Solid State NMR Study of [ε-^{13}C]Lys-bacteriorhodopsin: Schiff Base Photoisomerization

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ABSTRACT Previous solid state ^{13}C-NMR studies of bacteriorhodopsin (bR) have inferred the C=N configuration of the retinal-lysine Schiff base linkage from the [14-^{13}C]retinal chemical shift (1–3). Here we verify the interpretation of the [14-^{13}C]-retinal data using the [ε-^{13}C]lysine 216 resonance. The ε-Lys-216 chemical shifts in bR_{555} (48 ppm) and bR_{568} (53 ppm) are consistent with a C=N isomerization from *syn* in bR_{555} to *anti* in bR_{568}. The M photointermediate was trapped at pH 10.0 and low temperatures by illumination of samples containing either 0.5 M guanidine-HCl or 0.1 M NaCl. In both preparations, the [ε-^{13}C]Lys-216 resonance of M is 6 ppm downfield from that of bR_{568}. This shift is attributed to deprotonation of the Schiff base nitrogen and is consistent with the idea that the M intermediate contains a C=N *anti* chromophore. M is the only intermediate trapped in the presence of 0.5 M guanidine-HCl, whereas a second species, X, is trapped in the presence of 0.1 M NaCl. The [ε-^{13}C]Lys-216 resonance of X is coincident with the signal for bR_{568}, indicating that X is either C=N *anti* and protonated or C=N *syn* and deprotonated.

INTRODUCTION

Bacteriorhodopsin (bR)[1] is a 26-kDa protein located in the plasma membrane of the Halobacterium halobium. The protein contains a retinal chromophore Schiff base (SB) linked to Lys-216 and extrudes protons from the cell when exposed to light. Since its discovery (4, 5), bR has been the focus of intense experimental scrutiny. Recent electron cryomicroscopy results together with other biophysical data have formed the basis for a detailed model of the protein in the resting state (6). Optical and solid state NMR studies have indicated that the retinal chromophore isomerizes and the Schiff base protonation state changes during the photocycle (2, 3, 7, 8, 9). In the dark-adapted state, bR exhibits a broad absorption maximum at 560 nm due to an equilibrium mixture of two components: bR_{555} and bR_{568}, where the subscripts indicate the absorption maxima of the components. On extended exposure to light, bR_{555} is converted to bR_{568} so that light-adapted bR consists exclusively of bR_{568}. If the bR_{568} is illuminated at low temperature, the yellow M_{412} photocycle intermediate, containing a deprotonated Schiff base, can be trapped.

In order to fully characterize the role that the chromophore plays in the proton-pumping mechanism of bR, it is important to know the configuration about the C=N bond in the SB linkage and the protonation state of the SB for the various intermediates in the photocycle. Chemical shifts of the retinal carbons are sensitive to both of these features. Earlier solid state NMR studies of bR regenerated with retinal specifically ^{13}C-labeled at the 12-, 13-, and 15-positions confirmed earlier indications of a protonated, 13-*cis* chromophore in bR_{555} and a protonated, 13-*trans* chromophore in bR_{568} (1, 3, 10). In addition, results for bR regenerated with [14-^{13}C]retinal suggested a C=N *syn* Schiff base linkage in bR_{555} and a C=N *anti* Schiff base in bR_{568} (1).

Similar studies have also been performed under conditions which favor the accumulation of the M photointermediate. In the NMR samples prepared in 0.5 M guanidine-HCl at pH 10.0, a single yellow species, (previously designated M(GdnHCl)) (2), accumulates. The chemical shifts for this M intermediate suggested that it contains a 13-cis, C=N *anti* retinal chromophore with a deprotonated Schiff base. On the other hand, samples prepared in 0.1 M NaCl at pH 10.0 were found to contain a different species. Since the ^{13}C chemical shift observed for the 13-position in these samples was characteristic of a deprotonated SB, the ^{13}C chemical shift at the 14-position was interpreted as indicating a C=N *syn* SB configuration (2). However, by utilizing difference spectroscopy to remove natural abundance signals, we have since found that two photoproducts coexist at low temperature in 0.1 M NaCl at pH 10.0 (11). One of these is identical to the M component seen in guanidine-HCl. Since it can account for the presence of a deprotonated SB, it is possible that the second species, X, may be C=N *anti* and protonated rather than C=N *syn* and deprotonated.

The inference of the C=N configuration from the [14-^{13}C]retinal chemical shift is based on the effect of the

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[1] *Abbreviations used in this paper:* 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; CP, cross-polarization; Lys, lysine; MAS, magic angle spinning; NMR, nuclear magnetic resonance; Tyr, tyrosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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C=N configuration on steric interactions between the proton bonded to 14-C and the protons of the ε-carbon of Lys-216. When the C=N bond is *syn*, the steric interaction is expected to transfer electron density from the protons to the carbons and thus shift the carbon signals upfield relative to those for a C=N *anti* species. Intrinsic to the argument are mutual effects at the 14-position of the retinal and the ε-position of lysine 216. Therefore, a clear test of the steric interpretation would be a correlation between the [14-^{13}C]retinal chemical shift and the [ε-^{13}C]Lys-216 chemical shift. Such a test will distinguish steric effects from other chemical shift effects.

In the present study, we report the chemical shift of [ε-^{13}C]Lys-216 in the bR_{555}, bR_{568}, M, and X intermediates. Since earlier solid state NMR studies characterize distinct [14-^{13}C]retinal resonances for each of the four species, we use the [14-^{13}C]retinal resonance as a marker to identify the species present under a given set of experimental conditions. We find that the [ε-^{13}C]Lys-216 chemical shifts support the previous interpretations of the [14-^{13}C]retinal chemical shifts, indicating a C=N *syn* bond in bR_{555} and a C=N *anti* bond in bR_{568} and M. The data for another photoproduct, tentatively designated X, indicate either a protonated, C=N *anti* or an unprotonated, C=N *syn* Schiff base.

MATERIALS AND METHODS

Preparation of bR samples

Preparation of [ε-^{13}C]lysine-labeled bR

ε-^{13}C-Labeled L-lysine was synthesized according to the procedure of Raap et al. (12). The labeled lysine was incorporated into bacteriorhodopsin by growing *H. halobium* (JW-3) in a defined medium similar to that of Gochnauer and Kushner (13) except that all the D-amino acids and NH_4Cl were removed, and the L-lysine was replaced with one-tenth as much L-[ε-^{13}C]lysine and a trace amount of L-[ε-$^{3}H_2$]lysine for monitoring the incorporation. The purple membrane was isolated by the method of Oesterhelt and Stoeckenius (14). Amino acid analysis showed no scrambling of radioactive label to other amino acid residues.

Bleaching and regeneration

The 14-^{13}C-labeled retinal was synthesized as described by Pardoen et al. (15). Incorporation of the 14-^{13}C-labeled retinal into [ε-^{13}C]Lys-bR was accomplished by bleaching the purple membrane with 1 M hydroxylamine-HCl (pH 8.0) in the dark for 27 h at 35 ± 3°C. The extent of bleaching was determined to be 97% by monitoring the decrease in the absorption maximum of the chromophore bound to the protein at 560 nm. The bleached membrane was washed once in 5 mM HEPES containing 1 mM NaN_3, pelleted for 45 min at 30,000 *g*, and resuspended at 1 mg/3 ml in deionized water.[2] Aliquots of [14-^{13}C]retinal dissolved in dry ethanol at a concentration of 3 mg/ml were added to the bR suspension. The correct amount of retinal 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 90%, by use of the absorbance at 560 nm relative to the absorbance at 280 nm, a relative absorbance maximum for the protein. The regenerated membrane solution was centrifuged for 30 min at 30,000 *g* and washed 16 times in a 2% aqueous solution of bovine serum albumin (Sigma Chemical Company, St. Louis, MO) to remove the excess retinal and retinal oxime remaining from the bleaching process. Between washes, the membrane suspension contained 10 mM NaN_3 and was stored at 10°C. The bR was finally washed twice with deionized water in order to remove bovine serum albumin, resuspended in 5 mM HEPES, 10 mM NaN_3 (pH 7.0), pelleted for 45 min at 30,000 *g*, and stored at 10°C.

Sample conditions and trapping of the photocycle intermediates

The [ε-^{13}C]Lys,[14-^{13}C]retinal bR sample was prepared in three different ways for the spectra shown in Figs. 1 to 3. (*a*). For the spectra shown in Figs. 1, 2 *a*, and 2 *b*, bR was washed with a 5 mM HEPES solution at a pH of 7.1. (*b*). For the spectrum in Fig. 2 *c*, bR was washed with a 0.5 M guanidine-HCl solution at pH 10.0. (*c*). For the spectra in Figs. 3 *a* and 3 *b*, the sample was washed with a 0.1 M solution of NaCl at pH 10.0. In each case the bR suspension was centrifuged at 30,000 *g* for 2 h, and the resultant pellet transferred to a 7-mm cylindrical single crystal sapphire rotor (Doty Scientific, Columbia, SC). The temperature of the sample during data acquisition was controlled by the temperature of the spinning gas.

The light-adapted spectrum (Fig. 2 *b*) was obtained by illuminating the sample at 0°C for 1 h with a 500-watt incandescent lamp and a water filter to eliminate near IR components. The M photointermediate (Figs. 2 *c* and 3 *a*) was trapped by cooling the light-adapted bR sample to the range of −40 to −70°C and illuminating at these temperatures for a few hours with light of wavelengths greater than 540 nm. The sample temperature was maintained below −50°C during transfer into the NMR probe, and data acquisition was performed at a temperature of −80°C. The spectrum of the thermal decay products in 0.1 M NaCl (Fig. 3 *b*) was obtained after warming the sample to −25°C for 1 h, and then recooling to a temperature less than −40°C during data acquisition. The methods described here are slight variations of the techniques reported earlier (2, 11).

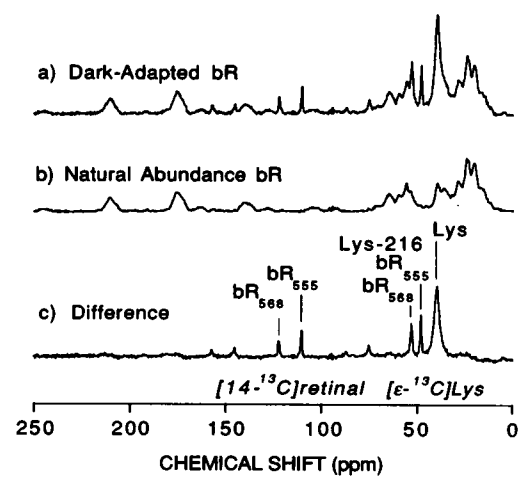


FIGURE 1 ^{13}C MAS spectra of dark-adapted bR collected at −80°C with a sample spinning speed of 2.8kHz. (*a*) [14-^{13}C]retinal, [ε-^{13}C]Lys-bR, (*b*) unlabeled bR, and (*c*) the difference spectrum obtained upon subtraction of (*b*) from (*a*). The signals at 40, 48, and 53 ppm are due to the [ε-^{13}C]Lys label, and the signals in the 100- to 130-ppm range arise from the [14-^{13}C]retinal marker resonances. Rotational sidebands are observed 35 pm upfield and downfield of the centerbands.

[2] Ethanol adversely affects the regeneration process. The 1-mg aliquots of bleached membrane were diluted to 3 ml with deionized water before addition of the retinal/ethanol solution in order to keep the ethanol concentration at a minimum.

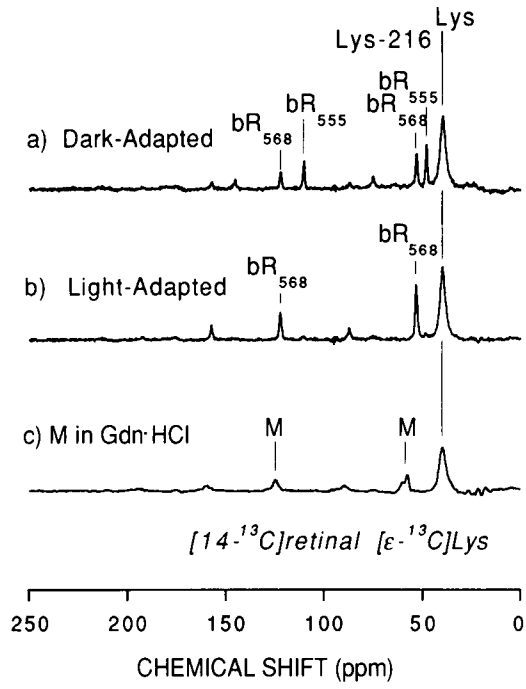


FIGURE 2 Difference spectra of [14-^{13}C]retinal, [ε-^{13}C]Lys-bR at −80°C in (*a*) the dark-adapted state, (*b*) the light-adapted state, and (*c*) the M state trapped in guanidine-HCl. The single [14-^{13}C]retinal resonance at 122 ppm in spectrum (*b*) confirms that all the bR_{555} has been converted to bR_{568} in the light-adapted sample. The lysine signal in *a* and *b* at 53 ppm is therefore assigned to bR_{568}, and the 48-ppm signal in *a* is assigned to bR_{555}. The single [14-^{13}C]retinal resonance at 125 ppm in spectrum *c* indicates the sole presence of the M photointermediate. The lysine signal in *c* at 59 ppm is therefore attributed to M. (The shoulder on this peak is a spinning sideband of the 14-^{13}C signal.)

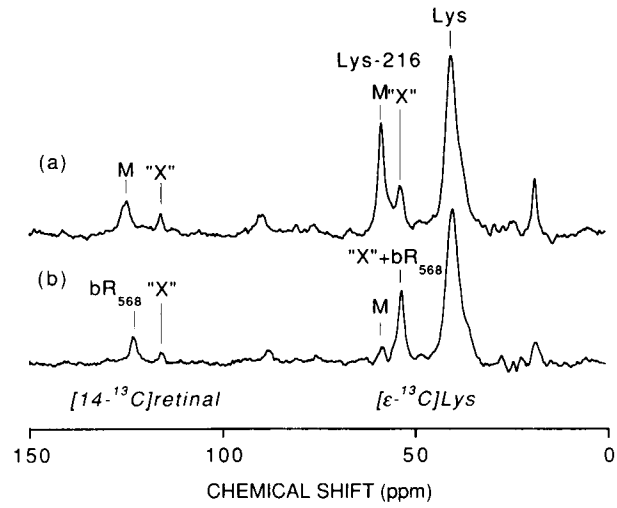


FIGURE 3 Difference spectra of [14-^{13}C]retinal, [ε-^{13}C]Lys-bR samples prepared in 100 mM NaCl at pH 10.0. (*a*) Difference spectrum of sample after illumination at cold temperatures and (*b*) difference spectrum of the same sample after partial thermal decay at −25°C for an hour. Both spectra were obtained at low (< −40°C) temperatures. The two signals due to the [14-^{13}C]retinal marker indicate the presence of two species, M (125 ppm) and X (115 ppm). We also observe [ε-^{13}C]Lys-216 signals at 59 and 53 ppm. The line at 59 ppm is attributed to M. The absence of a [14-^{13}C]retinal signal at 122 ppm indicates that there is no bR_{568} present in the sample. This allows the line at 53 ppm to be assigned to X. After slight warming, the M signal is reduced and a [14-^{13}C]retinal signal due to bR_{568} appears. The [14-^{13}C]-retinal signal due to X persists suggesting that, under the given conditions, X is more thermally stable than M.

Solid state NMR spectroscopy

Magic angle spinning (MAS) NMR spectra were obtained on a home-built spectrometer operating at a field of 7.4T (^{13}C and ^{1}H frequencies of 79.9 and 317.6 MHz, respectively), using a standard cross-polarization pulse sequence (16) with a mix time of 2 msec, sample rotation rates of 2.8–3.2 kHz, and continuous proton decoupling during data acquisition. The proton 90° pulse length was typically 3.8 μs. A spinning speed controller (17) was used to maintain the spinning speed of the samples to within ±5 Hz during the 7–10 h of data acquisition for each spectrum. Acquisition time was 20 msec, and typically, 10,000–20,000 transients were accumulated per spectrum. The recycle delay was 3 sec, which was adequate to allow for full relaxation (>5 ^{1}H T_1 values). The spectra shown in Figs. 1–3 were obtained at −40 to −80°C, to stabilize photointermediates.

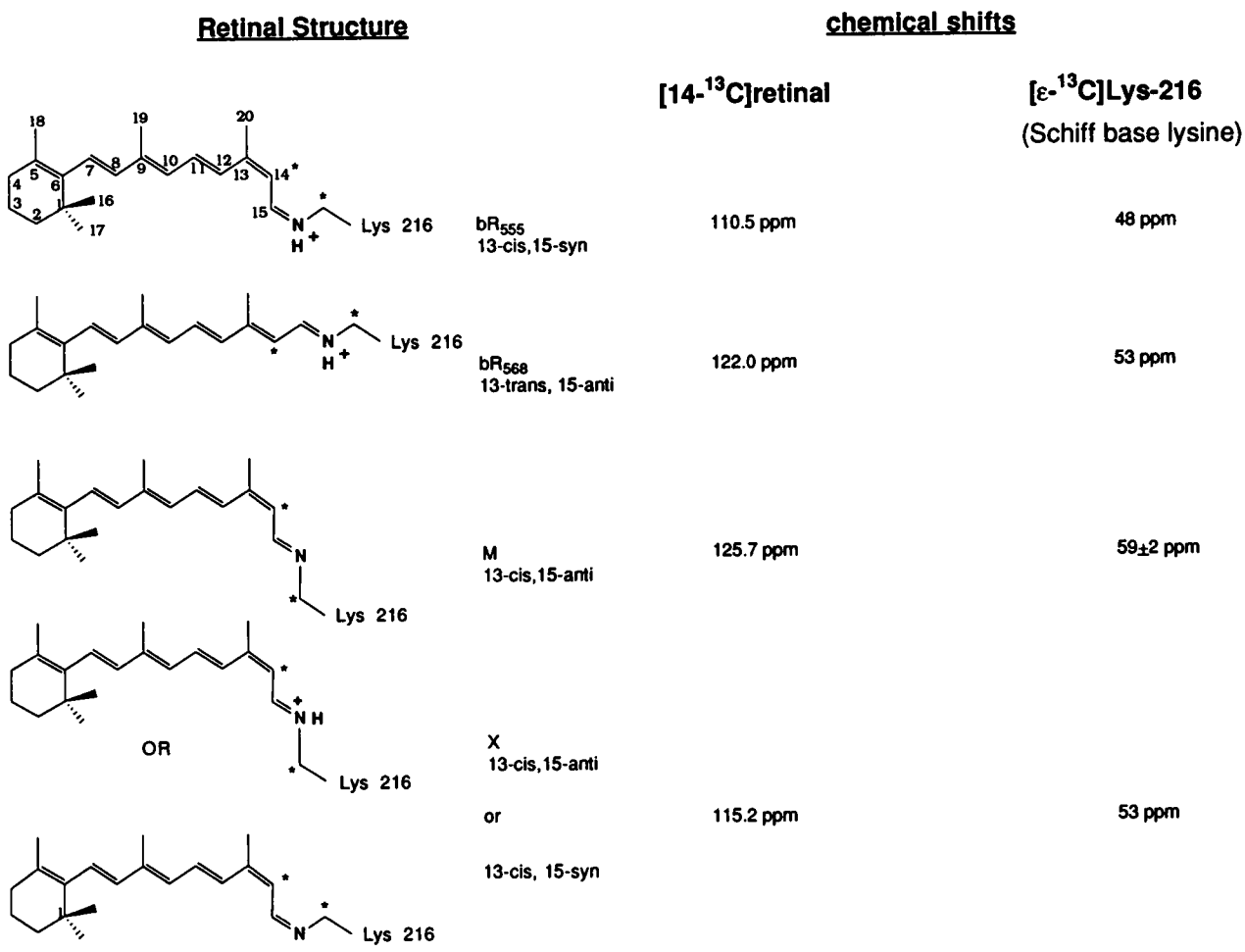
All isotropic shifts are reported relative to tetramethylsilane. Difference spectra were obtained by subtracting the spectrum of a frozen natural abundance (unlabeled) bR sample from each of the spectra of the frozen [ε-^{13}C]Lys, [14-^{13}C]retinal-bR photointermediates. This method permits the observation of the signal due only to the label in the sample.

RESULTS AND DISCUSSION

A compilation of [ε-^{13}C]Lys-216 isotropic chemical shifts derived from Figs. 1–3 appears in Scheme 1 along with the [14-^{13}C]retinal marker shifts for bR_{555}, bR_{568}, M, and X. The retinal configurations for bR_{555}, bR_{568}, and M are also shown, based on interpretation of the present data, as discussed below, and earlier solid state NMR and Raman scattering studies (1, 2, 3, 9, 10, 18, 19). Two possible retinal configurations for X consistent with the present data are also depicted.

Fig. 1 shows solid state ^{13}C-NMR MAS spectra of dark-adapted bR at a temperature suitable for trapping M. The difference spectrum shows signals due only to the [ε-^{13}C]Lys and [14-^{13}C]retinal labels. The resonance at 40 ppm is assigned to the ε-^{13}C label in the six free lysine residues, because it is the strongest signal. Furthermore, it has the expected chemical shift (20), and, as we shall see below, it is the only signal that is unperturbed by light adaptation or Schiff base deprotonation. The signals at 110.5 and 122 ppm have previously been assigned to the 14-^{13}C of retinal in bR_{555} and bR_{568}, respectively (3). The remaining signals at 48 and 55 ppm are assigned to the ε-^{13}C label in lys-216, based on the chemical shifts observed in protonated retinal Schiff bases (21, 22). The ^{13}C chemical shifts in Fig. 1 are not significantly different (<1 ppm) from those observed at room temperature.

The relative intensities of the peaks at 48 and 53 ppm suggest that they should be assigned to bR_{555} and bR_{568}, respectively. This assignment is verified by light adaptation. The difference spectrum of light-adapted [ε-^{13}C]Lys, [14-^{13}C]retinal-bR in Fig. 2 *b* shows resonances due to the [14-^{13}C]retinal marker and the [ε-^{13}C]Lys label. The [14-^{13}C]retinal marker indicates that the sample has been converted completely to the bR_{568} form. This enables a confirmation of the [ε-^{13}C]Lys-216 peak at 53 ppm as being due



SCHEME 1

to bR_{568}. Thus, the peak at 48 ppm in the dark-adapted spectrum in Figs. 1 *c* and 2 *a* is assigned to bR_{555}. It is evident that light adaptation results in an 11.5-ppm downfield shift of the 14-^{13}C resonance and a 5-ppm downfield shift of the ε-^{13}C resonance. This simultaneous downfield shift of both resonances supports the interpretation given earlier of the 14-^{13}C chemical shifts in terms of a steric interaction between the protons of the ε-C and the 14-C in the C=N *syn* configuration of the chromophore which is relieved in the C=N *anti* configuration. The smaller magnitude of the steric effect in the ε-C is expected due to the nonplanar character of this group.

Fig. 2 *c* shows a difference spectrum of [ε-^{13}C]Lys-bR obtained following illumination at low temperatures in the presence of 0.5 M guanidine-HCl at pH 10.0. Under these conditions, the sample is completely yellow, which is consistent with complete conversion to the M_{412} photointermediate. The presence of a single resonance at 125 ppm, due to the [14-^{13}C]retinal marker confirms the presence of a single species, M. The single [ε-^{13}C]Lys-216 resonance at 59 ppm in the difference spectrum in Fig. 2 *c* is therefore assigned to the M intermediate. Thus, both the 14-^{13}C and the ε-^{13}C chemical shifts are even further downfield in M than in bR_{568}. This is consistent with the known deshielding effects of Schiff base deprotonation on both of these carbons (21, 22) and with the idea that the M intermediate contains a C=N *anti* chromophore (2, 9).

The difference spectrum of the [ε-^{13}C]Lys,[14-^{13}C]-retinal-bR photointermediates trapped at low temperatures in 0.1 M NaCl at pH 10.0 is shown in Fig. 3 *a*. The 110–130-ppm region of the spectrum reveals two signals due to the [14-^{13}C]retinal marker: a signal at 125 ppm corresponding to M, and a signal at 115 ppm corresponding to a species designated as X (previously referred to as M(NaCl)) (2). In the alkyl region of the spectrum in Fig. 3 *a*, two [ε-^{13}C]Lys-216 resonances are observed at 59 and 53 ppm. The [ε-^{13}C]Lys-216 resonance at 59 ppm is assigned to M based on the spectrum in Fig. 2 *c*. The position of the other resonance at 53 ppm is coincident with that of bR_{568}, but the absence of a [14-^{13}C]retinal signal at 122 ppm due to bR_{568} allows us to conclude that no bR_{568} is present in the sample. This implies that the [ε-^{13}C]Lys-216 signal at 53 ppm in the spectrum in Fig. 3 *a* is attributable to X.

It was previously assumed that X was the only photointermediate trapped in NaCl at a pH of 10.0 (2). The use of difference spectroscopy in more recent studies has revealed

that both M and X are present under these conditions, and M is the more prominent component of such samples (11). The position of the [ϵ-^{13}C]Lys-216 signal in X at 53 ppm is consistent with at least two possible SB structures. If X has a deprotonated SB linkage, then the [ϵ-^{13}C]Lys-216 resonance, 6 ppm upfield from that in M, and the [14-^{13}C]retinal resonance, 10 ppm upfield from that in M, suggest that it has a C=N *syn* configuration about the SB. This was the conclusion drawn previously by (2, 3) based on the [14-^{13}C]-retinal data alone. But since our studies now find that M can account for the presence of a deprotonated species, it is possible that X is a protonated species. In that case, a C=N *anti* configuration would also be consistent with [ϵ-^{13}C]Lys-216 resonance at 53 ppm which is essentially identical to that in bR_{568}, with a protonated SB linkage in a C=N *anti* configuration. However, the [14-^{13}C]retinal chemical shift in X would then be anomalously low. Since the present results are inconclusive, further studies are underway to determine the structure of the SB in X and identify its role in the photocycle of bR.

Fig. 3 *b* shows results of thermal decay of the species M and X present in 0.1 M NaCl (Fig. 3 *a*). The sample from Fig. 3 *a* was warmed to −25°C for 1 h and then recooled to a temperature below −40°C for the duration of data acquisition. In this spectrum, the [14-^{13}C]retinal peak at 125 ppm due to the M intermediate is diminished in intensity and a signal due to bR_{568} appears at 122 ppm. The signal at 115 ppm due to the species X persists after warming the sample, although a reduction in its intensity as compared to that in Fig. 3 *a* is observed. This demonstrates that under the given conditions X is more thermally stable than M. Thawing the sample at room temperature for 20 s followed by recooling to less than −40°C for data acquisition results in a complete decay of both M and X to bR_{568} (spectrum not shown).

CONCLUSIONS

The ^{13}C isotropic chemical shift of [ϵ-^{13}C]Lys-216 is sensitive to its proximity to the Schiff base linkage to the retinal chromophore and is therefore distinguishable from the isotropic chemical shifts of the remaining six labeled lysine residues in the protein. Further, the chemical shift of [ϵ-^{13}C]-Lys-216 exhibits a 5-ppm upfield shift due to a steric interaction with the C_{14}-H group on the retinal when the SB is C=N *syn* as opposed to C=N *anti*. The C=N configuration of the retinal-lysine SB linkage is therefore *anti* in bR_{568} and *syn* in bR_{555}. The [ϵ-^{13}C]Lys-216 chemical shift is also sensitive to the protonation state of the Schiff base linkage, deprotonation causing a 6-ppm downfield shift of the [ϵ-^{13}C]Lys-216 resonance in the M intermediate relative to bR_{568}.

A yellow photointermediate is trapped upon illumination at low temperatures in the presence of 0.5 M guanidine-HCl at pH 10.0, and it appears that this M intermediate has a C=N *anti* chromophore. In contrast, two species are trapped upon illumination at low temperatures in the presence of 0.1 M NaCl at pH 10.0. One species is the same as the M intermediate trapped in the presence of guanidine-HCl. The configuration of the second species, X, is not yet determined, and further investigations are in progress. In particular, solid state ^{15}N-NMR studies are currently being conducted to assess the protonation state of the X intermediate.

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