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.1,2 Bathorhodopsin is a highly energetic species that stores about 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,3,4 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 13C chemical shifts in the retinylidene side chain of 13C-labeled bathorhodopsin and thereby obtain information on the electronic structure.3 However, only minor chemical shift changes were observed relative to rhodopsin.5

A more recent 2.7Å 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. The structural data are from the Protein Data Bank entry 2g87 (see ref 4). In this communication, we report on new measurements of the 13C chemical shifts in bathorhodopsin, showing the numbering of the carbon sites and the electronic structure of the protonated Schiff base linkage to the K296 side chain. The structural data are from the Protein Data Bank entry 2g87 (see ref 4).

The new data were generated using 13C2-labeled isotopomers of rhodopsin, prepared by total organic synthesis of 13C2-retinals,11 followed by regeneration of 13C2-rhodopsin using bleached opsin pigments isolated from bovine retinas, and reconstitution with natural-composition lipid membranes. Four 13C2-labeled rhodopsin isotopomers were studied, namely, [9,10-13C2], [11,12-13C2], [12,13-13C2], and [14,15-13C2]-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 ∼200 µm which were then mixed with ∼100 µm 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.

13C 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 R209", combined with phase-cycling to remove signals that do not pass through double-quantum coherence, achieves a clean suppression of the natural 13C background signals from the protein and the lipid. The spectra in Figure 2 show clearly resolved signals from the 13C 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 ± 5 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 configuration of the chromophore.

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).
The temperature was found to exceed the temperature of the exiting bathorhodopsin signals are indicated by asterisks. The new results are clearly more reliable and after (below) 10 h of illumination with 420 nm light. All spectra are the Fourier transforms of ~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 peaks are indicated by asterisks.

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 207Pb chemical shift of lead nitrate, using the narrow proton resonance of the endohedral dihydrogen fullerene complex H2@C60 as an independent chemical shift reference. 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 1991 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 13C peaks, indicating the generation of bathorhodopsin. The ~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 13C 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.

The isotropic 13C 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.

Acknowledgment. This research was supported by BBSRC (UK), EPSRC (UK), NWO (NL), CMSB (NL), and EC E-MeP (NL). We thank M. Carravetta, P. Jansson, J. James, H. J. M. de Groot, I. Heinmaa, D. Sebastiani, and K. Komatsu for technical and experimental help, data, discussions, and samples.

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.

Table 1. Isotopic 13C Chemical Shifts of Rhodopsin (δrho) and Bathorhodopsin (δbatho) As Found in This Work

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<th>δbatho/ppm</th>
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</tr>
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</table>

*The last column shows the isomerization shift, Δδ = δbatho − δrho. 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.

References


JA803801U
Supporting information for

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


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<td>S2</td>
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<tr>
<td>S3</td>
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</tbody>
</table>
(S1) Full reference abbreviated in main text

Ref. 14


Ref. 15


(S2) Materials and Equipment

(S2.1) Samples

Four samples have been prepared and used: [9,10-^{13}C_2]-, [11,12-^{13}C_2]-, [12,13^{13}C_2]-, [14,15-^{13}C_2]-retinylidene-rhodopsin.
(S2.1.1) Isotopically-labelled retinals

A series of doubly $^{13}$C-labelled retinals (see Fig. S1) were prepared by organic synthesis as described in ref [1] and ref [2]. The labeled all-trans-retinals were isomerised to 11-cis-retinal by dissolving them in a solution of dry acetonitrile under controlled illumination conditions. The desired isomer was isolated and purified by high-performance liquid chromatography (HPLC).

![Figure S1. Structure and labeling of retinals used in the synthesis of retinylidene-rhodopsin compounds.](image)

(S2.1.2) Isotopically-labelled rhodopsin

The doubly labeled retinylidene-rhodopsin samples used for the experiments were prepared by incorporating the labeled 11-cis-retinal into the opsin, isolated from fresh cattle eyes. Details of the methodology can be found in [3]. The insertion of the chromophore (11-cis-retinal) into the protein opsin was verified by optical measurements, due to the difference in optical absorbance between rhodopsin and 11-cis-retinal (respectively 498 nm and 380 nm in detergent solution). The w/w ratio protein to lipid was in the range 1:1 to 1:1.3 while the molar ratio protein to lipid was in the range 1:60 to 1:80. All the sample handling was carried out in a darkened room under dim red light. On storage, the samples were kept in light-tight vials in a nitrogen atmosphere at -80 °C. This is to minimize the light-induced isomerization, prevent water absorption and possible oxidation reactions of the sample.

(S2.1.3) Rotor packing

In order to improve the light penetration into the center of the sample, the reconstituted sample was frozen in liquid nitrogen, ground in a custom-made aluminum mortar under liquid nitrogen, and mixed with diameter glass beads (average diameter ≤100µm, from Sigma Aldrich) in a ~2:1 w/w ratio. The mixture was transferred into a 4mm thin wall rotor (VARIAN pencil 2) using a precooled aluminum funnel. All the manipulations involving rhodopsin were done in dim red light with $\lambda \geq 700$nm.
(S2.2) NMR Equipment

(S.2.2.1) NMR Spectrometer

A 9.4T Varian Infinity+ solid state NMR spectrometer was used in conjunction with other, in some cases custom-made, parts designed to allow the in-situ cooling and illumination of rhodopsin samples. These extra parts are essentially a custom-modified VT stack, an exchange Dewar and a purpose-built two channel solid state 4mm probe to allow illumination of the spinning sample. The VT stack and exchange dewar are the core of the low-temperature set up. The VT stack has a built-in heat exchanger using the cold exhaust gas to cool the bearing and the turbine gases. This was done in order to reduce temperature gradients in the proximity of the sample. The exchange Dewar (capacity 160L) is used to cool down the nitrogen gas flow below 120K by thermal exchange with the liquid nitrogen inside the Dewar.

(S.2.2.2) NMR Probe

A 4mm NMR probe was built to allow both the illumination and the cooling of the spinning sample. 14 optical fibers enter the probe from below and are arranged in order to drive the light into the rotor. Bearing and drive pipelines were also customized to guide the spinning gas from the VT stack to the rotor. Dry nitrogen gas, produced by boiling liquid nitrogen, was used for all purposes (bearing, drive, and temperature control).

(S.2.2.3) Illumination

The illumination equipment consists of two 250 W Halogen lamps, two optical lenses, two optical filters and a 14 optical fibers. Filters and lenses were purchased from Edmund optics (York, UK). Light sources produce white light that is passed through the optical filters to select the proper wavelength range. 30 µW were measured at the end of one optical fiber with the two lamps both on. The attenuation by the rotor walls has also been measured to be around 90%. The total power transmitted to the sample is about 42 µW. Three different filters were used in turn: Band Pass filter centered on $\lambda = 480 \pm 5$ nm and $420 \pm 5$ nm; and a Low Pass filter with $\lambda \leq 495$nm. The illumination wavelength used ($\lambda = 420 \pm 5$) was chosen to maximize the light penetration in dense samples while minimizing the formation of isorhodopsin that would interfere with bathorhodopsin formation as well as with signal assignment [4]. Data on illumination with other wavelengths will be shown elsewhere.

(S3) Temperature calibration

In order to have the real and precise value of the sample temperature, a calibration procedure is needed. As explained in ref. [5], the $^{207}\text{Pb}$ resonance of lead nitrate ($\text{Pb(NO}_3)_2$) is a good probe in this regard since its chemical shift varies with temperature. Moreover, the $^{207}\text{Pb}$ resonance in lead nitrate is very narrow, and its chemical shift has a large temperature dependence that is fairly linear over the range
of -130 to 150 °C. Finally, lead nitrate provides a strong NMR signal close to the $^{13}$C Larmor frequency, so that measurements are quick and convenient.

The existing literature data on the $^{207}$Pb chemical shift in lead nitrate does not go down to sufficiently low temperature for our purposes. Fortunately, Ivo Heinmaa, (KBFI, Tallin - private communication) has made available data that show a linear relationship between chemical shift and temperature in the range 105K-300K. These data, obtained without spinning the sample, are reported in Fig. S2 for both the perpendicular and parallel component of the chemical shift tensor, determined by fitting the static powder lineshape. Since the sample is static in these measurements, frictional heating is eliminated and the sample temperature has an error margin of roughly 1K.

![Figure S2. $^{207}$Pb chemical shift as a function of sample temperature. Data from I. Heinmaa refer to perpendicular (circles) and parallel (boxes) component of the lead chemical shift tensor. Triangles are data acquired in static conditions on our equipment used for comparison. Dotted line is the best fit to isotropic chemical shift values (fitting function is reported in Eq. 1).](image)

The isotropic chemical shift fits the following equation:

$$\delta_{iso}(T) = a \cdot T + b$$

where $a = 0.7117$ ppm K$^{-1}$ and $b = -3704.3$ ppm. It is reported in Fig. S2 as a dotted line.

The $^{207}$Pb chemical shift scale was referenced to the calculated frequency of Me,Pb. This frequency was calculated from the $^{27}$Al resonance frequency in aqueous Al(NO$_3$)$_3$ solution using the frequency ratio as given in J. Magn. Reson. 156, 323-326 (2002) by R. K. Harris and E. D. Becker.
Several steps were followed to calibrate the temperature on our spinning samples. First we confirmed that the thermocouple reading out our gas temperature was calibrated correctly by taking $^1$H and $^{207}$Pb NMR spectra of a rotor containing Pb(NO$_3$)$_2$ as well as a small sealed glass capillary of 2-methylpentane, which freezes at 119 K. The freezing of the 2-methylpentane could be detected as a broadening of the proton NMR signal. This confirmed the good correspondence between the sample temperature and the gas readout temperature under static sample conditions. Further calibration points taken on our equipment for the perpendicular component of the $^{207}$Pb chemical shift tensor for Pb(NO$_3$)$_2$ are reported in Fig. S2 (triangles) and verify the Heinmaa data.

In the second step the temperature calibration was done while spinning at 7.00 kHz, using Eq.(1) to interpret the isotropic $^{207}$Pb chemical shift in terms of temperature. The temperature inside the NMR rotor depends on the cooling, bearing and drive gas flows as well as the spinning frequency.

In order to assess the temperature gradient inside the rotor, a 4mm thin-wall (0.55 mm wall thickness) rotor was filled with two different layers of Pb(NO$_3$)$_2$ as in Fig. S3 (mix 1 and 2).

These layers consisted of a 1:1 mixture of Pb(NO$_3$)$_2$ and NaCl. The NaCl was used to reduce the sample density and to ensure that the rotor was comparable in mass to the one used in the rhodopsin experiments. In addition, a sample of the endohedral fullerene complex H$_2$@C$_{60}$ was fitted inside the rotor. The $^1$H chemical shift of H$_2$@C$_{60}$ shows no measurable temperature dependence, and the proton line stays narrow even at low temperatures (see ref.14 of main text). This make it possible to correct any apparent variation of the chemical shift due to susceptibility changes in the probe components, shim coils, or surrounding gas, rather than to a change in the temperature. Finally, to ensure reproducibility, the mass of all the rotors was kept close to the one used for the calibration and reported in Fig. S3. Fig. S4 shows real versus readout temperature as found by spinning the sample at 7.00 kHz.
Figure S4. Real sample temperature (from the $^{207}$Pb chemical shift) versus thermocouple readout of the exit gas temperature, while spinning at 7.00 kH.""
The gas flows that give rise to the desired spinning frequency and stability for this rotor were then duplicated for the rhodopsin experiments. This procedure demanded that we did not use automatic control of the bearing and drive gas flows but instead regulated these flows manually.

We also confirmed in separate experiments that the radiofrequency irradiation has a negligible effect on the sample temperature in this temperature regime. This was done by monitoring the $^{207}$Pb chemical shift of a rotor containing lead nitrate in close contact with a frozen rhodopsin sample. The rf irradiation parameters were comparable to those used in the actual experiments. At these low temperatures, the mobile ions in the biological sample are immobilized and are not capable of absorbing energy from the electric field. The situation is very different when performing NMR at near-physiological conditions, where rf heating can be dramatic.

(S4) Chemical shift comparison

Table S1 compares our results with the earlier report of Smith et al.[6,7] and also the recent quantum calculations of Röhrig et al.[8] and Gascon et al.[9,10].

<table>
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<tr>
<th>δC</th>
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<th>δ$_{\text{batho}}$ /ppm (this work)</th>
<th>δ$_{\text{rho}}$ /ppm [6]</th>
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Table S1. Chemical shifts of the retinilydene chromophore of rhodopsin and bathorhodopsin from this work are compared with previous experimental data (III and IV) and results of QM calculations (V, VI, VII and VIII).

Table S2 and figure S6 compare the isomerization shifts ($\Delta$δ=δ$_{\text{batho}}$−δ$_{\text{rho}}$ – difference in chemical shifts between bathorhodopsin and rhodopsin) for our results, the earlier experimental [6,7] data and quantum mechanical calculations [8-10]. Some features of the calculations in ref.[8] do correlate well with the experimental results. For example the strong negative isomerization shift at position 14 is predicted well by the calculations in [9,10] and all calculations predict positive isomerization shifts at position 10. However, the large magnitude of the isomerization shift at position 10 is not predicted by any of the calculations. The strong alternation of the isomerization shifts at positions 13 and 14 is also not predicted.
Table S2. Experimental and theoretical isomerization shifts for bathorhodopsin with respect to rhodopsin.

<table>
<thead>
<tr>
<th>13C</th>
<th>This work</th>
<th>Smith et al. [6,7]</th>
<th>Röhrig et al. [8]</th>
<th>Gascon et al. [9,10]</th>
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Figure S6. Isomerization shifts for bathorhodopsin with respect to rhodopsin: data from this work (circles), Smith et al. [6,7] (open boxes), Röhrig et al. [8] (solid line) and Gascon et al.[9,10] (dashed line).

(S5) NMR techniques

Double quantum filtered dipolar recoupling technique was used to isolate the signal of the two 13C enriched sites in both retinal and retinilydene-samples. The actual pulse sequence is reported in Fig. S5. A 1H 90º pulse length of 2.5 µs was used for the initial excitation of 1H transverse magnetisation before CP. During a CP contact time of 2 ms, the 1H rf nutation frequency was constant at 50 kHz, while the 13C rf nutation frequency was ramped linearly from 41 to 82 kHz. The recycle delay was 5 s. Double-quantum recoupling was achieved using cycles of the R2O2.
symmetry-based recoupling sequence with the basic element \((90^\circ, 270^\circ, 180^\circ)\) [11,12].

The excitation time was of the same duration as the reconversion time and was optimized experimentally for each sample to give maximum signal, namely 400 µs (40 basic elements) [9,10, 13\textsuperscript{13}C\textsubscript{2}], 380 µs (38 elements), [11,12, 13\textsuperscript{13}C\textsubscript{2}], 400µs (40 elements) [12,13, 13\textsuperscript{13}C\textsubscript{2}], 400µs (40 elements) and 380µs (38 elements) [14,15, 13\textsuperscript{13}C\textsubscript{2}].

The nutation frequency on the \(^{13}\text{C}\) channel during the recoupling sequence was set to \(\omega^\text{nunt}/2\pi = (N/n)*\tau_{\text{rel}} = (20/2)*7\text{kHz} = 70\text{ kHz}\) without proton decoupling [13]. The DQ efficiency (as compared to a CP experiment) was around 35%. \(^1\text{H}\) decoupling during the acquisition of the \(^{13}\text{C}\) FID was achieved using the SPINAL-64 [14] sequence with a \(^1\text{H}\) rf nutation frequency of 80kHz. A total phase cycle of 16 steps was used in order to filter out double quantum signals.

![Figure S7. Double quantum dipolar recoupling sequence used to isolate couples of \(^{13}\text{C}\)-enriched nuclear resonances in rhodopsin samples.](image)

A typical timing for one of such illumination experiments consists of about 12h of continuous illumination of the sample, then 12k scans are acquired in about 18h with a recycle delay of 5 s and an acquisition time of 20.48 ms.

Liquid nitrogen in the exchange Dewar was refilled each 4h for a total consumption of about 350 l per experiment. Nitrogen gas used for sample spinning was consumed with a rate of about 36 l/h (~650 l per experiment).

References