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Solid State ^{13}C and ^{15}N NMR Investigations of the N Intermediate of Bacteriorhodopsin†

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ABSTRACT: Previous solid state ^{13}C NMR studies of bacteriorhodopsin (bR) have inferred the C=N configuration and the protonation state of the retinal–lysine Schiff base (SB) linkage from the [13-^{13}C]-retinal, [14-^{13}C]retinal, and [ϵ-^{13}C]lysine-216 chemical shifts in the bR_{555}, bR_{568}, and M_{412} states. Here we determine the C=N configuration and the protonation state of the N photointermediate that is cryotrapped along with the M photointermediate at high salt concentrations (0.1 M NaCl) and high pH (10.0). We obtained ^{13}C and ^{15}N SSNMR spectra of [ϵ-^{15}N]lysine bR and [12-^{13}C]- and [13-^{13}C]retinal bR for samples illuminated under the above conditions. Two species are observed, both of which decay to bR_{568} upon warming. One species has chemical shifts identical to those obtained previously for M thermally trapped in guanidine·HCl at high pH (Smith et al., 1989a; Farrar et al., 1993). In the other species, the [ϵ-^{15}N]lysine and 13-^{13}C chemical shifts indicate that the SB is protonated, the 12-^{13}C shift indicates a 13=14 *cis* configuration, and the previously published [14-^{13}C]- and [ϵ-^{13}C]lysine shifts indicate a C=N *anti* configuration. These results are consistent with other studies of the N photointermediate.

Bacteriorhodopsin (bR)[1] is an integral membrane protein in *Halobacterium halobium* that functions as a light-driven proton pump. Cryoelectron microscopy results indicate that the 26-kDa, 248 amino acid protein forms a bundle of seven-membrane spanning α-helices (Henderson & Unwin, 1975; Henderson et al., 1990) encapsulating a retinal chromophore that is covalently linked to Lys-216 via a protonated Schiff base (SB) (Lewis et al., 1974; Huang et al., 1982; Jubb et al., 1984; Seiff et al., 1985).

Light absorbed by the pigment drives the photoisomerization of the retinal chromophore that results in a unidirectional transport of protons across the membrane. The electrochemical gradient so generated is used for ATP synthesis by the cell. In recent years, resonance Raman and solid state NMR (SSNMR) spectroscopy have been used extensively to characterize changes in the chromophore during the photocycle of bR. These studies have shown that the functionally active bR_{568} form contains a protonated, *all-trans*,15-*anti* chromophore, while bR_{555} contains a protonated 13-*cis*,15-*syn* chromophore (Aton et al., 1977; Harbison et al., 1984a,b; Smith et al., 1989b; Thompson et al., 1992), and the M

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[1] Abbreviations: bR, bacteriorhodopsin; bR_{568}, *all-trans* component of dark-adapted bacteriorhodopsin and sole component of light-adapted bacteriorhodopsin; bR_{555}, 13-*cis* component of dark-adapted bacteriorhodopsin; M, deprotonated intermediate in the photocycle of bR; N, protonated intermediate in the photocycle of bR; CP, cross-polarization; MAS, magic angle spinning; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; $^{15}NH_4Cl$, ^{15}N-labeled ammonium chloride.

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photocycle intermediate contains a deprotonated 13-*cis*,15-*anti* chromophore (Lewis et al., 1974; Ames et al., 1989; Smith et al., 1989a; Farrar et al., 1993; Lakshmi et al., 1993).

Visible spectroscopy indicates that the deprotonation of the chomophore in the transition from the L state to the M state is reversed in the transition from the M state to the N state. The properties of the N intermediate have been studied by resonance Raman spectroscopy (Fodor et al., 1988) but not yet by NMR. A concensus has recently developed that N accumulates with M at low temperature and high pH if sufficient NaCl or KCl is present (Kouyama et al., 1988; Balashov et al., 1990, 1993). We report NMR results obtained under these conditions which indicate that it has a protonated, 13-*cis*,15-*anti* chromophore.

MATERIALS AND METHODS

Preparation of [ϵ-^{15}N]*Lysine-Labeled bR Samples* (*Opsin Labeling*). [ϵ-^{15}N]-labeled L-lysine was synthesized according to the procedure of Raap et al. (1990). The labeled lysine was incorporated into bacteriorhodopsin by growing *H. halobium* (JW-3) on a synthetic medium containing L-[ϵ-^{15}N]lysine, according to procedures described previously by Argade et al. (1981). The incorporation of labeled L-lysine into bR was monitored by incorporation of trace L-[ϵ-$^{3}H_2$]-lysine. The purple membranes were isolated by the method of Oesterhelt and Stoeckenius (1974). Amino acid analysis showed no scrambling of the radioactive label to other amino acid residues in the protein.

Preparation of 12-^{13}C-, 13-^{13}C-, and 14-^{13}C-Labeled bR Samples (*Retinal Labeling*). The 12-^{13}C-, 13-^{13}C-, and 14-^{13}C-labeled retinals were synthesized as described by Pardoen et al. (1984) and Lugtenburg et al. (1984). The natural abundance retinal in the bR was detached by incubation with 1 M hydroxylamine·HCl at pH 8.0, in the dark for 24 h at 36 ± 2 °C. The extent of bleaching was determined to be >90% by monitoring the decrease in the absorbance at 560 nm (a relative absorption maximum of the chromophore bound to the protein) compared to the absorbance at 280 nm (a relative absorption maximum of the opsin). The bleached membrane was washed once in 5 mM HEPES buffer containing 1 mM NaN_3, pelleted for an hour at 30000*g*, and resuspended at 1 mg/3 mL in deionized water. Aliquots of ^{13}C-labeled retinals dissolved in dry ethanol at a concentration of 3 mg/mL were added to the bR suspensions. The correct amount of labeled retinals for complete regeneration without excess was determined to be the amount that resulted in the maximum absorbance at 560 nm without an increase in the absorbance at 350 nm, the wavelength of maximum absorbance of unbound retinal. To ensure a high extent of regeneration, a 10% excess of retinal was added. The bleached membrane/retinal solution was incubated in the dark at 10 °C for 12 h. The extent of regeneration was determined to be approximately 90%, by use of the ratio of the absorbance at 560 nm to the absorbance at 280 nm. The regenerated membrane solution was centrifuged for 30 min at 30000*g* and washed repeatedly with a 2% aqueous solution of bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO) to remove the excess retinal and retinal oxime remaining from the bleaching process (approximately 20 washes). Between washes, the membrane suspensions were stored in 10 mM NaN_3 at 10 °C. The regenerated bR samples were finally washed with deionized water in order to remove BSA and were stored at 10 °C in 5 mM HEPES and 10 mM NaN_3 at pH 7.0. The procedure described herein is similar to the one used by Farrar et al. (1993) with minor variations.

Preparation of Photointermediates. The labeled bR samples were prepared for spectroscopy as follows: (1) For the dark- and light-adapted spectra, the bR sample was washed with a 5 mM HEPES solution at a pH of 7.1. (2) For the M + N spectra, the sample was washed with a 0.1 M solution of NaCl at pH 10.0.

In each case the labeled bR suspension was centrifuged at 30000*g* for 0.5 h, and the resultant pellet was transferred to a 7-mm cylindrical single crystal sapphire rotor with Kel-F endcaps (Doty Scientific, Columbia, SC). The temperature of the sample during data acquisition was controlled by the temperature of the spinning gas.

To light-adapt, the sample was illuminated at 0 °C for 1 h with a 500-W incandescent lamp and a water filter to eliminate near IR components. Mixtures of M and N were trapped by illuminating for a few hours, with light of wavelengths greater than 540 nm, at temperatures varying between −30 and −80 °C. These temperatures are not sufficiently low to trap the K and L intermediates (Maeda et al., 1991). The temperature in the illumination chamber was controlled by a flow of cold, dry nitrogen gas. The sample temperature was maintained below −50 °C during transfer into the NMR probe, and data acquisition, unless otherwise indicated, was performed at a temperature of −60 to −80 °C. The methods described here are slight variations of those reported earlier (Smith et al., 1989a; McDermott et al., 1991; Farrar et al., 1993; Lakshmi et al., 1993).

Solid State NMR Spectroscopy. Magic angle spinning (MAS) NMR spectra were obtained on a home-built spectrometer operating at a field of 7.4 T (^{15}N, ^{13}C, and ^{1}H frequencies of 32.2, 79.9, and 317.6 MHz, respectively), using a standard cross-polarization pulse sequence (Pines et al., 1973) with a mix time of 2 ms and continuous proton decoupling during data acquisition. The proton 90° pulse length was typically 4.1 μs. Acquisition time was 20 ms, and typically, 12 000–20 000 transients were accumulated per spectrum. The recycle delay was 3 s, which was adequate to allow for full relaxation (>5 ^{1}H T_1's). To stabilize the M and N photointermediates, the spectra were obtained at −40 to −80 °C.

^{15}N isotropic shifts are reported relative to 5.6 M aqueous $^{15}NH_4Cl$ in water (based on the use of solid $^{15}NH_4Cl$ as an external reference which itself has a chemical shift 14.4 ppm downfield of 5.6 M aqueous $^{15}NH_4Cl$). ^{13}C isotropic shifts are reported relative to TMS. ^{13}C difference spectra were obtained by subtracting a spectrum of a frozen natural abundance (unlabeled) dark-adapted bR sample from each of the spectra of the frozen ^{13}C-labeled bR photointermediates at identical spinning speeds. This method permits the observation of the signal due only to the label in the sample.

RESULTS

The chemical shifts from the spectra described below, along with previously published chemical shifts, are compiled in Table 1.

Solid State ^{15}N NMR of [ϵ-^{15}N]*Lysine bR*. Figure 1a shows a ^{15}N CPMAS spectrum of [ϵ-^{15}N]lysine bR in the M and N states trapped in 0.1 M NaCl at −60 °C. The six free lysine residues give rise to the intense resonance at 8.4 ppm. The strong resonance downfield from the free lysine peak, at 93.6 ppm, is due to the natural abundance background from the peptide backbone in the protein, and the remaining two smaller resonances at 151 and 295 ppm are attributed the to the [ϵ-^{15}N]-lysine-216 label in the protein. The ^{15}N chemical shift of the Schiff base is known to be extremely sensitive to the protonation

Table 1: Summary of Chemical Shifts (ppm) Observed in the Present Study, Along with Previously Published Shifts

	[ε-^{15}N]Lys-216	[13-^{13}C]retinal	[14-^{13}C]retinal	[ε-^{13}C]Lys-216	[12-^{13}C]retinal
bR_{555}, 13-*cis*,15-*syn*, protonated	150.7	168.7	110.5	48	124.4
bR_{568}, 13-*trans*,15-*anti*, protonated	143.4	164.7	122.0	53	134.3
M, 13-*cis*,15-*anti*, unprotonated	295.2	145.0	125.7	59 ± 2	126.4
N, 13-*cis*,15-*anti*, protonated	150.7	166.0	115.2	53	124.4

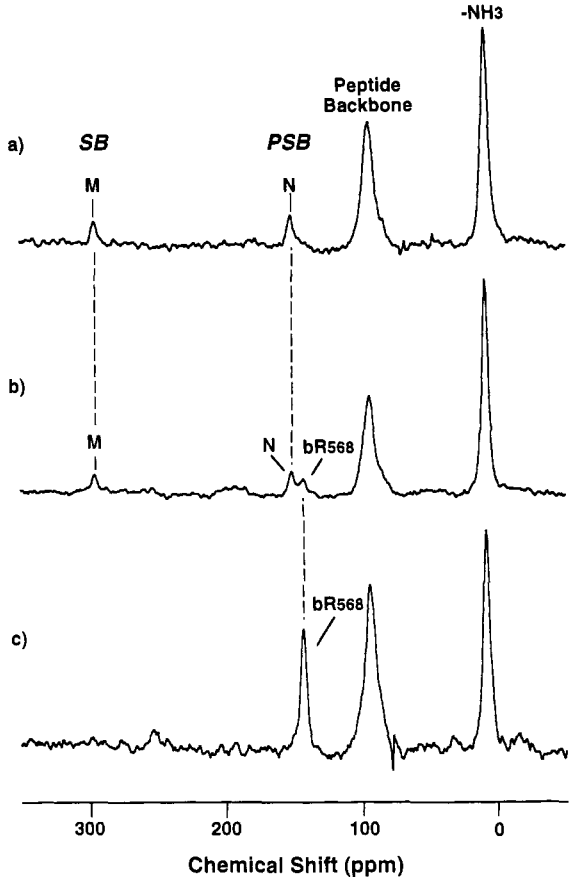

FIGURE 1: ^{15}N CPMAS spectra of [ε-^{15}N]lysine bR, with spinning side bands suppressed by variation of the spinning speed. (a) The M and N states. (b) Products of partial thermal decay obtained when the sample containing the M and N intermediates is warmed up to −30 °C. (c) Product of further thermal decay obtained when the sample containing the M and N intermediates is warmed to 0 °C for a couple of minutes and recooled during data acquisition.

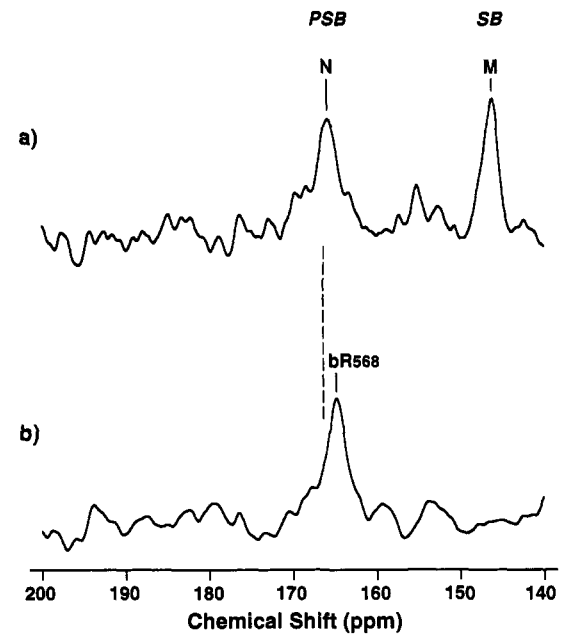

FIGURE 2: ^{13}C NMR difference spectra of [13-^{13}C]retinal bR spinning at 4.5 kHz. (a) The M and N states. (b) The thermal decay product obtained when the sample containing the M and N intermediates is warmed to 0 °C for a couple of minutes and recooled to −50 °C during data acquisition.

state of the SB nitrogen. Previous ^{15}N NMR studies conducted by Harbison et al. (1983) demonstrated that the protonation of the retinal *N*-butyl amine Schiff base results in a 145 ppm movement of the ^{15}N isotropic chemical shift, i.e., the chemical shift of an unprotonated SB nitrogen is ~300 ppm and that of the protonated SB salt (Cl^-) is ~155 ppm. On the basis of this information, we conclude that the 295 ppm resonance, in Figure 1a, is due to a species with a deprotonated Schiff base (by definition an M state).

The 150.7 ppm resonance corresponds to a protonated species, and since the chemical shift is identical to that of bR_{555} (Harbison et al., 1983), the possibility of incomplete conversion needs to be considered. Figure 1 panels b and c show spectra of the thermal decay products of M and N. In Figure 1b the sample has been warmed to −30 °C, and while some M and N remain, a resonance at 143.4 ppm is growing in. In Figure 1c it can be seen that the two resonances at 295.2 and 150.7 ppm evolve into the single resonance at 143.4 ppm on warming to 0 °C. This indicates that the entire sample is in the light-adapted (bR_{568}) state. It follows that there was no bR_{555} in Figure 1a and that another protonated species must have been present.

Differences in the integrated intensities of the SB signals in Figure 1a–c can be explained by variation in the effectiveness of cross-polarization at the increasing temperatures at which the series of spectra were collected. A similar series of spectra, in which the sample was recooled to −60 °C during data acquistion for each spectrum, yielded consistent intensities, but with lower signal-to-noise ratios.

Solid State ^{13}C NMR of [13-^{13}C]Retinal bR. The ^{15}N study indicated that a protonated species is trapped along with M in 0.1 M NaCl at high pH. Since the ^{15}N chemical shift is coincident with that of bR_{555}, it would be good to have an independent assessment of Schiff base protonation. In previous ^{13}C NMR studies conducted on Schiff base model compounds and bR, it was observed that the chemical shift of the C-13 position on the retinal chromophore in bR is sensitive to the protonation state of the Schiff base linkage (Shriver et al., 1976; Mateescu et al., 1984; Harbison et al., 1985; Smith et al., 1989a). This sensitivity can be explained by examining the resonance structures of the chromophore with a protonated SB linkage, where the positive charge partially resides on the C-13 position of the chromophore. Thus C-13 experiences relative deshielding when the Schiff base linkage is protonated in bR_{555} (168.7 ppm) and bR_{568} (164.7 ppm) compared to the unprotonated state in M (145 ppm).

As can be seen in the M + N spectrum for [13-^{13}C]retinal bR (Figure 2a), there are two resonances at 145 and 166 ppm. The resonance at 145 ppm can be attributed to the deprotonated M species (Smith et al., 1989a). The C-13 resonance at 166 ppm lies between the shifts for bR_{555} and bR_{568} and confirms the observation of a species with a protonated Schiff

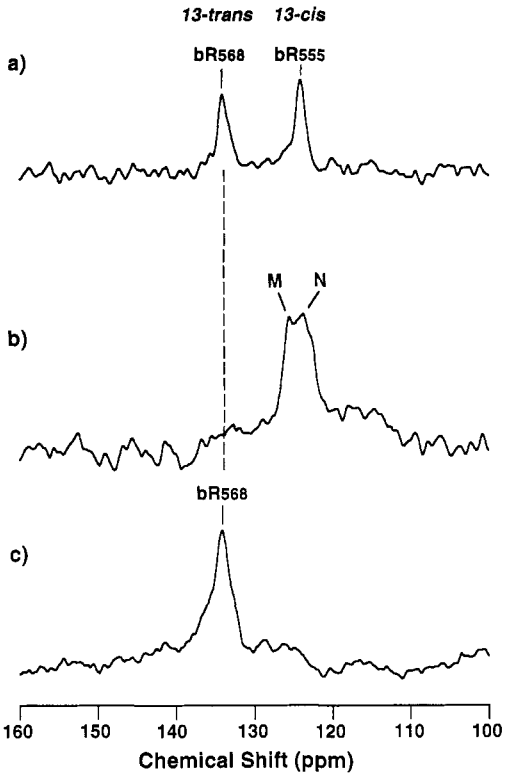

FIGURE 3: ^{13}C NMR difference spectra of [12-^{13}C]retinal bR spinning at 4.3 kHz. (a) The dark-adapted state. (b) The M and N states. (c) The thermal decay product obtained when the sample containing the M and N intermediates is warmed up to 0 °C for a couple of minutes and recooled to −50 °C during data acquisition.

base linkage. Again thermal decay yields exclusively bR_{568} (Figure 2b).

Solid State ^{13}C NMR of [12-^{13}C]Retinal bR. Previous ^{13}C NMR studies of [12-^{13}C]retinal bR and model compounds (Rowan & Sykes, 1974; Shriver et al., 1976; Harbison et al., 1984a, 1985; Mateescu et al., 1984) have shown that the chemical shift of C-12 position is sensitive to the configuration about the C13=C14 bond in the chromophore, due to steric interactions between the protons on C-12 and C-15 in the *cis* conformation. This is illustrated in Figure 3a, where the C-12 resonance of 13-*cis* bR_{555} (124.4 ppm) is upfield relative to that of *all-trans* bR_{568} (134.4 ppm) (Rowan & Sykes, 1974; Harbison et al., 1984a,b, 1985; Smith et al., 1989b).

Figure 3b shows a ^{13}C NMR spectrum of [12-^{13}C]retinal bR in the M and N states. It can be seen that this spectrum contains resonances at 124.4 and 126.4 ppm. The resonance at 126.4 ppm is due to the M state (Smith et al., 1989a). The similar shift of the other species indicates that it also contains a 13-*cis* chromophore. Again thermal decay produces only the signal of bR_{568} (Figure 3c).

DISCUSSION

Previous ^{13}C NMR studies of [ε-^{13}C]lysine, [14-^{13}C]retinal bR inferred the C=N configuration of the retinal–lysine Schiff base linkage in the dark-adapted and M states from the [14-^{13}C]retinal and [ε-^{13}C]lysine chemical shifts (Smith et al., 1989a,b; Farrar et al., 1993). Due again to steric interactions, there is an upfield shift in the C-14 and C-ε resonances in C=N *syn* bR_{555} (110 and 48 ppm, respectively) relative to C=N *anti* bR_{568} (122 and 53 ppm, respectively) or C=N *anti* M (125 and 59 ppm, respectively). These C=N configurations have been confirmed by rotational resonance NMR distance measurements (Thompson et al., 1992; Lakshmi et al., 1993). On the basis of these results, the C-14 and C-ε shifts at 115 and 53 ppm, for the species that accumulates with M in 0.1 M NaCl at high pH and low temperature, were suggested to be consistent with either a deprotonated C=N *syn* or a protonated C=N *anti* SB linkage (Farrar et al., 1993). Since the [13-^{13}C]retinal bR and [ε-^{15}N]-lysine bR chemical shifts reported above indicate that this species contains a protonated SB linkage, we conclude that the C-14 and C-ε shifts at 115 and 53 ppm indicate the absence of a γ-effect and are consistent with the presence of a C=N *anti* SB linkage. Thus, this species contains a 13-*cis*, C=N *anti* protonated chromophore.

Kouyama et al. (1988) have reported that the N intermediate accumulates at high pH and high ionic strength, whereas it is barely observed at low pH and low ionic strength, at physiological temperatures. Other studies have also found an intermediate that arises at alkaline pH on a time scale that places it between M and bR (Danchasky et al., 1986, 1987; Drachev et al., 1986). This intermediate is now recognized as being the N intermediate. Varo et al. (1990) state that transient concentrations of N increase and that of O decrease, with increasing pH. Pfefferlé et al. (1991) observed that N is the main photoproduct of a highly hydrated film of light-adapted bR at pH 10 and 274 K. The visible spectrum of N at pH 7 was identical with that at pH 10, which suggests that the N intermediate observed at neutral pH is identical to the one observed at high pH. These observations indicate that high pH, high salt conditions favor the accumulation of the N intermediate.

The decay of the M and N intermediates can be slowed by decreasing the temperature. The M intermediate is observed in 7–10 mM bicarbonate, at pH 10.2, and 0.2–0.7 M KCl, when illuminated at −60 °C. But subsequent heating of the sample to −30 °C and recooling to −60 °C results in a mixture of the M and N intermediates (Balashov et al., 1990). The N intermediate can also be accumulated and stabilized at −30 °C by warming the M intermediate obtained at −70 °C in 0.2 M KCl at pH 10 (Balashov et al., 1992). The illumination procedure used by the groups cited above, high pH (10.0–10.2) and high salt concentrations (0.2–0.7 M KCl) with temperature sweeping (i.e., warming the M intermediate from −70 to −30 °C), is identical to the technique employed by us in the present study. Thus, we believe that the intermediate trapped along with M in the present study is the N intermediate.

Previous studies by Ormos (1991) had suggested that at low temperatures normal N is not formed: the SB was thought to reprotonate from Asp-85 and produce no pumping. But more recently Ormos et al. (1992) have modified this scenario, based on recent data suggesting that the reprotonated species in their previously reported M-like spectra is entirely due to the N intermediate.

When NMR spectra were originally obtained for M cryotrapped at high pH in 0.1 M NaCl (Smith et al., 1989a), a species was observed which is different from M cryotrapped at high pH in the presence of guanidine·HCl. At the time this was thought to be a second form of M because no other species were observed. Subsequently, subtraction of the natural abundance signals, made possible by the development of precise spinning speed controllers, revealed that signals identical to those from M trapped in guanidine·HCl were also present in the NaCl samples (Farrar et al., 1993). This raised the possibility that the second species, tentatively identified as "X", might have a protonated chromophore and therefore might not be a second form of M. It is now apparent that "X" is the N intermediate.

Time-resolved resonance Raman studies have also found that the concentration of N is enhanced by elevating the pH to 9.5 in 3 M KCl (Fodor et al., 1988). The resonance Raman results suggest that the N intermediate contains a 13-*cis* chromophore with a protonated, C=N *anti* Schiff base linkage. This observation is in complete agreement with our present findings at low temperature.

CONCLUSIONS

^{15}N, 12-^{13}C, 13-^{13}C, 14-^{13}C, and ϵ-^{13}C NMR studies of the bR chromophore all show that a second species accumulates with M at high pH and low temperature in the presence of 0.1 M NaCl and that both species decay thermally to bR_{568}. The ^{15}N chemical shift, which is known to be extremely sensitive to the protonation state of the SB nitrogen, indicates that the Schiff base in this second intermediate is protonated. The 13-^{13}C chemical shift, which is also sensitive to the protonation state of the Schiff base, and the 12-^{13}C chemical shift, which is sensitive to the C13=C14 configuration, indicate that the intermediate contains a protonated 13-*cis* chromophore. Finally, given that the SB is protonated, the 14-^{13}C and ϵ-^{13}C chemical shifts must be interpreted to indicate a C=N *anti* configuration. Thus the species that coexists with M has a protonated, 13-*cis*, C=N *anti* chromophore.

There is past evidence of N accumulating under high pH and high ionic strength conditions. Lozier's and Ebrey's low temperature techniques for trapping N (Balashov et al., 1990, 1992) are identical to procedures employed here. On the basis of these and other reports on trapping the N intermediate under high salt and high pH conditions, we conclude that the intermediate trapped with M at low temperatures in the present study is the N intermediate. Furthermore, the 13-*cis*, protonated, C=N *anti* structure of the low temperature N chromophore, on the basis of the ^{13}C and ^{15}N SSNMR results presented here, matches the structure for the room temperature N intermediate determined by Fodor et al. (1988).

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