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Resonance Raman spectroscopy of specifically [ε-^{15}N]lysine-labeled bacteriorhodopsin

(purple membrane/Schiff base/retinylidene chromophore/bacterioopsin–chromophore interaction)

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ABSTRACT **The possible interaction of a second lysine with the retinylidene Schiff base of bacteriorhodopsin (Lewis, A., Marcus, M. A., Ehrenberg, B. & Crespi, H. (1978) *Proc. Natl. Acad. Sci. USA* 75, 4642–4646) has been investigated by specific incorporation of ^{15}N into the ε-amino groups of the lysine residues. Comparison of resonance Raman spectra of bacteriorhodopsin grown on 100%, 0%, and 50% labeled lysine demonstrates that ^{15}N isotope effects on the Schiff base vibration can be accounted for by ^{15}N labeling only at the Schiff base nitrogen. Our data also provide *in situ* confirmation of the linkage of the retinal chromophore with the ε-amino nitrogen of lysine.**

Understanding the molecular mechanism by which bacteriorhodopsin in the purple membrane of *Halobacterium halobium* functions as a light-transducing proton pump remains a key goal in membrane research (for a review, see ref. 1). One important area under active investigation is the nature of the interaction between the retinylidene chromophore of bacteriorhodopsin and the protein bacterioopsin (2, 3). It is believed that this interaction is responsible for the red shift of the absorption maximum of the major visible band of bacteriorhodopsin in its light-adapted state (bR_{570}) relative to that of the retinal chromophore (λ_{max} = 380 nm) and protonated retinylidene Schiff base model compounds (λ_{max} = 440 nm) (3). Because protonation of the Schiff base can only account partially for the red shift of bR_{570}, several types of secondary interactions have been postulated (2–5).

Lewis *et al.* (6) have proposed a model for proton pumping by bacteriorhodopsin that involves as a key element the direct interaction of a nitrogen-containing residue, most likely lysine, with the Schiff base of bR_{570}. This model is based partially on the evidence from resonance Raman spectroscopy that complete ^{15}N-labeling of bacteriorhodopsin lowers the protonated Schiff base vibration (C=N⊕H stretch) from 1642 (7) to 1627 cm^{-1}, whereas the deprotonated Schiff base vibration (C=N stretch) of the intermediate M_{412} is only shifted from 1619 (7) to 1614 cm^{-1}. It is implied that the labeling of a non-Schiff base nitrogen contributes to the 15-cm^{-1} isotopic shift in bR_{570} but does not contribute to the 5-cm^{-1} shift in M_{412}. Further, pH-dependent kinetic effects indicate that this nitrogen-containing group is likely to be lysine (6). Although this lysine interaction model is of interest, it is not clear how an isotopic substitution of a nonchromophoric atom could directly affect a chromophore vibration such as the C=N⊕H stretch. For this reason, we have attempted to test the hypothesis that the labeling of a nitrogen other than the Schiff base nitrogen in bacteriorhodopsin affects the Schiff base vibration.

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MATERIALS AND METHODS

Halobacterium halobium R_1 was grown in a synthetic medium similar to that of Gochnauer and Kushner (8): 430 mg of DL-alanine, 400 mg of L-arginine, 50 mg of L-cysteine, 1.3 g of L-glutamic acid, 60 mg of glycine, 440 mg of DL-isoleucine, 800 mg of L-leucine, 42.5 mg of L-lysine, 370 mg of DL-methionine, 260 mg of DL-phenylalanine, 50 mg of L-proline, 610 mg of DL-serine, 500 mg of DL-threonine, 200 mg of L-tyrosine, 1 g of DL-valine, 100 mg of adenylic acid, 100 mg of uridylic acid, 1 g of glycerol, 5 g of NH_4Cl, 2 g of KCl, 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 84.1 mg of $NaNO_3$, 40.8 mg of Na_2HPO_4, 50.7 mg of $NaH_2PO_4 \cdot H_2O$, 535 mg of sodium citrate dihydrate, 0.164 mg of $MnSO_4 \cdot H_2O$, 3.81 mg of $CaCl_2 \cdot 2H_2O$, 0.04 mg of $ZnSO_4$, 3.6 mg of $FeCl_2 \cdot 4H_2O$, and 0.032 mg of anhydrous $CuSO_4$ per liter, adjusted to pH 6.5. When [6-^{3}H]lysine was added to the medium, it was found that more than 95% of the radioactivity contained in the purple membrane appeared in the lysine peak upon amino acid analysis of the HCl hydrolyzate. Thus little, if any, label was transferred from lysine to other amino acid residues. Furthermore, the specific activity of the lysine in bacteriorhodopsin was comparable to that for lysine in the medium, indicating little, if any, synthesis of unlabeled lysine. Therefore, the incorporation of the tritium label is specific and efficient under our growth conditions.

For Raman spectroscopy, samples were prepared with media containing unlabeled lysine, [ε-^{15}N]lysine (^{15}N at the ε-amino position), or a 1:1 mixture of unlabeled lysine and [ε-^{15}N]lysine. Since the ^{15}N label is in a slightly different position from the ^{3}H label, the degree of labeling of the lysine in each of our Raman samples was checked by using gas chromatography/mass spectroscopy as follows. Lipids were removed from samples (about 10 nmol) of bacteriorhodopsin from bacteria grown on unlabeled lysine ([^{14}N]Lys-bR) and bacteriorhodopsin from bacteria grown on [ε-^{15}N]lysine ([^{15}N]Lys-bR) on a 37 × 1 cm column of Sephadex LH-20 (Pharmacia) eluted with 88% formic acid/ethanol, 30:70 (vol/vol), as described by Gerber *et al.* (9). The void-volume material was pooled, dried, and hydrolyzed under vacuum in 6 M HCl (Pierce) for 24 hr. The amino acids were converted to the *N*-heptafluorobutyryl isobutyl esters by the procedure of Pearce (10). Isotopic ratios were measured with an Hitachi RMU-6L mass spectrometer interfacing with a Perkin–Elmer 990 gas chromatograph. The amino acid deriv-

Abbreviations: [^{14}N]Lys-bR, bacteriorhodopsin from bacteria grown on unlabeled lysine; [^{15}N]Lys-bR, bacteriorhodopsin from bacteria grown on [ε-^{15}N]lysine; [^{14}N/^{15}N]Lys-bR, bacteriorhodopsin from bacteria grown on a 1:1 mixture of unlabeled lysine and [ε^{15}N]lysine; bR_{570}, light-adapted bacteriorhodopsin.

atives were separated on a 274-cm column packed with 3% (wt/wt) OV-101 (Supelco, Bellefonte, PA) that was programmed from 100°C to 300°C at 12°C/min. The mass spectrometer was electrically scanned from m/e = 517 to m/e = 524 at a rate of 1 Hz during elution of the lysine gas chromatographic peak. Data was collected with an IBM 1800 computer, and peak heights were measured with the aid of a PDP-11/45 computer as described by VanLangenhove *et al.* (11). The ratio of unlabeled lysine to [ε-^{15}N]lysine in bacteriorhodopsin from bacteria grown on a 1:1 mixture of unlabeled lysine and [ε-^{15}N]lysine ([^{14}N/^{15}N]Lys-bR) was determined by measuring the ratio of m/e = 520 ([^{14}N]lysine-HOC$_4$H$_9$) to m/e = 521 ([^{15}N]lysine-HOC$_4$H$_9$). This ratio was compared to the m/e = 520 to m/e = 521 ratio expected for various proportions of unlabeled lysine to [ε-^{15}N]lysine. The latter was calculated by adding the unlabeled lysine spectrum and [ε-^{15}N]lysine spectrum (obtained by shifting the spectrum of unlabeled lysine one mass unit higher) in various proportions. The relative error of these measurements was estimated to be ±5%.

Resonance Raman spectra were recorded with a Spex 1401 double monochromator interfaced with a NIC-1180 data system (Nicolet Instrument, Madison, WI). The spectra were recorded by using the 514.5-nm line from an Argon ion laser (Spectra Physics, model 164). A 1.5-ml purple membrane suspension in distilled water (OD$_{570}$ = 1.0) was inserted into a home-made rotating cell as described by Stockburger *et al.* (12). The separation of the beam from the axis of the cell was 2.3 cm, and the cell was rotated at 40 revolutions per second. The beam diameter at the sample was 40 μm, resulting in a total sample exposure time in the beam of approximately 7 μs and a time between exposures of 25 ms. The 100-μm input slit of the double monochromator was adjusted at the center of a magnified image (×5) formed by the collecting lens, resulting in the effective sample exposure time of 5.2 μs. The power at the sample was 10 mW. Under these conditions, the main contribution to the Raman spectrum was from bR$_{570}$. Care was taken to use only samples with small amounts of carotenoids, whose presence is evident from peaks at 1515 cm^{-1} and 1155 cm^{-1}. All the spectra were recorded at 2-cm^{-1} resolution and were multiply scanned to provide a high signal-to-noise ratio. The spectra were plotted without smoothing on a Calcomp plotter.

RESULTS

In Fig. 1, spectra A and B are those of [^{14}N]Lys-bR and [^{15}N]Lys-bR, respectively, in the range 800–1700 cm^{-1}. A single band at 1530 cm^{-1} in these spectra shows that the contribution is predominantly from bR$_{570}$. In contrast, a photosteady mixture of bacteriorhodopsin, which was obtained by using a fixed sample, contained several intermediates, and the corresponding spectrum displayed a complex of several overlapping peaks in the region between 1512–1567 cm^{-1}.[§] In Fig. 2, spectra A and B are those of the same samples in the region between 1600–1700 cm^{-1}. The C=N⊕H vibration, which appears at 1642 cm^{-1} in [^{14}N]Lys-bR, is found to be shifted to 1629 cm^{-1} in [^{15}N]Lys-bR. Because the same shift (within the spectral resolution) has been reported for fully ^{15}N-labeled bacteriorhodopsin (6), it can be concluded that the labeling of nitrogens other than that in the ε-amino group of the lysine residues has no effect on the Schiff base vibration. Furthermore, the results confirm directly that the linkage of the chromophore is through the ε-amino group of lysine.

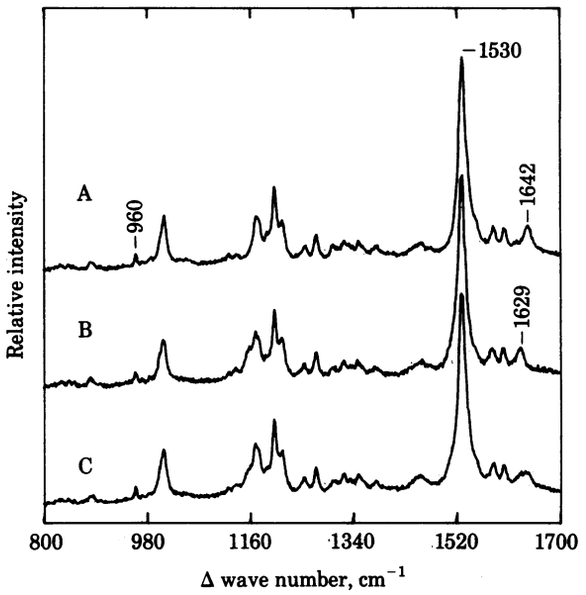

FIG. 1. Resonance Raman spectra of [^{14}N]Lys-bR (A), [^{15}N]Lys-bR (B), and [^{14}N/^{15}N]Lys-bR (C), recorded by scanning 20 times at a speed of 1 cm^{-1}/s. The band at 1530 cm^{-1} is assigned to C=C stretching vibration of bR$_{570}$ (7). A small shoulder at 1550 cm^{-1} may be due to the "X" intermediate (13) and has about 5% contribution to the spectrum. The shoulder at 1155 cm^{-1} in spectra B and C is due to carotenoids (14).

Is More Than One Lysine Involved in the Observed Isotopic Shift for bR$_{570}$? To test the hypothesis that the labeling of a second lysine contributes to the observed isotopic shift in spectra of [^{15}N]Lys-bR, we recorded the spectrum of purple membrane grown on a 1:1 mixture of unlabeled lysine and [ε-

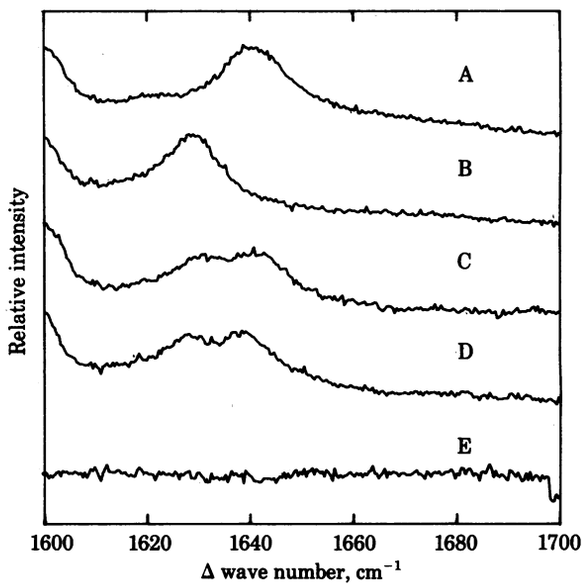

FIG. 2. Resonance Raman spectra of [^{14}N]Lys-bR (A), [^{15}N]Lys-bR (B), [^{14}N/^{15}N]Lys-bR (C), 56:44 mixture of [^{14}N]Lys-bR and [^{15}N]Lys-bR (D)—all recorded by scanning 300 times at 1 cm^{-1}/s. The shift of peaks to the right in spectrum C is due to a displacement of 2 cm^{-1} in starting the scan. Subtraction of spectrum D from spectrum C, after the peak positions in C were aligned with those in D, gives spectrum E. The abrupt intensity changes in E near 1700 cm^{-1} are subtraction artifacts.

[§] In this case the 1530 cm^{-1} peak can be fitted by a single Voigtian curve indicating the presence of a single intermediate. In contrast, a photosteady mixture of purple membrane produces a complex peak containing at least five separate components, which can be resolved by using a curve-fitting program (unpublished results).

^{15}N]lysine (Figs. 1C and 2C). Analysis by gas chromatography/mass spectrometry indicated that, in this preparation, 44% of the lysine residues were singly ^{15}N-labeled and 56% were unlabeled. Thus, each of the 7 lysines of bacteriorhodopsin have a 56% probability of containing unlabeled lysine and a 44% probability of containing [ε-^{15}N]lysine. The possible involvement of a second lysine requires consideration of "hybrids" containing both unlabeled and [ε-^{15}N]lysine, each present in either the primary Schiff base or the secondary lysine, as shown schematically in Table 1. In the absence of some mechanism by which labeling of the secondary nitrogen alters the vibrational frequency of the C=N⊕H vibration, we expect the Schiff base vibrational frequency for species (^{14}N, ^{14}N) to equal (^{14}N, ^{15}N) and species (^{15}N, ^{15}N) to equal (^{15}N, ^{14}N). In contrast, the postulated isotope effect due to labeling the secondary nitrogen would produce intermediate Schiff base vibrational frequencies for the hybrid species.

To see whether the intermediate Schiff base peaks exist, we compared a spectrum of a 56:44 mixture of [^{14}N]Lys-bR and [^{15}N]Lys-bR (Fig. 2, spectrum D) with the spectrum of [^{14}N/^{15}N]Lys-bR (Fig. 2, spectrum C). When spectrum D in Fig. 2 was subtracted from C, no difference was observed above the noise level (Fig. 2, spectrum E), indicating that these spectra are identical within the accuracy of our resonance Raman measurements. We also noted that the Raman spectra of 50:50, 44:56, and 56:44 mixtures of [^{14}N]Lys-bR and [^{15}N]Lys-bR clearly differed.

To determine the extent to which the above measurements can detect intermediate peaks in the Schiff base region of [^{14}N/^{15}N]Lys-bR, we simulated a set of "hybrid" Raman spectra by producing a well-defined splitting of two peaks at 1629 and 1642 cm^{-1} with appropriate intensities. Spectra A and B in Fig. 2 were first normalized and then added in the proportion 56:44, representing a case of no splitting (Fig. 3, spectrum A). In order to simulate a splitting, a composite spectrum was prepared by adding 31% of Fig. 2, spectrum A, 19% of Fig. 2, spectrum B, and 25% each of Fig. 2, spectra A and B shifted by varying amounts. One such composite with a splitting between (^{14}N, ^{14}N), (^{14}N, ^{15}N) and (^{15}N, ^{15}N), (^{15}N, ^{14}N) of 2 cm^{-1} is shown in Fig. 3, spectrum B. The subtraction of spectrum A from spectrum B in Fig. 3 is shown in Fig. 3, spectrum C. We found, in general, that a splitting of more than 2 cm^{-1} produced a difference which was well above noise (Fig. 3, spectrum C). Consequently, we set a limit of 2 cm^{-1} on the splitting of 1629 cm^{-1} and 1642 cm^{-1} bands.

The M_{412} Intermediate. By examining a photosteady mixture of purple membrane and subtracting a pure bR_{570} spectrum, we obtained the spectrum of the remaining intermediates, which, as revealed by curve fitting, comprises predominantly M_{412} (C=C stretch at 1567 cm^{-1}), L_{550} (C=C stretch at 1540 cm^{-1}) and the "X" intermediate (13) (C=C stretch at 1550 cm^{-1}). It is clear from this difference spectrum (unpublished data) that the deprotonated Schiff base vibration is shifted from 1622 to 1618 cm^{-1} upon [ε-^{15}N]lysine labeling. This again is in agreement (within the spectral resolution) with the reported results for fully ^{15}N-labeled bacteriorhodopsin (6) and indicates the involvement of only the ε-amino nitrogen of lysine in this shift.

Table 1. The four possible Schiff base species

Hybrid	Schiff base structure	Probability of occurrence
(^{14}N, ^{14}N)	C=^{14}N⊕H...^{14}NH$_2$−	56% × 56% = 31%
(^{14}N, ^{15}N)	C=^{14}N⊕H...^{15}NH$_2$−	56% × 44% = 25%
(^{15}N, ^{15}N)	C=^{15}N⊕H...^{15}NH$_2$−	44% × 44% = 19%
(^{15}N, ^{14}N)	C=^{15}N⊕H...^{14}NH$_2$−	44% × 56% = 25%

The structures and frequencies of occurrence of the four possible species of the Schiff base with a hypothetical secondary lysine interaction for a sample containing 44% [ε-^{15}N]lysine and 56% unlabeled lysine. The dotted line indicates the secondary interaction.

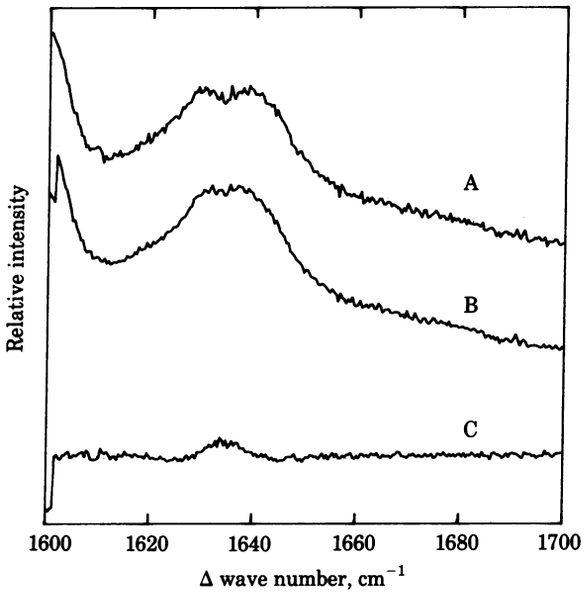

FIG. 3. Addition of spectra A and B in Fig. 2 in the proportion 56:44 (A); a composite spectrum containing 31% of Fig. 2, spectrum A, 19% of Fig. 2, spectrum B, 25% of Fig. 2, spectrum A shifted 2 cm^{-1} left, and 25% of Fig. 2, spectrum B shifted 2 cm^{-1} right (B); subtraction of spectrum A from spectrum B plotted to the same scale (C). The abrupt intensity changes in B and C near 1600 cm^{-1} are subtraction artifacts.

Comparison of [^{15}N]Lys-bR and Fully ^{15}N-Labeled Bacteriorhodopsin in Other Regions of the Spectrum. We observed no detectable shifts in the spectrum of [^{15}N]Lys-bR outside of the Schiff base region (Fig. 1, spectra A and B). In contrast, Marcus and Lewis (13) have reported a splitting of the 960 cm^{-1} peak into two components of about equal magnitude at 960 and 969 cm^{-1} in fully ^{15}N-labeled bacteriorhodopsin and to 960 and 985 cm^{-1} in fully ^{15}N-labeled bacteriorhodopsin suspended in ^2H_2O. This implies that a nitrogen-containing group other than lysine is producing the change Marcus and Lewis (13) have observed. However, this conclusion should be treated cautiously because the appearance of a peak near 970 cm^{-1} also could be due to increased contributions from a 972-cm^{-1} peak, which is present in the pure spectrum of M_{412}.

DISCUSSION

The labeling of the ε-amino nitrogen of lysine in bacteriorhodopsin leads directly to a shift in the frequency of the C=N⊕H stretching vibration in bR_{570}. This *in situ* measurement confirms that retinal is linked to the protein by a Schiff base formed by the ε-amino nitrogen of lysine. Previous studies have been based on chemical assays after bacteriorhodopsin has been denatured (2, 15).

Our results also indicate that, contrary to the previous suggestion (6), the shift from 1642 cm^{-1} to 1629 cm^{-1} upon ^{15}N labeling of bR_{570} can be accounted for, within our experimental error, by the presence of ^{15}N in the Schiff base linkage. However, this finding does not eliminate the possibility that a second lysine interacts with the Schiff base. In fact, the effect on the C=N⊕H stretching vibration of ^{15}N labeling of a nitrogen that

interacts with the Schiff base would be expected to be negligibly small.

The existence of a 13-cm^{-1} shift due to ^{15}N substitution in the Schiff base linkage alone is also supported by theoretical calculations and model compound studies. The normal mode calculations of Aton *et al.* (16) predict a shift in the protonated Schiff base vibration of 19 cm^{-1} upon ^{15}N labeling. In agreement, the resonance Raman spectrum of protonated retinylidenemethylamine exhibits a 13-cm^{-1} shift due to ^{15}N substitution (17).

The M_{412} intermediate, which contains an unprotonated Schiff base, exhibits only a 4- to 5-cm^{-1} shift upon ^{15}N substitution. Normal mode calculations also predict a much smaller shift (7 cm^{-1}) in the unprotonated Schiff base (16). In contrast, the unprotonated form of retinylidenemethylamine upon ^{15}N substitution exhibits the same shift as the protonated form; however, there is an indication that the Schiff base stretching vibration is modified in this case (17).

The absence of effects of [ϵ-^{15}N]lysine substitution on vibrations other than the Schiff base vibration indicates that other modes of the retinylidene chromophore are insensitive to an increase of one unit mass in the Schiff base. This is surprising for two reasons. (*i*) The 1254-cm^{-1} band has been assigned to the C=N—H bending vibration (12). Normal mode calculations predict a 7-cm^{-1} shift in this vibration upon ^{15}N substitution of the Schiff base nitrogen (Callender, R. H., personal communication), although this calculation is very sensitive to the bond parameters used. (*ii*) In fully ^{15}N-labeled bacteriorhodopsin, Marcus and Lewis (13) observed a splitting of the 960-cm^{-1} band (assigned to C—C—H out-of-plane bending) into bands of equal intensity at 960 and 969 cm^{-1}. This was given as evidence that there is a direct interaction of Schiff base nitrogen with a C—C—H bending vibration. In view of the fact that we do not observe this shift, it is necessary to postulate that a nitrogen other than those of the lysine side chains is somehow affecting the 960-cm^{-1} vibration. This could occur if a protein group is directly participating in the 960-cm^{-1} vibration and appears in the Raman spectrum because of resonant enhancement. Stockberger *et al.* (12), for example, have speculated that the 1583- and 1602-cm^{-1} bands of bR_{570} may have such a nonchromophoric origin.

In conclusion, we have demonstrated that the origin of the ^{15}N isotope effects on the C=N⊕H vibration in bR_{570} can be accounted for by a single substitution at the Schiff base linkage between retinal and lysine. However, this does not exclude the possibility that a second lysine or some other group interacts with this region of the chromophore. Comparison of our results with those of Marcus and Lewis (13) on fully ^{15}N-labeled bacteriorhodopsin raises the possibility of the existence of a direct interaction and possible participation of another nitrogen-containing protein group with the chromophore vibrations. However, further measurements should be made on fully ^{15}N-labeled bacteriorhodopsin in a pure bR_{570} state in order to confirm this conclusion. The utilization of specific isotopic labeling of protein groups in conjunction with resonance Raman spectroscopy appears to be a promising approach for future studies. For example, the claim that nonexchangeable hydrogens of the protein (other than those from the lysine side chain covalently attached to the Schiff base) interact with the Schiff base (6) can be verified using this approach.

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