Solid-State ^{13}C NMR of the Retinal Chromophore in Photointermediates of Bacteriorhodopsin: Characterization of Two Forms of M†

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ABSTRACT: Solid-state ^{13}C NMR spectra of the M photocycle intermediate of bacteriorhodopsin (bR) have been obtained from purple membrane regenerated with retinal specifically ^{13}C labeled at positions 5, 12, 13, 14, and 15. The M intermediate was trapped at −40 °C and pH = 9.5–10.0 in either 100 mM NaCl [M (NaCl)] or 500 mM guanidine hydrochloride [M (Gdn-HCl)]. The ^{13}C-12 chemical shift at 125.8 ppm in M (NaCl) and 128.1 ppm in M (Gdn-HCl) indicates that the C_{13}=C_{14} double bond has a cis configuration, while the ^{13}C-13 chemical shift at 146.7 ppm in M (NaCl) and 145.7 ppm in M (Gdn-HCl) demonstrates that the Schiff base is unprotonated. The principal values of the chemical shift tensor of the ^{13}C-5 resonance in both M (NaCl) and M (Gdn-HCl) are consistent with a 6-s-trans structure and a negative protein charge localized near C-5 as was observed in dark-adapted bR. The ~5 ppm upfield shift of the ^{13}C-5 M resonance (~140 ppm) relative to ^{13}C-5 bR_{568} and bR_{548} (~145 ppm) is attributed to an unprotonated Schiff base in the M chromophore. Of particular interest in this study were the results obtained from ^{13}C-14 M. In M (NaCl), a dramatic upfield shift was observed for the ^{13}C-14 resonance (115.2 ppm) relative to unprotonated Schiff base model compounds (~128 ppm). In contrast, in M (Gdn-HCl) the ^{13}C-14 resonance was observed at 125.7 ppm. The different ^{13}C-14 chemical shifts in these two M preparations may be explained by different C=N configurations of the retinal–lysine Schiff base linkage, namely, syn in NaCl and anti in guanidine hydrochloride.

The retinal chromophore of bacteriorhodopsin (bR)[1] is buried within the interior of the protein and drives the transport of protons across the bacterial cell membrane upon absorption of light [for reviews, see Birge (1981) and Stoeckenius and Bogomolni (1982)]. Determining the structure of the intermediates in the photoreaction cycle of bR (Figure 1) is crucial to unraveling the mechanism of proton pumping. The light-adapted form of the pigment (bR_{568}) has an all-trans protonated Schiff base (PSB) chromophore which isomerizes to the 13-cis configuration in the primary photoproduct K (Hsieh et al., 1981; Braiman & Mathies, 1982). The retinal–lysine Schiff base linkage deprotonates in the formation of the M intermediate and subsequently reprotonates as the chromophore reisomerizes in the thermal conversion back to the native pigment (Lewis et al., 1974; Smith et al., 1983). These reactions have been the basis of models in which the retinal chromophore functions as a "proton switch" transferring the Schiff base proton across the retinal binding site (Schulten & Tavan, 1978; Stoeckenius, 1980; Smith et al., 1986).

Solid-state NMR studies of dark-adapted bR have established a method for probing the structure and environment of the retinal chromophore in bR (Harbison, 1984a,b, 1985a). The approach has been to ^{13}C label the retinal at specific positions and to compare the ^{13}C chemical shifts of the protein-bound retinal to the chemical shifts of retinal model compounds. These studies have shown that the ^{13}C chemical shifts obtained from the solid-state NMR spectrum are sensitive to isomerization of the retinal, as well as to charged protein residues in the retinal binding site. In order to address the structural changes involved in the bR photoreaction, the techniques developed on the dark-adapted protein can be applied to the *photointermediates* of bR.

Our initial focus is on the M intermediate(s) in the bR photocycle. The original model for the photocycle indicated that there was a single M intermediate located in an unbranched sequence between the L and N intermediates (Lozier et al., 1975). Subsequently, kinetic absorption studies have provided evidence for at least two M intermediates, often designated M_{slow} (or M_s) and M_{fast} (or M_f), that differ in their decay kinetics (Slifkin & Caplan, 1975; Lozier et al., 1976; Hess & Kuschmitz, 1977; Ort & Parson, 1978; Ohno et al., 1981; Li et al., 1984; Groma & Dancshazy, 1986). Recently, Kouyama et al. (1988) have shown that the M_s intermediate is a photoreaction product of the N intermediate. The conditions that favor the M_s intermediate are low temperature, high pH, high salt, and illumination (Kouyama et al., 1988). One of our interests is to investigate the differences in the structure of the retinal chromophore between these two M forms. Resonance Raman experiments under conditions that favor M_f have shown that this intermediate has a 13-cis chromophore (Braiman & Mathies, 1980), while Raman spectra of the L and N intermediates suggest that M_f has a

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[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared; Gdn-HCl, guanidine hydrochloride; MAS, magic angle spinning; NMR, nuclear magnetic resonance; ppm, parts per million; PSB, protonated Schiff base; rf, radio frequency; SB, unprotonated Schiff base.

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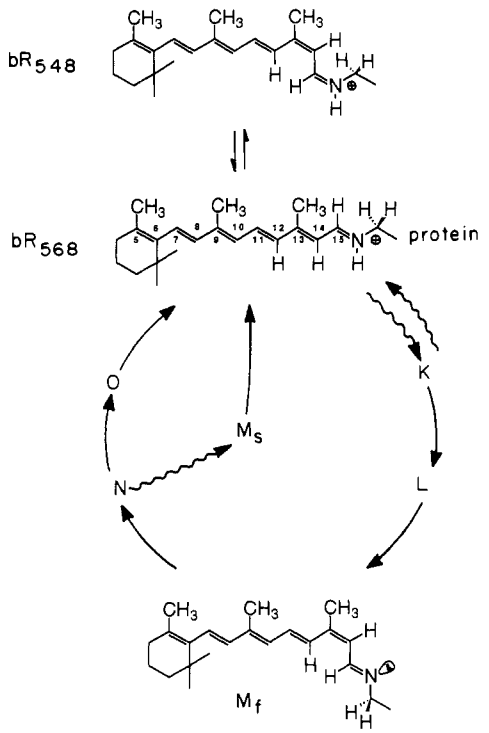

FIGURE 1: Photochemical reaction cycle of bR. Absorption of light by bR_{568} produces the primary photoproduct K. K decays thermally through a series of intermediates designated L, M_f, N, and O. Absorption of light by the N intermediate leads to the M_s intermediate which decays to bR_{568} (Kouyama et al., 1988). Dark-adapted bR is a 60:40 mixture of bR_{548} and bR_{568} in thermal equilibrium.

C=N anti Schiff base bond (Smith et al., 1984; Fodor et al., 1988). In contrast, the structure of the M_s form has not been addressed.

A second area of interest is to investigate the protein environment in M_f and M_s. The deprotonation–reprotonation reactions involving the Schiff base nitrogen of the M intermediates are thought to be key elements in the proton translocation mechanism. Deprotonation is presumed to be caused by a decrease in the p*K* of the Schiff base nitrogen as a result of a change in the protein environment. FTIR and transient UV absorption studies have provided evidence that a tyrosine deprotonates during the formation of M_f (Bogomolni et al., 1978; Hess & Kuschmitz, 1979; Dollinger et al., 1986; Braiman et al., 1987), leading to the proposal that a tyrosinate is *required* for Schiff base deprotonation (Kalisky et al., 1981). El-Sayed and co-workers have argued that deprotonation of a tyrosine apparently does not precede deprotonation of the Schiff base in M_s but that another protein group (positively charged) is responsible for the decrease in the p*K* of the Schiff base nitrogen in this case (Hanamoto et al., 1984). As yet there is still no direct evidence for either a tyrosinate or a positively charged protein group *interacting* with the Schiff base in either M intermediate.

In this paper we present results of our first solid-state ^{13}C NMR experiments on the M intermediate. Spectra were obtained at −40 °C and pH = 9.5–10.0 in either 100 mM NaCl or 500 mM guanidine hydrochloride. The conditions using NaCl favor the M_s branch of the photocycle (Kouyama et al., 1988). We present results for samples specifically ^{13}C labeled at positions 5, 12, 13, 14, and 15 of the retinal chromophore. The chemical shifts of the ^{13}C-13 and ^{13}C-15 resonances are sensitive to the protonation state and environment of the Schiff base. The ^{13}C-5 resonance is sensitive to the conformation of the C_6–C_7 bond. This bond has a twisted s-cis structure in most retinal model compounds but was found to be planar s-trans in bR_{568} and bR_{548} (Harbison et al., 1985a). The chemical shifts of the ^{13}C-12 and ^{13}C-14 resonances can be used to determine the configurations of the C13=C14 and C=N bonds, respectively.

Materials and Methods

The ^{13}C-labeled retinals were synthesized as previously described (Pardoen et al., 1984, 1985; Lugtenburg, 1985). Incorporation of labeled retinal into the protein was accomplished by bleaching native purple membrane with 0.5 M hydroxylamine hydrochloride (pH = 8) and light (>440 nm) followed by regeneration with an equimolar ratio of [^{13}C]-retinal to protein (Oesterhelt & Schuhmann, 1974). Excess retinal from the regeneration and retinal oxime generated by the bleaching process were removed by washing the membrane 10–15 times with a 2% solution of bovine serum albumin (Sigma Chemical Co.).

Solid-state NMR spectra of dark-adapted bR are typically taken with wet pellets of purple membrane containing ~80 mg of protein. In contrast, the M spectra were obtained from a suspension of purple membrane containing 25–40 mg of protein. The lower concentration used for the M experiments decreased the optical density of the sample and increased the fraction of bR converted to M. We noticed that the amount of water was important in the bR → M conversion as indicated by the intensity of the M peaks in the NMR spectra and the yellow color of the sample. However, quantitative studies were not undertaken. It was necessary to increase the pH to 9.5–10.0 and lower the sample temperature to approximately −40 °C in order to slow the decay of M (Ort & Parson, 1978; Li et al., 1984) sufficiently that the lifetime of the intermediate was greater than 8 h, the minimum time required to acquire data. The M intermediate was trapped in either NaCl (100 mM) or Gdn-HCl (500 mM). Gdn-HCl (pH = 10) was originally used to generate M for chromophore extraction experiments which demonstrated that M (Gdn-HCl) contained retinal in the 13-cis configuration (Pettei et al., 1977). In our NMR experiments using Gdn-HCl, we were able to convert close to 100% of the purple membrane to M; the samples were distinctly yellow. In contrast, the proportion of M trapped in NaCl ranged from ~30 to 90%; the NMR samples were generally yellowish brown. Because of their very high optical density, absorption spectra of the NMR samples were not obtained. However, FTIR spectra of M at pH = 9.5 (Engelhard et al., 1984) and resonance Raman spectra at pH = 9.7 in 2 M Gdn-HCl (Braiman & Mathies, 1980) are very similar to those at pH = 7, indicating that high pH and high Gdn-HCl does not adversely affect the chromophore or protein. It is worth noting that, even in the presence of high concentrations of Gdn-HCl (8 M) and alkaline pH (pH > 8), bR is still able to pump protons upon illumination (Yoshida et al., 1980). This suggests that M (Gdn-HCl) is a "functional" M intermediate.

Low-temperature trapping of the M intermediate was accomplished in a glovebox temporarily placed over the NMR probe. The sample was first light adapted at ~0 °C, and then the temperature was lowered to −40 °C with cold N_2 gas. At this temperature, illumination of the sample using light focused from a slide projector (>530 nm) converted bR_{568} to the M intermediate. The sample was then inserted into the probe at approximately −40 °C and the probe cover replaced. Maintaining a dry N_2 atmosphere prevented condensation and problems associated with rf arcing within the probe.

Solid-state MAS NMR spectra were obtained on a home-built spectrometer operating with ^{13}C and ^{1}H frequencies of

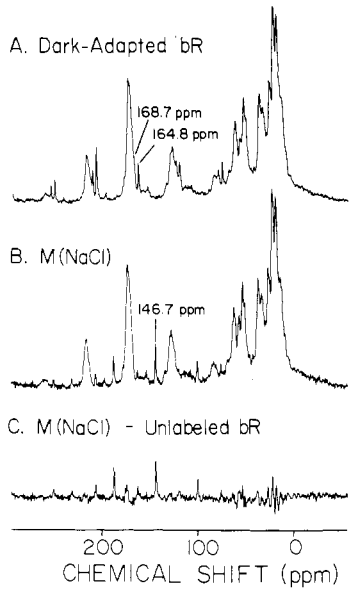

FIGURE 2: ^{13}C MAS spectra of ^{13}C-13 dark-adapted bR (A) and M (B). The difference spectrum between (B) and unlabeled bR is shown in (C). The M spectrum was obtained in 100 mM NaCl (pH = 9.5).

79.9 and 317.6 MHz, respectively. The ^{13}C and ^{1}H $\pi/2$ pulse lengths were ~6.0 and 3.0 μs, respectively. A standard cross-polarization pulse sequence was used with a mix time of 2 ms, an acquisition time of 10 ms, and a recycle delay of 2 s. Typically, 50 000–70 000 scans were averaged for each spectrum. In the ^{13}C-5 and ^{13}C-13 spectra, the spinning speed was maintained to within ~2 Hz with a spinning speed controller (de Groot et al., 1988), and difference spectra were employed to examine the resonance due to the retinal label in more detail.

All chemical shifts are referenced to external TMS in the solid-state NMR spectra and to internal TMS in the solution NMR spectra.

Results

Resonance Raman spectra obtained at pH = 7 and chemical extraction studies using Gdn-HCl have shown that the retinal chromophore in M has a C_{13}=C_{14} cis bond and an unprotonated Schiff base (Lewis et al., 1974; Pettei et al., 1977; Braiman & Mathies, 1980). Our first solid-state NMR studies of M using the [12-^{13}C]- and [13-^{13}C]retinal-labeled pigments were selected to reexamine these features of the chromophore's structure and were carried out at pH ≃ 9.8 in 100 mM NaCl. Figure 2 presents the ^{13}C NMR spectra of [13-^{13}C]retinal-labeled dark-adapted bR (A) and [13-^{13}C]retinal-labeled M (NaCl) (B). Subtraction of a spectrum of *unlabeled* dark-adapted bR from the ^{13}C-13 M spectrum removes the natural abundance ^{13}C resonances and accentuates the lines due to the label in M (Figure 2C). Dark-adapted bR has two components, bR_{568} and bR_{548}, responsible for the two sharp retinal centerbands at 164.8 and 168.7 ppm, respectively (Figure 2A), each of which is flanked by sharp rotational sidebands spaced at the spinning frequency. The ^{13}C-13 resonance is sensitive to Schiff base protonation and exhibits an 18-ppm difference between the protonated (162 ppm) and unprotonated (144 ppm) 13-*cis*-retinylidene–propylimine Schiff bases (Mateescu et al., 1984). The chemical shift observed for M (NaCl) at 146.7 ppm is close to that observed for the unprotonated Schiff base. Furthermore, analysis of the sideband intensities for ^{13}C-13 M (NaCl) can be used to extract the principal values of the chemical shift tensor (Herzfeld & Berger, 1980). These are presented in Table I and are very similar to those seen in the unprotonated *all-trans*-retinylidene–*n*-butylimine Schiff base (Harbison et al., 1985b).[2]

Table I: Isotropic Chemical Shifts and Shift Tensor Values in [13-^{13}C]Retinal Model Compounds and M (NaCl)

compound	σ_1	σ_{33}	σ_{22}	σ_{11}
13-cis PSB[a]	162.2	269	188	30
all-trans PSB[a]	161.8	267	189	29
all-trans SB[b]	144	237	166	29
M (NaCl)[c]	146.7	240	169	30

[a] The Schiff bases were prepared with *n*-butylamine and protonated with HCl. [b] Data from Harbison et al. (1985b). [c] The shift tensor values in M were determined from the sideband intensities of a single spectrum (Figure 2c). Errors are estimated to be ±5 ppm on the basis of analyses of other [^{13}C]bR spectra having comparable signal-to-noise ratios.

Table II: Isotropic Chemical Shifts and Shift Tensor Values in [5-^{13}C]Retinal Model Compounds, Dark-Adapted bR, and M (NaCl)

compound	σ_1	σ_{33}	σ_{22}	σ_{11}
6-*s-trans*-retinoic acid[a]	135.9	237	143	27
6-*s-cis*-retinoic acid[a]	128.8	217	141	28
6-s-cis Schiff base[a]	126	202	143	33
bR_{568}/bR_{548}[b]	144.8	237	170	28
M (NaCl)[c]	139.4	232	161	26

[a] Data from Harbison et al. (1985b). [b] Data from Harbison et al. (1985a). [c] The shift tensor values in M were the average of the values determined from the sideband intensities of two spectra. Errors are estimated to be ±10 ppm on the basis of analyses of other [^{13}C]bR spectra having comparable signal-to-noise ratios.

Figure 3 presents solid-state ^{13}C spectra of ^{13}C-12 dark-adapted bR (A) and ^{13}C-12 M (NaCl) (B). The ^{13}C-12 chemical shift is sensitive to the configuration of the C_{13}=C_{14} bond, and the difference in chemical shift (10.1 ppm) observed between the two components (bR_{568} and bR_{548}) of ^{13}C-12 bR has previously been attributed to a difference in the C_{13}=C_{14} geometry (Harbison et al., 1984a). The ^{13}C-12 chemical shift for the M intermediate is observed at 125.8 ppm, close to that of the 13-cis unprotonated Schiff base model compound (~127 ppm). In contrast to the ^{13}C-13 resonance, the ^{12}C-12 resonance is not very sensitive to protonation of the Schiff base, shifting ~3 ppm upfield upon protonation of the 13-cis Schiff base. *Together, the ^{13}C-12 and ^{13}C-13 spectra demonstrate that the intermediate we are observing is a 13-cis unprotonated Schiff base.*

The spectra of ^{13}C-5 dark-adapted bR and ^{13}C-5 M (NaCl) are presented in panels C and D of Figure 3, respectively. The corresponding shift tensor elements are compared with those for model compounds in Table II. The downfield shift tensor element is sensitive to the conformation of the C_6–C_7 bond (Harbison et al., 1985b). For both bR_{568} and bR_{548}, this element occurs at 237 ppm in the dark-adapted spectrum. In M (NaCl), it is found at 232 ppm, consistent with a similar 6-s-trans conformation. The middle shift tensor element is shifted downfield relative to that in the model compounds in M (NaCl), as it is in bR_{568} and bR_{548}. This suggests that the electrostatic environment near C-5 in M (NaCl) is similar to that in bR_{548} and bR_{568}.

Solid-state MAS spectra have also been obtained of ^{13}C-15 dark-adapted bR and ^{13}C-15 M (NaCl) (Figure 3, panels E and F). The ^{13}C-15 resonance is not as sensitive as the ^{13}C-13

[2] The chemical shift values reported here are for the 13-cis SB in solution (Mateescu et al., 1984). Solid-state experiments have not been reported on the 13-cis SB since it has not been possible to obtain crystalline samples. The similarity in the shift tensor values between the 13-cis and all-trans PSBs in Table I suggests that the shift tensor in the 13-cis SB should be similar to that of the all-trans SB.

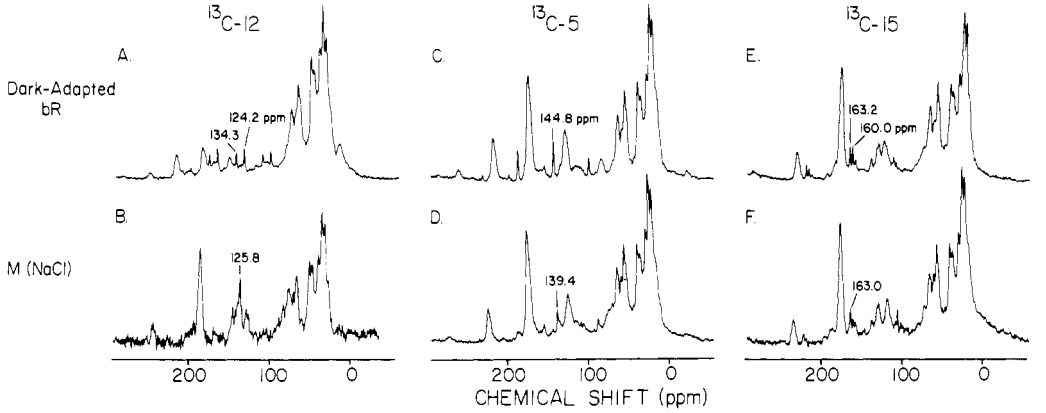

FIGURE 3: ^{13}C MAS spectra of ^{13}C-12 dark-adapted bR (A), ^{13}C-12 M (B), ^{13}C-5 dark-adapted bR (C), ^{13}C-5 M (D), ^{13}C-15 dark-adapted bR (E), and ^{13}C-15 M (F). The M spectra were obtained in 100 mM NaCl (pH = 9.5).

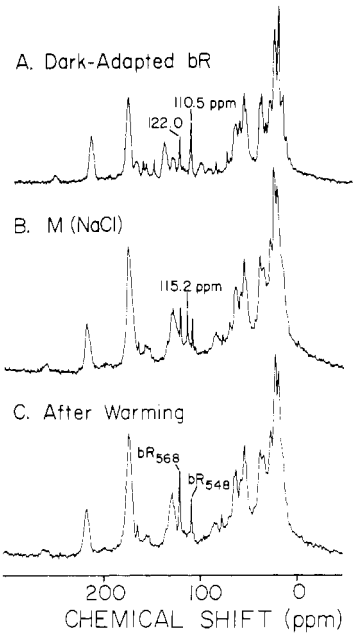

FIGURE 4: ^{13}C MAS spectra of ^{13}C-14 dark-adapted bR (A) and ^{13}C-14 M (B). After acquisition of the M spectrum, the sample was warmed to 10 °C for ~5 min to allow the M intermediate to decay and then recooled to −40 °C. The spectrum shown in (C) was obtained after this procedure. The M spectra were obtained in 100 mM NaCl (pH = 10).

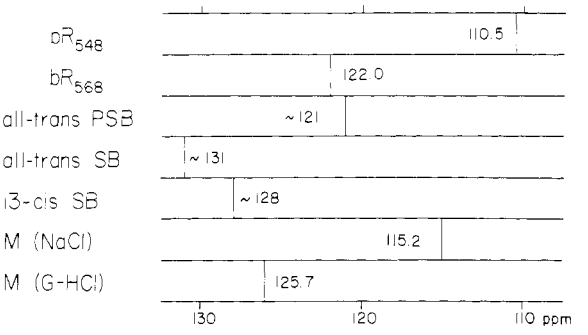

FIGURE 5: Comparison of ^{13}C-14 chemical shifts observed in bR_{568}, bR_{548}, M (NaCl), M (Gdn-HCl) and several retinal model compounds. The retinal model compound data are taken from Shriver et al. (1976), Mateescu et al. (1984), and Harbison et al. (1985b).

resonance to the protonation state of the Schiff base. The ^{13}C-15 resonances in the dark-adapted spectrum are at 160.0 (bR_{568}) and 163.2 (bR_{548}), while in the M spectrum the ^{13}C-15 resonance appears at 163.0 ppm. The ^{13}C-15 M (NaCl) resonance is slightly higher in frequency than that in the 13-cis unprotonated Schiff base model compound (158.4 ppm).

In order to establish that the intermediate we have trapped is obtained by photolysis of bR_{568} rather than bR_{548}, a series of spectra were obtained of ^{13}C-14 bR. In the ^{13}C-14 derivative, the bR_{568} and bR_{548} components are clearly resolved and exhibit intense centerbands at moderate spinning speeds due to their narrow chemical shift tensors. Figure 4 presents solid-state spectra of ^{13}C-14 dark-adapted bR (A) and ^{13}C-14 M (NaCl) (B). The ^{13}C-14 resonances in the dark-adapted spectrum are at 122.0 (bR_{568}) and 110.5 ppm (bR_{548}). The isotropic chemical shift for ^{13}C-14 M (NaCl) is observed at 115.2 ppm together with resonances from unphotolyzed bR_{568} and bR_{548}. Following the acquisition of the M spectrum, the sample was warmed to 10 °C for ~5 min to allow the M intermediate to decay and then was recooled to −40 °C to prevent dark adaptation of the purple membrane. The spectrum obtained following this procedure is presented in Figure 4C and clearly shows that the M line has disappeared and that the downfield NMR resonance assigned to bR_{568} has gained intensity compared to that of bR_{548}. This implies that the intermediate we are trapping decays to, and is therefore part of, the bR_{568} photocycle.

Our previous ^{13}C NMR studies of dark-adapted bR demonstrated that the ^{13}C-14 chemical shift can provide important information on the configuration of the C=N bond. As seen in Figure 4A, there is a large difference (12 ppm) in chemical shift between bR_{568} and bR_{548} which has previously been attributed to a difference in C=N geometry (Harbison et al., 1984b). In order to address the C=N configuration in M, we compare in Figure 5 the ^{13}C-14 chemical shifts observed in bR and M with those of several retinal model compounds. Figure 5 first shows the large upfield shift (12 ppm) in the ^{13}C-14 resonance in bR_{548} relative to bR_{568}. This shift is similar to that seen at C-12 upon isomerization from all-trans to 13-cis, where it is attributed to steric interaction between the C_{12}–H and C_{15}–H protons in the cis conformation. In a series of ketimine model compounds, this "γ-effect" (steric interaction between protons on carbons that are three bonds apart) generated shifts of 5–6 ppm (Harbison et al., 1984b). At C-14 the γ-effect would involve interaction between the C_{14}–H proton and the methylene protons of lysine across a syn C=N bond. Figure 5 also compares the bR_{568} resonance (122.0 ppm) with that of all-trans PSB model compounds (120–122 ppm), showing that the ^{13}C-14 resonance does not shift appreciably when the retinal PSB is incorporated into the protein. This is at first surprising since the absorption band (λ_{max}) of the retinal PSB shifts from ~440 nm in the solution to 568 nm in the pigment, indicating that the ^{13}C-14 chemical shift is insensitive to delocalization of the conjugated π-electrons. In

Table III: Summary of M (NaCl) and M (Gdn-HCl) Chemical Shifts (ppm)

	M (NaCl)	M (Gdn-HCl)
^{13}C-5	139.4	139.8
^{13}C-12	125.8	128.1
^{13}C-13	146.7	145.7
^{13}C-14	115.2	125.7
^{13}C-15	163.0	162.5

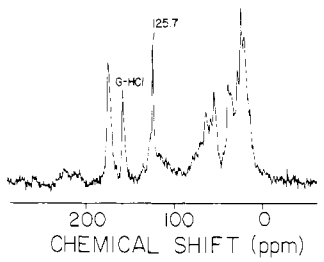

FIGURE 6: ^{13}C MAS spectrum of ^{13}C-14 M in 500 mM guanidine hydrochloride (pH = 10).

contrast, deprotonation of the all-trans PSB results in a large downfield shift to 130–132 ppm (Shriver et al., 1976; Harbison et al., 1985b). Finally, comparison of the all-trans and 13-cis Schiff bases indicates a slight upfield shift (~2–4 ppm) due to isomerization. In summary, these results suggest that *deprotonation* and the *γ-effect* have the most dramatic effects on the ^{13}C-14 chemical shift. Comparison of the chemical shifts of the 13-cis unprotonated Schiff base and M (NaCl) reveals a large upfield shift in the ^{13}C-14 resonance. Accordingly, these data argue for a C=N syn bond in M (NaCl). However, two additional effects that are not considered in the model compounds, conformational distortion (twists) in the retinal chain and charged protein residues in the retinal binding site, could also influence the ^{13}C-14 chemical shift (see discussion below).

The spectra shown thus far were recorded at pH = 9.5–10 in 100 mM NaCl. For comparison, we have also obtained spectra in 500 mM Gdn-HCl (pH = 10). These data along with the corresponding data of M (NaCl) are summarized in Table III. In general, the M (Gdn-HCl) spectra exhibited resonances close to those observed for M (NaCl). The ^{13}C-13 resonance at 145.7 ppm in Gdn-HCl demonstrates that the Schiff base is again unprotonated, while the ^{13}C-12 resonance at 128.1 indicates that the chromphore is 13-cis. Interestingly, the ^{13}C-14 resonance (Figure 6) at 125.7 ppm in Gdn-HCl is 11 ppm downfield of the M (NaCl) resonance. On the basis of the above analysis, the 125.7-ppm chemical shift argues for a C=N anti configuration in M (Gdn-HCl). Thus, the different chemical shifts at ^{13}C-14 argue for two distinct M forms. It is important to note that in both NaCl and Gdn-HCl *sharp single* resonances are observed with line widths comparable to those for the bR_{548} and bR_{568} species.

Discussion

The ^{13}C spectra of M presented above demonstrate that high-resolution solid-state NMR can be used to study the structure of *intermediates* in the bR photocycle. The M intermediates (M_s and M_f) are of particular interest because of their potential importance in the proton-pumping mechanism of bR. The present study was directed at characterizing the structures of the C_6–C_7, C_{13}=C_{14}, and C=N bonds and the protein environment surrounding the retinal. Solid-state spectra were obtained under conditions that favor trapping of the M_s intermediate (low temperature, high pH, and constant illumination), and also of the M intermediate stabilized in guanidine hydrochloride. Since bR pumps protons in the presence of Gdn-HCl, it is expected that M (Gdn-HCl) is a "functional" intermediate.

C_6–C_7 Conformation in M. Our previous work on dark-adapted bR demonstrated that the C_6–C_7 bond was s-trans (Harbison et al., 1985a), in contrast to the 6-s-cis structure found in most retinal model compounds (Honig et al., 1971) and in the visual pigment rhodopsin (Smith et al., 1987a). This conclusion was based on several NMR measurements, including analyzing the isotropic chemical shift of the ^{13}C-5 resonance in terms of the three principal values of the ^{13}C-5 chemical shift tensor. Determining the C_6–C_7 conformation in M is important for two reasons. First, since the energy difference between the planar 6-s-trans and twisted 6-s-cis conformations is calculated to be ~2.5 kcal/mol (Langlet et al., 1970), it is possible that the C_6–C_7 single bond changes conformation during the photocycle. Second, a planar C_6–C_7 bond would contribute to the opsin shift observed in M (the red shift in the visible absorption band of the pigment relative to SB model compounds). The retinal absorption band of M has a λ_{max} of ~412 nm compared with 370 nm for the free 6-s-cis *n*-butylamine Schiff base.

In order to interpret the ^{13}C-5 chemical shift in M, it is necessary to reexamine the ^{13}C-5 spectra of dark-adapted bR where the ^{13}C-5 resonance was observed at 144.8 ppm, a downfield shift of ~16 ppm from 6-s-cis retinal model compounds. This large shift was attributed to s-cis → s-trans isomerization (~7 ppm) and a negative protein charge near C-5 (~9 ppm). These two effects appear as localized changes in the chemical shift tensor (Table II). Comparing the shift tensors in Table II, we see that in the retinoic acid model compounds C_6–C_7 isomerization generates a localized shift (20 ppm) in the σ_{33} tensor element, while only the σ_{22} tensor element moves significantly (17 ppm) between 6-s-*trans*-retinoic acid and bR_{568}, presumably due to electrostatic interactions with the protein.

The ^{13}C-5 isotropic shift exhibited by M (NaCl) at 139.4 ppm is ~5 ppm upfield from the ^{13}C-5 resonance in dark-adapted bR. A slight upfield shift, relative to bR, might be expected in M due to deprotonation of the Schiff base (Shriver et al., 1976). As seen in Table II, the 5 ppm shift between M and bR is resident in both σ_{22} and σ_{33}. When the shift tensor elements are compared between M and the unprotonated 6-s-cis, all-trans Schiff base, a large (30 ppm) shift is observed in the σ_{33} element. This shift provides the best support for a 6-*s-trans*-retinal chromophore in M. The conclusion that M (NaCl) has a 6-s-trans chromophore implies that (1) the C_6–C_7 bond has not isomerized in this part of the photocycle and (2) a portion of the opsin shift in M can be attributed to a 6-s-trans bond.

C=N Configuration in M. The ^{13}C-14 chemical shift in M (NaCl) at 115 ppm is ~13 ppm upfield of the ^{13}C-14 resonance in the 13-cis (C=N anti) unprotonated Schiff base model compound. This upfield shift is very suggestive of a C=N syn bond in this intermediate. However, there are two other effects that might influence the ^{13}C-14 shift, a twist in the C_{13}=C_{14}—C_{15} bonds or a protein charge in close proximity to C-14. It is known from resonance Raman spectra of M (pH = 7) that the retinal chromophore is not planar. Intensity in the C_{14}–H hydrogen out-of-plane wagging vibration at ~790 cm^{-1} argues for a twist in the C_{13}=C_{14}—C_{15} region of the chromophore (Smith et al., 1987c). We can gauge the influence of this distortion on the ^{13}C-14 chemical shift by looking at the adjacent ^{13}C-13 and ^{13}C-15 resonances which shift ~3 and 5 ppm, respectively, and suggest that a twist in the retinal chain of M is at best only partially responsible for

the large observed shift of 13 ppm at C-14. The relatively small shifts of the ^{13}C-13 and ^{13}C-15 resonances also indicate that *a negative protein charge near C-13 or C-15 cannot be responsible for the ^{13}C-14 shift.* A much larger downfield shift at C-13 or C-15 would be expected to accompany the large upfield shift observed at C-14 if the latter was in response to a shift in electron density from the odd- to even-numbered carbon atoms. A positive charge close to C-14 could in principal generate the observed shift, although in this case it is difficult to estimate the change in chemical shift at C-13 and C-15.

It is notable that the upfield shift in the ^{13}C-14 resonance and the downfield shift in the ^{13}C-13 resonance, in M (NaCl) compared to 13-cis unprotonated Schiff bases, are similar to those observed in bR_{548} compared to 13-cis protonated Schiff bases. The chromophore in bR_{548} has a C=N syn bond and exhibits a large upfield shift (12 ppm) in the ^{13}C-14 resonance due in part to a γ-effect (Harbison et al., 1984b). A 6.5-ppm downfield shift is observed in the ^{13}C-13 resonances of bR_{548} relative to that of the 13-cis PSB. This shift can be attributed in part to increased π-electron delocalization in the pigment ($\sim$3 ppm) and in part ($\sim$3.5 ppm) to conformational distortion in the chromophore.[3]

Spectra of ^{13}C-14 M obtained in 500 mM guanidine hydrochloride (pH = 10) shed additional light on the origin of the ^{13}C-14 frequency observed in M (NaCl). Interestingly, the M species trapped in guanidine exhibits a ^{13}C-14 resonance at 126 ppm, close to the frequency observed in the 13-cis unprotonated SB model compound at 128 ppm. One explanation for the downfield shift of the ^{13}C-14 M (Gdn-HCl) resonance relative to ^{13}C-14 M (NaCl) is that the chromophore is C=N anti in M (Gdn·HCl). The observation that the ^{13}C-13 and ^{13}C-15 chemical shifts in the Gdn-HCl form of M are similar to those in the NaCl form of M confirms that the effects at ^{13}C-14 are localized. This is important since one might expect that if the difference between the two M forms involved the position of a protein charge near C-14, a larger difference would be observed in the ^{13}C-13 and ^{13}C-15 resonances, while a steric interaction directed along the ^{13}C–H bond of C-14 would generate localized changes in predominantly the ^{13}C-14 chemical shift.

The observation of a C=N syn structure in M (NaCl) shows that the retinal binding site can accommodate and stabilize a *13-cis C=N syn* unprotonated Schiff base in the bR photocycle. Ebrey and co-workers previously showed that both pH and temperature can influence the amplitudes of the different M forms (M_{fast} and M_{slow}) observed in kinetic absorption studies (Li et al., 1984). On the basis of their findings, the M_{slow} component is favored at low temperatures (<25 °C) and low pH (<9). Above pH = 9 the amplitudes of M_{fast} and M_{slow} are comparable. Also, Li et al. correlated the temperature and pH dependence of M_{slow} with proton pumping. Recently, a new model of the bR photocycle has been presented that indicates that M_s is produced by photolysis of the N intermediate. Resonance Raman spectra have shown that the N intermediate has a 13-cis, C=N anti protonated Schiff base chromophore (Fodor et al., 1988). These results raise the possibility that absorption of a photon by N leads to isomerization about the C=N bond *alone*. Additional studies are now needed on an "L-like" intermediate that may be positioned between N and M_s (Kouyama et al., 1988) to better resolve the structural changes in this branch of the photocycle.

[3] The ^{13}C-13 resonance shifts $\sim$3 ppm between bR_{568} and the all-trans PSB. This shift is attributed predominantly to increased π-electron delocalization since the chromophore is not conformationally distorted as indicated by weak hydrogen out-of-plane vibrations in the Raman spectrum (Smith et al., 1987b). In contrast, the ^{13}C-13 resonance shifts $\sim$6.5 ppm between bR_{548} and the 13-cis PSB. Resonance Raman spectra of bR_{548} exhibit an intense C_{14}–H wagging vibration at 800 cm^{-1} similar to that in M, arguing that the chromophore is conformationally distorted in this region (Smith et al., 1987c). Consequently, the difference in the ^{13}C-13 chemical shift between bR_{548} and the 13-cis PSB may be due to this distortion.

Conclusions

The results presented here demonstrate that it is possible to trap the M intermediate of bR in two distinct forms and obtain their NMR spectra at low temperature. Both M species contain an unprotonated 13-*cis*-retinal chromophore with a planar 6-s-trans bond. The most dramatic difference in these M intermediates is in the chemical shift at ^{13}C-14 which is most readily explained by a difference in the configuration of the C=N bond. The observation of a C=N syn chromophore in the M (NaCl) intermediate in bR suggests that this bond is photochemically isomerized when the N intermediate absorbs light. In contrast, the M (Gdn-HCl) form has an anti C=N bond. Since bR in Gdn-HCl has been shown to actively pump protons, M (Gdn-HCl) in the bR (Gdn-HCl) photocycle may be analogous to the M_f intermediate in the bR (NaCl) photocycle. Finally, the solid-state NMR studies of M presented here provide a basis for directly probing the protonation state of protein residues at this stage of the photocycle. Studies on the protonation state of tyrosine residues in M are in progress.

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