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Structure and Protein Environment of the Retinal Chromophore in Light- and Dark-Adapted Bacteriorhodopsin Studied by Solid-State NMR†

Steven O. Smith,‡,§ Huub J. M. de Groot,‡,∥ Ronald Gebhard,⊥ Jacques M. L. Courtin,⊥ Johan Lugtenburg,⊥ Judith Herzfeld,∥ and Robert G. Griffin*,‡

Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, Department of Chemistry, Rijksuniversiteit te Leiden, 2300 RA Leiden, The Netherlands, and Department of Chemistry, Brandeis University, Waltham, Massachusetts 02254-9110

Received March 13, 1989; Revised Manuscript Received June 20, 1989

ABSTRACT: Our previous solid-state ^{13}C NMR studies on bR have been directed at characterizing the structure and protein environment of the retinal chromophore in bR_{568} and bR_{548}, the two components of the dark-adapted protein. In this paper, we extend these studies by presenting solid-state NMR spectra of light-adapted bR (bR_{568}) and examining in more detail the chemical shift anisotropy of the retinal resonances near the ionone ring and Schiff base. Magic angle spinning (MAS) ^{13}C NMR spectra were obtained of bR_{568}, regenerated with retinal specifically ^{13}C labeled at positions 12–15, which allowed assignment of the resonances observed in the dark-adapted bR spectrum. Of particular interest are the assignments of the ^{13}C-13 and ^{13}C-15 resonances. The ^{13}C-15 chemical resonance for bR_{568} (160.0 ppm) is upfield of the ^{13}C-15 resonance for bR_{548} (163.3 ppm). This difference is attributed to a weaker interaction between the Schiff base and its associated counterion in bR_{568}. The ^{13}C-13 chemical shift for bR_{568} (164.8 ppm) is close to that of the *all-trans*-retinal protonated Schiff base (PSB) model compound (~162 ppm), while the ^{13}C-13 resonance for bR_{548} (168.7 ppm) is ~7 ppm downfield of that of the 13-cis PSB model compound. The difference in the ^{13}C-13 chemical shift between bR_{568} and bR_{548} is opposite that expected from the corresponding ^{15}N chemical shifts of the Schiff base nitrogen and may be due to conformational distortion of the chromophore in the C_{13}=C_{14}—C_{15} bonds. We have also obtained spectra of dark-adapted bR regenerated with retinal ^{13}C labeled at positions 1–4, 16, and 17. The chemical shifts of the ^{13}C-1 (34.5 ppm), ^{13}C-2 (42.7 ppm), ^{13}C-3 (18.6 ppm), and ^{13}C-4 (34.6 ppm) resonances in bR are very close to their values in the all-trans PSB model compound. This indicates that the perturbation previously observed near the β-ionone ring is localized near C-5. The spin–lattice relaxation times (T_1) of the ^{13}C-16,17 bR resonances of ~1.2 s are closer to the T_1's of 6-s-trans model compounds than to those of 6-s-cis model compounds, confirming the previous solid-state NMR determination of a 6-s-trans chromophore in the protein.

Absorption of light by the retinal chromophore in bacteriorhodopsin (bR)[1] initiates a cyclic photochemical reaction which drives the transport of protons across the cell membrane of *Halobacterium halobium* [for reviews, see Birge (1981) and Stoeckenius and Bogomolni (1982)]. The structure of bR's retinal prosthetic group and its interaction with specific binding site protein residues determine in large part its efficiency as a light-energy converter. Spectroscopic methods have revealed many of the details of the proton translocation process. Resonance Raman (Smith et al., 1985; Terner & El-Sayed, 1985; Stockburger et al., 1986) and FTIR spectroscopy (Engelhard et al., 1985; Dollinger et al., 1986; Braiman et al., 1987) have been used to study chromophore structural changes and protein protonation changes during the bR photocycle, while visible absorption studies of bR regenerated with dihydro derivatives of retinal have provided evidence for protein charges (or dipoles) near the β-ionone ring of the chromophore (Nakanishi et al., 1980; Lugtenburg et al., 1986; Spudich et al., 1986). Solid-state NMR spectroscopy has also been used to probe the structure of bR and has provided specific information on the chromophore's C_6–C_7 single-bond conformation and C=N double-bond configuration, as well as the location of specific protein perturbations in the retinal binding site

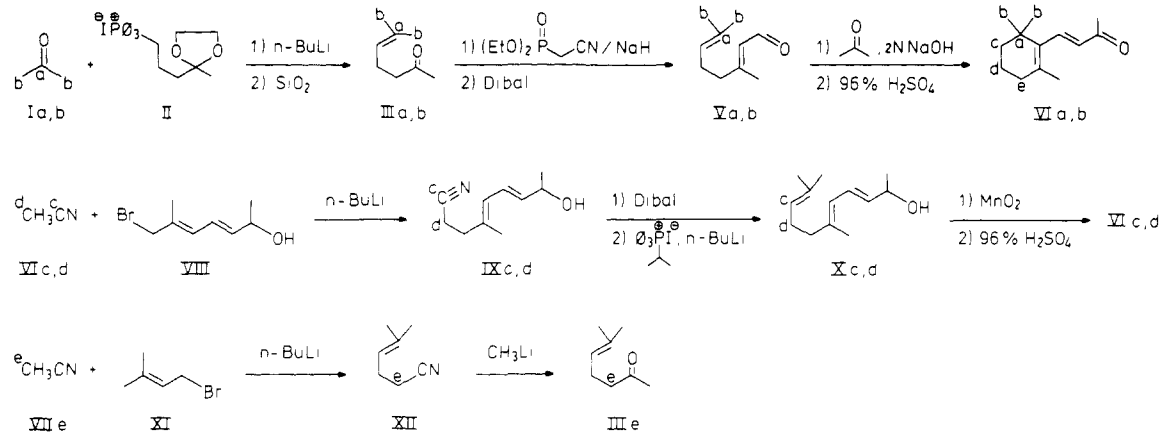
† This research was supported by the National Institutes of Health (GM-36810, GM-23289, RR-00995), the Netherlands Foundation for Chemical Research (SON), and the Netherlands Organization for the Advancement of Pure Research (ZWO). S.O.S. was supported by a USPHS postdoctoral fellowship (GM-10502).

‡ Massachusetts Institute of Technology.
§ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511.
∥ Brandeis University.
⊥ Rijksuniversiteit te Leiden.

[1] Abbreviations: bR, bacteriorhodopsin; bR_{568}, light-adapted bacteriorhodopsin; bR_{548}, 13-cis component of dark-adapted bacteriorhodopsin; MAS, magic angle spinning; PSB, protonated Schiff base; ppm, parts per million; SB, unprotonated Schiff base; TMS, tetramethylsilane.

0006-2960/89/0428-8897$01.50/0

Scheme I



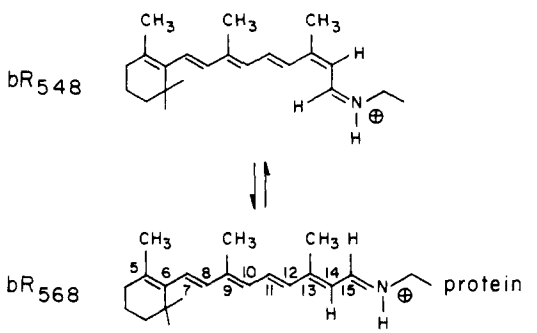


FIGURE 1: Structure of the retinal chromophore in dark-adapted bacteriorhodopsin.

(Harbison et al., 1983, 1984b, 1985a).

Our previous ^{13}C and ^{15}N solid-state NMR studies of bR were focused on the chromophore in the *dark-adapted* form of the pigment (Figure 1). Dark-adapted bR consists of two components, bR_{568} and bR_{548}, in thermal equilibrium.[2] The pigment converts to nearly 100% bR_{568} upon light adaptation. The retinal chromophore, which is attached to lysine-216 through a protonated Schiff base linkage (Lewis et al., 1974; Bayley et al., 1981; Rothschild et al., 1982), has an all-trans configuration in bR_{568}. In the thermal $bR_{568} \rightarrow bR_{548}$ conversion, the retinal chromophore isomerizes about both the $C_{13}=C_{14}$ and C=N double bonds (Smith et al., 1984; Harbison et al., 1984b). The bR_{548} pigment has no direct role in the proton pumping process, although it is thought that the protein mechanism that *thermally* converts bR_{548} to bR_{568} may be important in the thermal reactions of the photocycle. As a result, we have been interested in the structure and protein environment of the retinal chromophore in both the bR_{548} and bR_{568} pigments.

In this paper, we present NMR spectra of fully light-adapted bR_{568}. The light-adapted spectra allow us to assign the ^{13}C resonances previously observed in the dark-adapted pigment for positions C-12 through C-15. In addition, we extend our previous solid-state NMR studies on dark-adapted bR by presenting ^{13}C spectra of the dark-adapted protein regenerated with ^{31}C-1, ^{13}C-2, ^{13}C-3, ^{13}C-4, and ^{13}C-16,17 retinals. The ^{13}C-1 through ^{13}C-4 bR spectra show that the perturbation of the ionone ring is localized at the C-5 position, while the T_1 relaxation data of the ^{13}C-16,17 resonances confirm a 6-s-trans chromophore in bR. With the ionone ring labels, we have completed a series of solid-state NMR experiments on dark-adapted bR in which each carbon position of the retinal chromophore has been individually substituted with ^{13}C.

Materials and Methods

The ^{13}C-12, ^{13}C-13, ^{13}C-14, and ^{13}C-15 retinals were synthesized according to published procedures (Pardoen et al., 1984, 1985; Lugtenburg, 1985). The synthesis of the ^{13}C-1, ^{13}C-2, ^{13}C-3, ^{13}C-4, and ^{13}C-16,17 retinals will be described in detail elsewhere (Gebhard et al., 1989); here, we provide a brief description. Scheme I describes the synthetic routes used to introduce ^{13}C labels at the required positions in the retinal skeleton on the basis of simple and commercially available 90% ^{13}C-labeled starting materials: [2-^{13}C]acetone (Ia), [1,3-^{13}C]acetone (Ib), [1-^{13}C]acetonitrile (VIIc), and [2-^{13}C]acetonitrile (VIId,e) (Merck Sharp & Dohme Isotopes).

When [1-^{13}C]acetone (Ia) is treated with the Wittig reagent (II) in the presence of *n*-butyllithium (*n*-BuLi), a double bond is formed with the ^{13}C label at the future C-1 position of the retinal. After deketalization with the aid of SiO_2, the resulting ^{13}C-labeled ketone (IIIa) was subjected to a Horner–Emmons reaction with a C-2 phosphonate (IV). Subsequent reduction with diisobutylaluminum hydride (Dibal) yielded ^{13}C-labeled citral (Va). Citral was easily converted into 1-^{13}C-labeled β-ionone (VIa) via successive aldol condensation with acetone and cyclization in 96% sulfuric acid (Smit et al., 1959). Via the same procedure, [1,3-^{13}C]acetone (Ib) was converted into di-^{13}C-labeled β-ionone (VIb). Both β-ionones (VIa and VIb) were then subjected to the well-known four-step procedure (Pardoen et al., 1985), consisting of two Horner–Emmons reactions with a C-5 phosphonate and Dibal reductions, to yield [1-^{13}C] and [16,17-^{13}C]retinals in 20% yield.

For the introduction of the ^{13}C label at the C-2 or C-3 positions of retinal, the anion of [1-^{13}C]- or [2-^{13}C]acetonitrile (VIIc,d) formed with *n*-BuLi was treated with hydroxy bromide VIII (prepared by allylic bromation and Dibal reduction of 6-methyl-3,5-heptadienone). Dibal reduction of the resulting nitrile (IXc,d), followed by condensation with the Wittig salt of isopropyl iodide, yielded the pseudo ionoles (Xd,c). Oxidation with manganese dioxide and cyclization in 96% sulfuric acid yielded the β-ionones (VIc,d), which were converted to [2-^{13}C]- and [3-^{13}C]retinal in the same way as IIIa and IIIb. The yield was 10% on the basis of the acetonitriles.

To introduce the ^{13}C label at the C-4 position of retinal, commercially available (Fluka AG) 1-bromo-3-methyl-2-butene (VIII) was treated with the anion of [2-^{13}C]acetonitrile (IX) formed with *n*-BuLi. The resulting ^{13}C-labeled nitrile

[2] Recent studies on the isomer ratio in dark-adapted bR indicate a ratio of 67:33 for the 13-cis and all-trans components, respectively (Scherrer et al., 1989). These results, along with measured absorption spectra of bacteriorhodopsin regenerated with 13-*cis*-retinal, yield a λ_{max} for the 13-cis component of 555 nm. Consequently, the two components of dark-adapted bR may alternatively be designated bR_{568} and bR_{555}.

(IX) was reacted with excess methyllithium to provide the ketone (IIIc), which was converted into [4-^{13}C]retinal in the same way as IIIa and IIIb. The overall yield of [4-^{13}C]retinal was 17%.

The labeled retinals were incorporated into bR by bleaching native purple membrane followed by regeneration with retinal (Oesterhelt & Schuhmann, 1974). The purple membrane was bleached in 0.5 M hydroxylamine hydrochloride (pH 8 and 35–40 °C). The bleaching process was done either in the dark (12–16 h) or with light from a slide projector with a 440-nm cut-off filter (2–3 h). The bleached membrane was pelleted, washed two to three times to remove excess hydroxylamine, and resuspended in 10 mM Hepes buffer (pH 7). The pigment was regenerated by addition of retinal in ethanol (~3 mM). Excess retinal from the regeneration and retinal oxime produced by the bleaching process were removed by washing the membrane 10–15 times with a 2% solution of bovine serum albumin (Sigma Chemical Co.). The purple membrane was then washed twice in 10 mM Hepes buffer (pH 7) and pelleted. Comparison of the NMR spectra of labeled bR obtained by the bleaching/regeneration procedure with those of the corresponding labeled protein obtained by regenerating bacterioopsin ("white membrane") from a retinal-deficient strain of *H. halobium* as was done in our previous solid-state NMR studies (Harbison et al., 1984a,b, 1985a) showed no significant differences.

High-resolution solid-state ^{13}C NMR spectra of bR can be obtained by MAS and cross-polarization techniques. These methods are well suited for studying integral membrane proteins that have restricted motion and are not able to rapidly reorient relative to an external magnetic field. In such systems, the ^{13}C NMR resonances broaden due to anisotropy in the dipolar and chemical shift interactions. MAS averages these interactions and breaks up a broad ^{13}C NMR resonance into a sharp centerband at the isotropic chemical shift flanked by rotational sidebands that are spaced at the spinning frequency. The sidebands contain information concerning the chemical shift anisotropy which often aids in the interpretation of the NMR data. Since the sidebands move when the spinning speed changes, high-resolution *difference* spectra depend on an accurate spinning-speed controller. Difference spectra are important because they can be used to remove the natural abundance signal from the spectrum and give the pure spectrum of labeled groups. After the signal of interest is isolated, it may be possible to measure the intensities of the individual sidebands and analyze them according to the procedure of Herzfeld and Berger (1980). However, if the difference spectrum is noisy or if there is overlap between the lines, it is necessary to analyze the entire spectrum by combining the line intensities according to Herzfeld and Berger with empirical line widths in a least-squares fit. A complete description of the program that we use to implement this approach will be presented elsewhere (de Groot et al., unpublished results).

Both light- and dark-adapted solid-state MAS spectra of hydrated purple membrane were obtained on a home-built spectrometer using a standard cross-polarization pulse sequence with a mix time of 2 ms, an acquisition time of 10 ms, and a recycle delay of 2 s. Typically 20 000–30 000 scans were averaged for each spectrum, and the spinning speed was maintained to within ~3 Hz by a spinning-speed controller (de Groot et al., 1988). The ^{13}C frequency was 79.9 MHz, and the ^{1}H $\pi/2$ pulse length was ~3.0 μs. The carbon T_1 of ^{13}C-16,17 bR was measured with a modified inversion–recovery pulse sequence adapted from a sequence proposed by Torchia (1978) and used previously to measure the methyl group T_1 values in bR and retinal model compounds (Harbison et al., 1985a). All chemical shifts are referenced to external TMS.

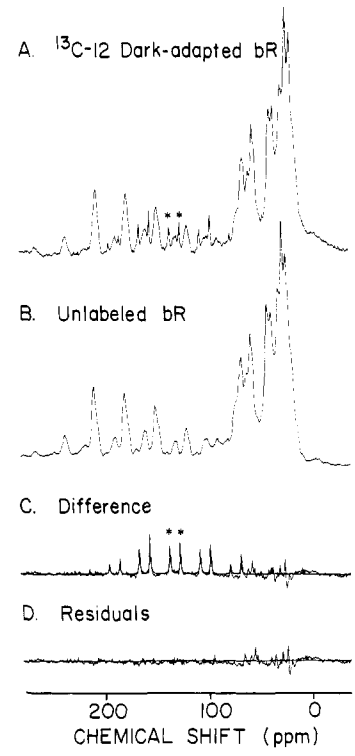


FIGURE 2: ^{13}C MAS spectra of ^{13}C-12 dark-adapted bR (A) and unlabeled dark-adapted bR (B). The difference between (A) and (B) yields a spectrum of only the labeled resonance. The difference spectrum and a simulated fit are both shown in (C). The residuals between the real and simulated data are shown in (D). The centerbands of the ^{13}C-12 resonances are marked with asterisks.

The purple membrane was light adapted by irradiating the sample in the NMR rotor (7-mm sapphire rotors, Doty Scientific, volume ≃ 300 μL) with light focused from a slide projector at ~0 °C. The sample was then inserted into the NMR probe, and the temperature was maintained below −20 °C to keep the purple membrane from dark adapting. Typically, the amount of bR used for dark-adapted experiments (~80 mg) is greater than the amount used for light-adapted experiments (~40 mg). The lower concentration used in the light-adapted experiments allows a suspension of bR to be placed in the NMR rotor rather than a pellet. This facilitates complete light adaptation of the sample.

Results

An analysis of the isotropic and anisotropic chemical shifts in [^{13}C]retinal-labeled bR can provide information on the structure of the retinal chromophore and its interaction with the surrounding protein. The chemical shift anisotropy can in turn be extracted from the intensities of the rotational sidebands in a MAS spectrum (Herzfeld & Berger, 1980). However, in many instances when there is overlap between the [^{13}C]retinal resonances and the natural abundance background from the protein, it is difficult to accurately measure sideband intensities. Recently, a least-squares fitting method has been developed for determining the chemical shift anisotropy in this situation (de Groot et al., unpublished results). These methods are used here to establish the principal values of the chemical shift tensor for retinal positions C-12 through C-15.

Figure 2 illustrates the least-squares fitting approach for analyzing MAS spectra. Figure 2A is the proton-decoupled

Table I: Isotropic Chemical Shifts and the Three Principal Values of the Chemical Shift Tensors for bR_{568} and bR_{548} at C-12, C-13, C-14, C-15, and the Schiff Base Nitrogen

compound	σ_I	σ_{11}	σ_{22}	σ_{33}
^{13}C-12				
bR_{568}	134.3	58 (6)	135 (8)	210 (6)
bR_{548}	124.2	35 (5)	132 (7)	206 (5)
^{13}C-13				
bR_{568}	164.8	22 (5)	209 (5)	264 (4)
bR_{548}	168.7	26 (4)	217 (5)	262 (4)
^{13}C-14				
bR_{568}	122.0	50 (3)	134 (2)	182 (3)
bR_{548}	110.5	45 (3)	107 (4)	179 (3)
^{13}C-15				
bR_{568}	160.0	76 (3)	170 (2)	236 (3)
bR_{548}	163.2	51 (3)	182 (3)	256 (4)
^{15}N				
bR_{568}	143.5	14 (8)	157 (8)	260 (7)
bR_{548}	150.6	13 (8)	178 (8)	260 (7)

[a] Chemical shifts measured in ppm relative to external TMS (^{13}C) and NH_4Cl (^{15}N). Numbers in parentheses represent experimental errors.

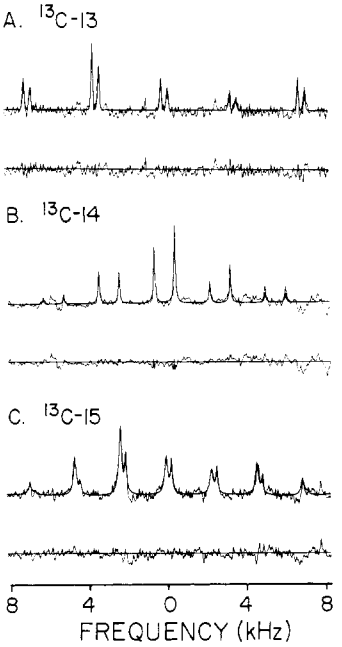


FIGURE 3: ^{13}C MAS difference spectra along with simulated fits for ^{13}C-13 dark-adapted bR (A), ^{13}C-14 dark-adapted bR (B), and ^{13}C-15 dark-adapted bR (C). The residuals between the real and simulated data are shown below each spectrum. The zero frequency in these figures is taken as the isotropic shift for each labeled position.

MAS ^{13}C spectrum of dark-adapted bR containing retinal ^{13}C-labeled at position 12. Because only a limited amount of sample was available, the spectrum was recorded at low temperature (ca. −60 °C) to improve the signal-to-noise ratio. The sharp lines at 134.3 and 124.2 ppm (marked with asterisks) are from the ^{13}C-12 resonance and arise from the two components, bR_{568} and bR_{548}, in the dark-adapted sample. The rotational sidebands associated with these resonances are clearly observed in the difference spectrum (Figure 2C). The remaining lines in the spectrum are natural abundance ^{13}C resonances from the protein. The first step in the fitting procedure is to obtain a spectrum of unlabeled bR at the same temperature and use it to subtract the natural abundance background from the spectrum of the labeled bR. The difference spectrum (Figure 2C) which contains only the labeled resonances is then fit with the least-squares fitting routine. The fitting program minimizes the sum of the squares of the differences between the data and the calculated spectrum. These

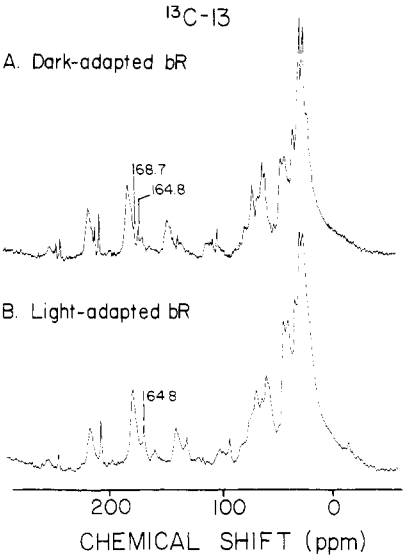


FIGURE 4: ^{13}C MAS spectra of ^{13}C-13 bR in the dark-adapted (A) and light-adapted (B) states.

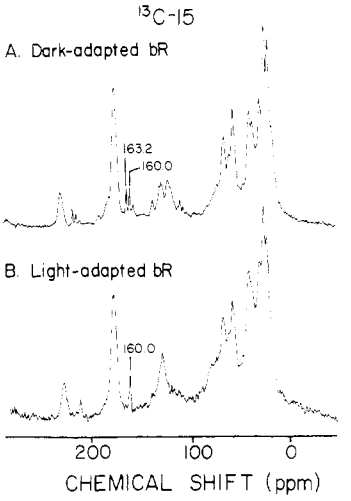


FIGURE 5: ^{13}C MAS spectra of ^{13}C-15 bR in the dark-adapted (A) and light-adapted (B) states.

differences or "residuals" are shown in Figure 2D. The principal values of the ^{13}C-12 shift tensor that are obtained with this approach are given in Table I.

Figure 3 presents the difference spectra, fits, and residuals for dark-adapted bR regenerated with ^{13}C-13-, ^{13}C-14-, and ^{13}C-15-labeled retinal. As in the ^{13}C-12 bR spectrum, there are two components corresponding to bR_{568} and bR_{548} in each of these spectra. The principal values of the chemical shift tensors for these resonances are given in Table I.

The assignment of the two components observed in the dark-adapted spectrum to bR_{568} and bR_{548} is important before attempting to interpret any of the chemical shift differences. Originally, we based our assignments on the ratio of the peak intensities (Harbison et al., 1984a,b, 1985a) since it is known that the ratio of bR_{548}:bR_{568} is about 3:2. However, in our dark-adapted spectra the ratio has varied between 50:50 and 60:40, and therefore, it has not been possible to conclusively assign all of the ^{13}C resonances. A direct method for assigning the dark-adapted resonances is to light adapt the sample, thereby converting the bR_{548}/bR_{568} mixture entirely to bR_{568}. Two positions that have previously not been assigned are ^{13}C-13 and ^{13}C-15. Figure 4 presents spectra of dark-adapted (A) and light-adapted (B) bR regenerated with ^{13}C-13 retinal. In the dark-adapted spectrum, the two retinal resonances are

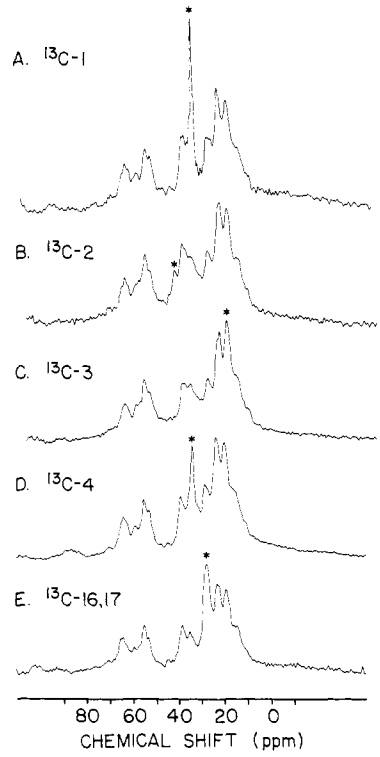


FIGURE 6: ^{13}C MAS spectra of dark-adapted bR regenerated with ^{13}C-1 (A), ^{13}C-2 (B), ^{13}C-3 (C), ^{13}C-4 (D), and ^{13}C-16,17 (E) retinals. The spectra were obtained at −20 ± 5 °C.

observed at 168.7 and 164.8 ppm. Comparison of the retinal resonances in spectra A and B can be used to assign the bR_{568} resonance to the upfield line at 164.8 ppm. Similarly, Figure 5 presents spectra of bR regenerated with ^{13}C-15 retinal in the dark-adapted (A) and light-adapted (B) states. In the dark-adapted spectrum, resonances are observed at 163.3 and 160.0 ppm. By comparison of the light- and dark-adapted spectra, the resonance corresponding to bR_{568} can be assigned to the upfield resonance at 160.0 ppm. Light-adapted spectra of ^{13}C-12 bR and ^{13}C-14 bR have also been obtained and confirm the previous assignments of the 134.3 ppm (^{13}C-12) and 122.0 ppm (^{13}C-14) resonances to bR_{568}.

Solid-state NMR spectra of bR regenerated with ^{13}C-1, ^{13}C-2, ^{13}C-3, ^{13}C-4, and ^{13}C-16,17 retinals are shown in Figure 6. Difference spectra (not shown) between the labeled and unlabeled spectra clearly show the labeled retinal resonances for ^{13}C-1, ^{13}C-2, ^{13}C-3, ^{13}C-4, and ^{13}C-16,17 bR at the indicated positions. The differences in intensities of the lines corresponding to the labels in these spectra are due partially to differences in the completeness of regeneration. The most unusual feature of the five ^{13}C bR spectra in Figure 6 is the intensity and narrow width of the ^{13}C-1 resonance, which may be due to the absence of an attached proton and associated dipolar broadening. The line width (fwhm) is ~50 Hz for this resonance, in contrast to ~200 Hz for the ^{13}C-2, ^{13}C-3, and ^{13}C-4 resonances. For comparison, the line widths of the ^{13}C resonances along the conjugated retinal chain are typically 80–100 Hz. The larger line widths for positions 2–4 are most likely due to motion of the ionone ring. The ^{13}C-1 through ^{13}C-4 spectra were all obtained at low temperature (less than −15 °C) to slow any possible motion. Low temperature had the most dramatic effect for the ^{13}C-4 resonance, which was not observable at room temperature, an effect which we are currently investigating further.

Previous studies of the spin–lattice relaxation times (T_1) of the C-16, C-17, and C-18 methyl groups on the ionone ring indicated that the ^{13}C T_1 values are sensitive to the conformation about the C_6–C_7 bond (Harbison et al., 1985a). Spectra obtained of ^{13}C-16,17 bR with 50-Hz resolution did not reveal a splitting between the two resonances. Table II presents the T_1 data for ^{13}C-16,17 bR and several 6-s-cis and 6-s-trans model compounds. The 6-s-cis geometry occurs in most retinal derivatives, while the 6-s-trans structure has been observed in crystal structures of 13-*cis*-retinal (Simmons et al., 1981) and retinoic acid (Stam & McGillivary, 1963).

The results presented above, along with previously reported data (Harbison et al., 1984a,b, 1985a), complete a series of solid-state NMR experiments on bacteriorhodopsin where each position of the retinal chromophore has been selectively labeled

Table II: ^{13}C T_1 Values of the ^{13}C-16 and ^{13}C-17 Methyl Groups in bR Compared with Those of 6-*s-cis*- and 6-*s-trans*-Retinal Derivatives

compound	position 16	position 17
6-s-cis[a]	0.15–0.31	0.18–0.28
6-s-trans[a]	1.05	1.32
bR[b]	1.2	1.2

[a] Data from Harbison et al. (1985b). [b] The ^{13}C-16 and ^{13}C-17 resonances were not resolved.

Table III: Summary of ^{13}C Chemical Shifts for bR_{568} and bR_{548} and for *all-trans*- and 13-*cis*-Retinal Protonated Schiff Base Model Compounds

	bR_{568}[a]	bR_{548}	6-s-cis, all-trans PSB[b]	6-s-cis, 13-cis PSB[a]
^{13}C-1	34.5	34.5[a]	34.4	34.4
^{13}C-2	42.7	42.7[a]	40.0	41.6
^{13}C-3	18.6	18.6[a]	20.5	19.4
^{13}C-4	34.6	34.6[a]	34.2	33.5
^{13}C-5	144.8	144.8[c]	128.8	131.8
^{13}C-6	135.4	134.9[c]	138.9	137.7
^{13}C-7	129.5	130.7[c]	128.8	132.0
^{13}C-8	132.7	131.6[c]	140.8	137.3
^{13}C-9	146.4	148.4[c]	142.1	145.6
^{13}C-10	133.0	129.7[d]	135.0	129.1
^{13}C-11	139.1	135.4[d]	138.9	137.0
^{13}C-12	134.3	124.2[d]	135.0	125.1
^{13}C-13	164.8	168.7[c]	161.8	162.2
^{13}C-14	122.0	110.5[e]	122.6	118.2
^{13}C-15	160.0	163.2[c]	167.0	165.5
^{13}C-16	28.9	28.9[a]	28.9	29.0
^{13}C-17	28.9	28.9[a]	31.7	29.0
^{13}C-18	22.0	22.0[c]	23.3	22.0
^{13}C-19	11.3	11.3[d]	14.0	13.3
^{13}C-20	13.3	22.0[f]	14.0	22.0

[a] Data from this work. [b] Data from Harbison et al. (1985b). [c] Data from Harbison et al. (1985a). [d] Data from Harbison et al. (1984a). [e] Data from Harbison et al. (1984b). [f] A. McDermott and H. de Groot, unpublished results.

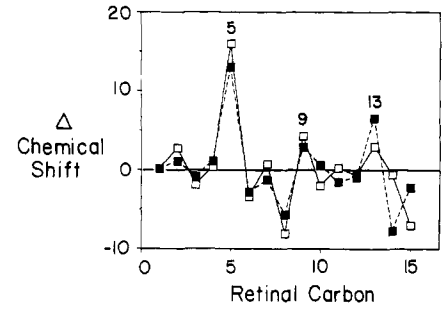


FIGURE 7: Plot of the isotropic chemical shift difference between bR_{568} and the all-trans PSB model compound (solid line, open boxes) and between bR_{548} and the 13-cis PSB model compound (dashed line, closed boxes), as a function of chromophore position (with atoms numbered as in Figure 1).

with ^{13}C. These results are summarized in Table III and also illustrated in Figure 7, where chemical shifts are plotted for (1) bR_{568} relative to *all-trans*-retinal PSB (*n*-butylimmonium chloride salt) and (2) bR_{548} relative to 13-*cis*-retinal PSB (*n*-butylimmonium chloride salt).

Discussion

Our previous solid-state NMR studies have focused on the dark-adapted form of bacteriorhodopsin (Harbison et al., 1983, 1984a,b, 1985a; de Groot et al., 1989). These studies have provided evidence for (1) a weak interaction between the Schiff base nitrogen and its associated counterion, (2) an s-trans conformation about the C_6–C_7 retinal bond, (3) syn and anti conformations about the C=N bond, and (4) a protein-induced perturbation of the β-ionone ring of the retinal chromophore. In this paper, we present solid-state ^{13}C NMR spectra of light-adapted bR. These studies demonstrate the ability to photoconvert bR_{548} to bR_{568} in the solid-state NMR experiment and provide a direct method for assigning the [^{13}C]retinal resonances.

The resonance assignments, along with two recent improvements for determining the principal values of the chemical shift tensor in complex protein samples (MAS difference spectroscopy and least-squares fitting of the difference spectrum), allow us to more closely examine the ^{13}C-12 to ^{13}C-15 resonances of the retinal. The C_{13}=C_{14} and C_{15}=N bonds are known to isomerize during the thermal $bR_{568} \rightarrow bR_{548}$ conversion, while the C_{13}=C_{14} bond isomerizes during the bR photocycle. Our immediate aim is to determine how the chemical shift tensor changes for these positions in bR_{568} and bR_{548}. Such information should provide a more detailed picture of the structure of the retinal in light- and dark-adapted bR, as well as forming a basis for further studies of the bR photointermediates.

We also present ^{13}C NMR spectra of dark-adapted bR selectively labeled on the β-ionone ring. These spectra more accurately define the perturbation of the β-ionone ring and also provide additional support for a 6-s-trans bond in the bR chromophore.

Chemical Shifts of ^{13}C-12-Labeled bR. One of the largest differences in the retinal ^{13}C chemical shifts between the all-trans and 13-cis PSB model compounds (Table III) occurs at position 12. A comparable upfield shift of ~6–8 ppm has been observed in going from the all-trans to the 13-cis configuration in several other retinal derivatives (Englert, 1975) and is thought to arise from steric interaction between the proton attached to C-12 and the proton on C-15 *across the cis* C_{13}=C_{14} bond. An analysis of the chemical shift tensors of ^{13}C-12 *all-trans*- and 13-*cis*-retinal showed that this "γ-effect" is localized in the σ_{11} element, which is oriented perpendicular to the C=C bond (Wolff et al., 1977; Harbison et al., 1985b).[3] Such localized shifts have also been observed in σ_{11} (associated with a γ-effect) in aromatic compounds (Maricq & Waugh, 1979) and polyacetylene (Mehring et al., 1982; Terao et al., 1984), as well as in the ^{13}C-12 resonance of bR_{548} (Harbison et al., 1984a). Our analysis of the ^{13}C-12 dark-adapted bR spectrum confirms that the upfield movement of the isotropic chemical shift of bR_{548} is predominantly in the σ_{11} element (Table I).

Chemical Shifts of ^{13}C-13-Labeled bR. The position of the ^{13}C-13 resonance in retinal Schiff bases is sensitive to changes at the Schiff base nitrogen. For example, protonation of the Schiff base generates a positive charge at the imine nitrogen and causes a 17 ppm downfield shift in the ^{13}C-13 resonance from ~145 ppm in the unprotonated SB to ~162 ppm in the PSB. This shift of the ^{13}C-13 resonance in the PSB results from partial positive charge character established at position 13 due to delocalization of positive charge within the conjugated π-system (Shriver et al., 1976). A further increase in π-electron (or positive charge) delocalization results when the distance is increased between the Schiff base nitrogen and its associated counterion. In a series of retinal PSB model compounds containing bulky counterions, the ^{13}C-13 resonance was observed to shift ~5 ppm further downfield to 166–168 ppm (Childs et al., 1987).

The ^{13}C-13 resonances in dark-adapted bR, which are observed at 164.8 and 168.7 ppm, can be assigned to bR_{568} and bR_{548}, respectively, on the basis of the light-adapted spectrum presented in Figure 4. The 164.8 ppm chemical shift observed in bR_{568} is ~3 ppm downfield of the all-trans PSB chloride salt. This difference in chemical shift between bR_{568} and the PSB is attributed to a weaker interaction between the Schiff base and its counterion in bR and is in agreement with ^{15}N NMR studies which show extremely low ^{15}N chemical shifts in bR_{548} and bR_{568} (Harbison et al., 1983; de Groot et al., 1989).

The ^{13}C-13 resonance in bR_{548} is observed at 168.7 ppm, 6.5 ppm downfield from that of the 13-cis PSB model compound and also 3.5 ppm downfield from the corresponding resonance in bR_{568}. These results would argue for increased π-electron delocalization in bR_{548}, possibly as a result of a weaker Schiff base–counterion interaction. However, the ^{15}N chemical shift in bR_{548} (150.6 ppm) is higher in frequency than that in bR_{568} (143.5 ppm), arguing for a stronger Schiff base–counterion interaction and *decreased* delocalization of the positive charge from the Schiff base nitrogen into the retinal chain. Consequently, the observed chemical shift at 168.7 ppm suggests that the Schiff base environment in bR_{548} is not the sole determinant of the ^{13}C-13 shift. Two possible causes for the unexpected downfield ^{13}C-13 resonance are a twist in the planar retinal chain or a protein charge near C-13 or C-14. We previously attributed a large shift in the σ_{22} element of C-5 to a nearby charge or dipole (Harbison et al., 1985a). The magnitude of the shift (~9 ppm) is similar to the shifts seen in C-13 (~7 ppm) and C-14 (~11 ppm). A second possibility is that a twist in the retinal chain in the C_{13}=C_{14}—C_{15} bonds may produce the observed shifts. Resonance Raman spectra of bR_{548} exhibit an intense vibrational line at 800 cm^{-1} which has been assigned to the out-of-plane wagging vibration of the proton attached to C-14 (Smith et al., 1987). Intensity in this Raman line is interpreted as a distortion in the normally planar structure of the retinal chromophore. Currently, we cannot distinguish between these possibilities on the basis of NMR.

Chemical Shifts of ^{13}C-14-Labeled bR. The largest difference in chemical shift between bR_{568} and bR_{548} is observed at C-14. This difference (~11 ppm) was previously attributed to a γ-effect associated with a syn C=N bond in bR_{548} (Harbison et al., 1984b). By analogy with position 12, the downfield shift in the ^{13}C-14 resonance might be expected to be localized in the σ_{11} tensor element. However, the γ-effect at C-14 would originate from the ϵ-CH_2 group of lysine, which has a different geometry from that of the CH group of a polyene. In fact, our recent measurements of ^{13}C-14 bR using

[3] σ_{11} is oriented perpendicular to the plane of the retinal polyene chain. σ_{22} and σ_{33} lie in the plane of the retinal polyene chain, with σ_{22} parallel and σ_{33} perpendicular to the C=C bond. These orientations were derived from a single-crystal study of dihydromuconic acid (Wolff et al., 1977). Note that the convention for assignment of σ_{11} and σ_{33} used here is the reverse of that used in the single-crystal study.

difference methods and least-squares data analysis have shown that only the σ_{22} element changes significantly upon isomerization (Table I; de Groot et al., 1989). Recent spectra of dark-adapted bR labeled with ^{13}C at the ϵ-CH group of lysine exhibit an upfield shift (a possible γ-effect) in the bR_{548} resonance (M. Farrar et al., unpublished results). These results would support the argument that a γ-effect is present at C-14.

Chemical Shifts of ^{13}C-15-Labeled bR. The isotropic shift of the ^{13}C-15 resonance appears to be largely insensitive to changes in the retinal's structure or environment, although small shifts in the ^{13}C-15 resonance have been correlated with larger shifts in the ^{15}N resonance. In model compound studies, it was noted that the ^{13}C-15 chemical shift moved upfield in the protonated *N*-butylamine Schiff base salts in going from chloride (167 ppm) to bromide (165 ppm) to iodide (163.5 ppm) (Harbison et al., 1985b, unpublished results) in parallel with the larger ^{15}N shifts (chloride, 171.7 ppm; bromide, 166.1 ppm; iodide, 154.5 ppm). The ^{13}C-15 resonance in bR_{568} at 160.0 ppm is further upfield than that of most retinal PSB model compounds. Recently, retinal PSB's formed with a *tert*-butyl group and a weakly electronegative perchlorate counterion yielded a ^{13}C-15 resonance at 160 ppm (Childs et al., 1987). These results suggest that an upfield shift in the ^{13}C-15 resonance occurs as the polarization of the polyene system is increased, probably because the interaction between the Schiff base and its counterion is weakened. A similar process may account for the difference in isotropic chemical shifts between bR_{548} and bR_{568}.

Chemical Shifts of the β-Ionone Ring Carbons. As mentioned above, the chemical shift data on the β-ionone ring carbons reported here (C1–C4 and C16 and C17) complete our studies of chemical shifts in retinal. More importantly, however, these data bear on two interesting points. First, it has recently been observed (Childs et al., 1987 that the C-2 chemical shift is sensitive to 6-s-cis versus 6-s-trans isomerization. Specifically, the C-2 shift in the 6-s-cis forms of retinal occurs in the range of 38–40 ppm, whereas for the 6-s-trans forms there is a downfield movement of ~5 ppm to ~43 ppm. The shift we observe for the C-2 in bR is 42.7 ppm, indicating the presence of a 6-s-trans conformer.

Second, the chemical shift data permit us to further characterize the protein perturbation suggested to be in the neighborhood of the β-ionone ring. Absorption spectra of bacteriorhodopsin regenerated with various dihydroretinals provided the evidence for a negative protein charge near the ionone ring (Nakanishi et al., 1980). In addition, solid-state NMR spectra of bR containing ^{13}C-5-labeled retinal (Harbison et al., 1985a) show a 16 ppm downfield shift of the ^{13}C-5 resonance which was suggested to arise from a combination of 6-s-cis → trans isomerization and a negative protein charge near C-5. The absence of any large shifts at C-6 and C-7 was used to infer that the putative negative charge near C-5 had a positive protein counter charge near C-7. Our present data on ^{13}C-1 and ^{13}C-4 bR argue that the protein-induced deshielding effect is localized at C-5. Furthermore, the absence of a shift at C-1 argues against theoretical predictions which place the positive counterion to the C-5 charge near this position (Spudich et al., 1986). Interestingly, the ^{13}C-1, ^{13}C-4, and ^{13}C-18 chemical shifts are not influenced by the putative protein charge near C-5.

^{13}C T_1 of ^{13}C-16,17 Bacteriorhodopsin. Our previous NMR studies on the C_6–C_7 conformation in bR used three lines of evidence to argue for a 6-s-trans structure (Harbison et al., 1985a). These were (1) the chemical shift of ^{13}C-5, (2) the chemical shift of ^{13}C-8, and (3) the ^{13}C T_1 of the ^{13}C-18 methyl group. Another diagnostic of the C_6–C_7 conformation observed in retinal model compounds is the ^{13}C T_1 of the ^{13}C-16,17 methyl groups. In the 6-s-cis geometry, the ^{13}C T_1 is ~0.15 s, while the 6-s-trans model compounds exhibited T_1's on the order of 1.0–1.3 s (Harbison et al., 1985a). However, the corresponding ^{13}C-16,17-labeled bR derivative was not available for comparisons at that time. In the present paper we present ^{13}C T_1 data for ^{13}C-16,17-bR. The measured value of ~1.2 s is much closer to that of the 6-s-trans than to that of the 6-s-cis model compounds (Table II). A slight upfield shift is also observed.

Conclusions

Solid-state NMR spectra of light-adapted bacteriorhodopsin have allowed us to assign the resonances observed in the dark-adapted spectrum to bR_{568} and bR_{548}. Analysis of the ^{13}C chemical shift anisotropy of the retinal resonances indicates that several factors may be important in generating the differences in isotropic chemical shifts between these pigments. First, the difference in chemical shift at C-12 results from the cis C_{13}=C_{14} bond in bR_{548}, as shown by the large change in the σ_{11} tensor element. Second, the downfield shift in the σ_{22} tensor element of ^{13}C-13 of bR_{548} is attributed to either a twist in the C_{13}=C_{14}—C_{15} region of the chromophore or a negative protein charge (or dipole) near C-13. A twist in the bR_{548} chromophore is in agreement with previous resonance Raman studies. Third, the upfield shift in the σ_{22} element of ^{13}C-14 bR_{548} may result from steric interaction with the ϵ-CH_2 group of lysine across the syn C=N bond.

Solid-state NMR spectra of dark-adapted bacteriorhodopsin regenerated with [^{13}C]retinal labeled on the ionone ring indicate that the perturbation observed previously is localized at the C-5 position. The spin–lattice relaxation times of ^{13}C-16,17 bR provide additional support for an s-trans geometry about the C_6–C_7 bond.

These studies complete a series of solid-state NMR experiments on dark-adapted bR, in which each carbon position of the retinal chromophore has been individually substituted with ^{13}C.

Acknowledgments

We thank J. van Haveren, J. B. Shadid, and C. J. van Haeringen for their assistance in the synthesis of the labeled retinal molecules and M. Farrar for obtaining spectra of [2-^{13}C]- and [3-^{13}C]bR.

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