted all-*E* configuration (Figure 1). The resultant photostate is called *bathorhodopsin* since its optical absorption is red-shifted relative to rhodopsin.^{1,2} Bathorhodopsin is a highly energetic species that stores about $\frac{2}{3}$ of the absorbed photon energy.¹ Under physiological conditions, bathorhodopsin is converted rapidly into a series of less energetic photostates, culminating in the active state *metarhodopsin-II*, which is capable of binding and activating many copies of the G-protein transducin, a process that leads to a modulation of the transmembrane potential within the cell and induction of an optical nerve signal.²

Since bathorhodopsin is stable at temperatures below 125 K,¹ its structure and properties may be studied by illuminating rhodopsin at low temperature and using techniques such as X-ray diffraction and nuclear magnetic resonance. An early solid-state NMR study attempted to measure ^{13}C chemical shifts in the retinylidene side chain of ^{13}C -labeled bathorhodopsin and thereby obtain information on the electronic structure.³ However, only minor chemical shift changes were observed relative to rhodopsin.³

A more recent 2.7 Å X-ray structure of bathorhodopsin has verified the distorted all-*E* configuration of the chromophore and also identified various structural changes in the immediate protein environment, including a small displacement of the negatively charged Glu-113 counterion relative to the positively charged nitrogen atom of the protonated Schiff base (PSB) linking the chromophore to the lysine-296 side chain of the opsin protein.⁴ The electronic structure of bathorhodopsin has been modeled using density functional theory,^{5,6} QM/MM,^{7–9} and molecular dynamics¹⁰ calculations, but in general, the level of agreement between the reported NMR chemical shifts and the calculated values has been quite poor.

In this communication, we report on new measurements of the ^{13}C chemical shifts in the retinylidene chromophore of bathorhodopsin, performed with modern double-quantum recoupling techniques and with great care paid to the calibration of the real sample temperature and to the illumination methodology. In contrast to the 1991 results,³ the new values indicate a considerable perturbation in bathorhodopsin. For example, we observe a +9.4

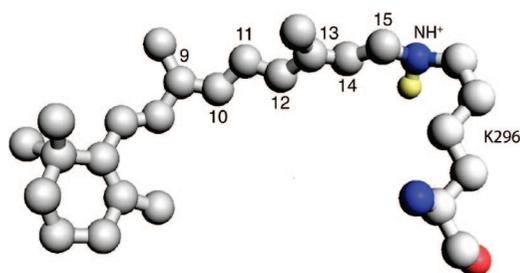


Figure 1. Sketch of the distorted all-*E* retinylidene chromophore in bathorhodopsin, showing the numbering of the carbon sites and the protonated Schiff base linkage to the K296 side chain. The structural data are from the Protein Data Bank entry 2g87 (see ref 4).

ppm deshielding shift of the ^{13}C isotropic chemical shift of site C10 when bathorhodopsin is generated.

The new data were generated using $^{13}\text{C}_2$ -labeled isotopomers of rhodopsin, prepared by total organic synthesis of $^{13}\text{C}_2$ -retinals,¹¹ followed by regeneration of $^{13}\text{C}_2$ -rhodopsin using bleached opsin pigments isolated from bovine retinas, and reconstitution with natural-composition lipid membranes. Four $^{13}\text{C}_2$ -labeled rhodopsin isotopomers were studied, namely, [9,10- $^{13}\text{C}_2$], [11,12- $^{13}\text{C}_2$], [12,13- $^{13}\text{C}_2$], and [14,15- $^{13}\text{C}_2$]-retinylidene rhodopsin. Since the reconstituted samples are optically dense, the light penetration was improved by grinding frozen rhodopsin samples in liquid nitrogen to form particles of diameter $\sim 200\ \mu\text{m}$ which were then mixed with $\sim 100\ \mu\text{m}$ diameter glass beads. The rhodopsin/glass bead mixtures were packed into 4 mm diameter thin-wall zirconia rotors for the magic-angle-spinning NMR experiments. All procedures were performed in dim red light to avoid premature isomerization.

^{13}C NMR spectra of the labeled chromophores were recorded using the double-quantum filtered pulse sequence reported in ref 12. Symmetry-based double-quantum recoupling using pulse sequences with the symmetry R20₂,⁹ combined with phase-cycling to remove signals that do not pass through double-quantum coherence, achieves a clean suppression of the natural ^{13}C background signals from the protein and the lipid. The spectra in Figure 2 show clearly resolved signals from the ^{13}C labels of the chromophore.

Bathorhodopsin was generated by illuminating the rhodopsin samples in situ in a custom-built NMR probe at a sample temperatures between 110 and 125 K. The light was generated by two 250 W halogen lamps passed through $420 \pm 5\ \text{nm}$ interference filters (Edmund optics, UK) and led into the sample region by 14 optical fibers. The illumination wavelength was chosen so as to improve the light penetration into the sample and to minimize the undesirable photoisomerization of bathorhodopsin into the 9-*Z*

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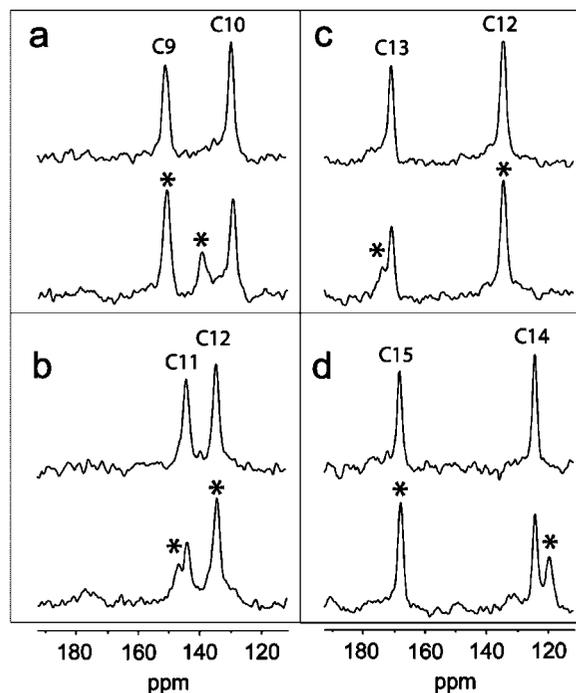


Figure 2. Double-quantum filtered ^{13}C NMR spectra of (a) [9,10- $^{13}\text{C}_2$], (b) [11,12- $^{13}\text{C}_2$], (c) [12,13- $^{13}\text{C}_2$], (d) [14,15- $^{13}\text{C}_2$] rhodopsin before (above) and after (below) 10 h of illumination with 420 ± 5 nm light. All spectra are the Fourier transforms of $\sim 12\,000$ NMR signals acquired at a temperature < 120 K with magic-angle spinning at 7.00 ± 0.05 kHz. The positions of the bathorhodopsin signals are indicated by asterisks.

isomer *isorhodopsin*.¹³ These conditions were optimized by numerical finite-element simulations of the light penetration through the optically dense sample, as described elsewhere.

The sample temperature is difficult to determine in magic-angle-spinning experiments, due to frictional heating and warming caused by the rotor bearing and drive nitrogen gas streams. As described in the Supporting Information, the sample temperature was calibrated during the illumination and acquisition of the NMR data using the ^{207}Pb chemical shift of lead nitrate,¹⁴ using the narrow proton resonance of the endohedral dihydrogen–fullerene complex $\text{H}_2@C_{60}$ as an independent chemical shift reference.¹⁵ The sample temperature was found to exceed the temperature of the exiting nitrogen gas by up to 40 K. The new results are clearly more reliable than those obtained in 1991³ using a vulnerable experimental and data subtraction procedure.

As shown in Figure 2, double-quantum filtered NMR spectra obtained after illumination show a clear splitting of at least one of the ^{13}C peaks, indicating the generation of bathorhodopsin. The $\sim 35\%$ bathorhodopsin yield is probably limited by the partial penetration of light into the optically dense particles and by secondary photoisomerization of bathorhodopsin.

The new ^{13}C peaks that appeared after illumination were replaced by broader signals at positions closer to the rhodopsin peaks when the temperature of the illuminated sample was allowed to warm above 125 K for several hours. This observation supports their assignment as being due to bathorhodopsin.

Table 1. Isotropic ^{13}C Chemical Shifts of Rhodopsin (δ_{rho}) and Bathorhodopsin (δ_{batho}) As Found in This Work^a

^{13}C site	$\delta_{\text{rho}}/\text{ppm}$	$\delta_{\text{batho}}/\text{ppm}$	$\Delta\delta/\text{ppm}$
9	148.9	149.6	+ 0.7
10	127.9	137.3	+ 9.4
11	141.4	144.4	+ 3.0
12	131.8	132.8	+ 1.0
13	167.4	171.2	+ 3.8
14	122.3	117.5	-4.8
15	165.4	164.4	-1.0

^a The last column shows the isomerization shift, $\Delta\delta = \delta_{\text{batho}} - \delta_{\text{rho}}$. All chemical shifts have a confidence limit of ± 0.5 ppm and are referenced indirectly to TMS using the rhodopsin shift data in ref 16.

The isotropic ^{13}C chemical shifts of the C9 to C15 sites in bathorhodopsin are summarized in Table 1. The +9.4 ppm isomerization shift of C10 is particularly striking. The isomerization shifts tend to be in the deshielding direction toward the center of the chain but in the shielding direction at the end of the chain. Comparisons with previous NMR data and with numerical shift calculations are presented in the Supporting Information.

We are currently in the process of interpreting the bathorhodopsin chemical shifts. The isomerization shifts of the odd-numbered carbons might be interpreted in terms of the small displacement of the negatively charged counterion from the vicinity of the positively charged protonated Schiff base linkage, while the isomerization shifts of the even-numbered carbons are due to the torsional twists.

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Supporting Information Available: Full refs 15 and 16, sample preparation, NMR equipment, temperature calibration data, chemical shift comparisons, and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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