Participation of bacteriorhodopsin active-site lysine backbone in vibrations associated with retinal photochemistry

Y. Gat*, M. Grossjean*, I. Pinevsky†, T. Rothman‡, H. Sigrist‡, A. Lewis†‡§, and M. Sheves*

*Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel; †Department of Applied Physics, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; ‡Department of Applied Physics, Cornell University, Ithaca, NY 14853; †The Hadassah Laser Center, Department of Ophthalmology, Hadassah, Hebrew University Hospital, Ein Kerem, Israel; and §Institute of Biochemistry, University of Berne, Friedrichstrasse 3, CH-3012 Berne, Switzerland

Communicated by G. Stork, October 28, 1991

ABSTRACT Bacteriorhodopsin (bR) has been biosynthetically prepared with lysine deuterated at its α carbon (Ca—H). The labeled membranes containing bR were investigated by difference Fourier transform infrared (FTIR) spectroscopy. It has been derived from K/bR and M/bR difference spectra (K and M are photocycle intermediates) that several bands previously assigned to the retinal chromophore are coupled to the Ca—H. The vibrational modes that exhibit this coupling are principally associated with C15—H and N—H vibrations. [Ca—H]Lysine-labeled bR was fragmented enzymatically, and bR structures were regenerated with the Ca—H label either on lysine-216 and -172 or on the remaining five lysine residues of the protein. FTIR studies of the regenerated bR system, together with methylation of all lysines except the active-site lysine, reveal that the changes observed due to backbone labeling arise from the active-site lysine. The intensity of the C15—H out-of-plane wag is interpreted as a possible indication of a twist around the C15—N bond.

Bacteriorhodopsin (bR), a 26-kDa pigment, acts as a light-driven proton pump in the cell membrane of Halobacterium halobium (1). bR is composed of a chromophore (retinal) covalently linked to an amino acid polypeptide chain through the terminal group of a lysine residue. The configuration of the chromophore of bR588 (light-adapted, proton-pumping active form) is an all-trans-retinal, and the linkage is through a protonated Schiff base (RSBH+) (2) (Fig. 1). Time-resolved absorption spectra on the light-adapted form reveals a photolytic that is kinetically coupled to proton transport (3, 4). In this photocycle, spectrally distinct intermediates appear, which are designated J625, K630, L650, M412, N420, and O440. Resonance Raman (RR) and Fourier transform infrared (FTIR) spectroscopy have been shown to be powerful tools for obtaining structural information on the light-induced structural alterations of the active-site retinal and various amino acids (5, 6). In all of these investigations, the possibility of structural alterations in the active-site lysine has not been addressed. McMaster and Lewis (7) have focused on the problem of lysine, and they showed that numerous bands in the previously investigated FTIR difference spectra associated with light absorption by the chromophore involved lysine. In this paper, we extend these measurements to include contributions to the FTIR spectra of the backbone of lysine. We prove that these contributions arise from the lysine that is complexed to the retinal chromophore, and we show that active-site lysine backbone vibrations are strongly coupled to modes that have been assigned previously to the retinal. Furthermore, we observe that the nature of the coupling to the backbone is altered in going from bR to K.

MATERIALS AND METHODS

Lysine deuterated at the α carbon (Ca—H) was synthesized in a similar fashion to the method of Johns and Whelan (8). Incorporation into bR was carried out by growing halobacteria on a defined medium in which lysine was replaced by d1,3-[2-2H]lysine (9). To investigate the isotopic lysine labeling after incorporation into bR, the labeled membranes fragments were hydrolyzed (HCl), and the amino acids were protected as the n-butyl ester and trifluoroacetate. The resulting mixture was analyzed by a gas chromatography/mass spectrometry system (GC/MS Finnigan model 4500). We have observed that the molecular peaks of N/C-protected lysine and the main fragment, which includes the backbone moiety, are shifted by one mass unit, indicating the presence of deuterium in the backbone. The analysis indicated ca. 95% incorporation of deuterated lysine into the bR.

FTIR measurements were performed on an IBM IR98 spectrometer. The details of the methods used, such as signal averaging, etc., have been described (7).

Regeneration of hybrid bR was carried out as described below. bR fragments were produced from native and lysine-labeled bR with Staphylococcus aureus protease V8 purchased from Miles Laboratories. The protease V8 fragmentation and the characterization of the proteolytic fragments have been described (10). [Ca—H]Lysine-labeled fragments were combined with unlabeled complementary fragments (67 nmol each) and adjusted to a final fragment concentration of 16 μM by addition of 0.2% SDS in 10 mM sodium phosphate buffer (pH 6.0) (final volume, 4.2 ml). Fragments were mixed with 16 μl of DMPC (dimyristoyl phosphatidylcholine)/CHAPS 3-[3-cholamidopropyl]dimethylammonium]-1-propanesulfonate [8% (wt/vol) DMPC/3% (wt/vol) CHAPS] in 100 mM sodium phosphate buffer (pH 6.0) and all-trans-retinal (80 nmol in 30 μl of ethanol) and were

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incubated for 18 hr at 23°C. The lipid/protein/detergent mixture was completed by addition of purple membrane lipids (1.12 mg) and taurocholate (1.52 mg in 10 mM sodium phosphate buffer (pH 7)). Excess SDS was removed as described by Popot et al. (11) by dropwise addition of 180 µl of 4 M KCl. Precipitated potassium dodecyl sulfate was separated by centrifugation at 1500 × g for 4 min at ambient temperature. Samples were then dialyzed against five changes (1 liter each) of 150 mM KCl/30 mM potassium phosphate buffer, pH 6.0. Upon removal of buffer salts by dialysis for 60 min in H2O (two changes, 1 liter each), the same procedure described yielded 1.2 mg of regenerated bR protein.

Methylation of all six nonactive-site lysines was carried out as described by Longstaff and Rando (12), and the [C15–2H]lysine-labeled bR568 was synthesized by described methods (13).

RESULTS AND DISCUSSION

M/bR. In Fig. 2, bR568 → M412 difference spectra at 250 K of native and [Ca–2H]lysine-labeled pigments are shown. In these spectra, positive peaks originate from M412 and negative peaks are due to bR568. In FTIR spectra, it is generally observed that M412, which is an unprotonated Schiff base, has a much weaker absorption than bR and other protonated Schiff base intermediates. This weak intensity in the M/bR difference spectra provides a means of focusing on the bR alterations, since the bR peaks appear relatively unaffected by the M difference spectra absorption.

Fig. 2 shows that there are two spectral regions significantly different in the [Ca–2H]lysine-labeled sample when compared with native bR. One region that is not discussed in detail in this paper is the spectral alterations induced by the replacement of H by D occurring between 1030 cm⁻¹ and 1150 cm⁻¹. Both normal-mode calculations and vibrational studies on related molecules (14–16), the FTIR study on bR labeled with deuterated lysine (7), and recent observations of RR spectra of chromophores with a [Ca-2H]lysine labeled chain (17) suggest that bands in this wave-number range mainly stem from stretching vibrations of lysine's carbon skeleton. Thus, changes of band positions and relative intensities of lines in this region can be interpreted in terms of two mechanisms: (i) mutual couplings between lysine's C–C stretching modes, which are changed by replacing the hydrogen at the Ca position by a deuterium; and (ii) couplings between lysine's stretching modes and Ca–H modes. A detailed investigation of the relative weight and role of these two mechanisms requires characterization of the C–C stretching nature of all bands in this frequency range as a result of [13C labeling.

A second region of alterations, on which this paper focuses, are due to replacement of lysine Ca—H by Ca—2H occurring between 1300 and 1400 cm⁻¹ (Fig. 3). A striking effect is seen in this region on the prominent band at 1348 cm⁻¹. This band loses intensity, and a new peak appears at 1339 cm⁻¹ in the 2Ha spectrum (Fig. 3, spectrum B). The band at 1348 cm⁻¹ in the native membrane shifts to 1346 cm⁻¹ upon resuspension in 2H2O, and the latter shifts to 1338 cm⁻¹ upon 2Hz labeling (Fig. 3, spectrum D). The RR bR 1348 cm⁻¹ band observed with the native chromophore has been assigned to both N—H and C15—H rocks, and the 1346 cm⁻¹ band in the 2H2O spectra (see Fig. 3, spectrum C) is assigned to the 135 cm⁻¹ band in the FTIR spectra (C, 15, 16, 19).

It is necessary to establish the origin of the 1348 cm⁻¹ mode in the FTIR data before the alterations in this mode with deuteration at Ca—H can be interpreted. With bR enriched with 15N-lysine, it was observed that the 1348 cm⁻¹ bR band in the FTIR spectra shifts to 1346 cm⁻¹ (data not shown). This is similar to what is observed by RR spectroscopy (18) in which such a shift in vibrational frequency has been associated with an N—H rock. Subsequent resumption of this 15N-enriched bR in 2H2O shifts the 1346 cm⁻¹ band to 1343 cm⁻¹. Once again similar shifts occur in the RR experiments. To probe for C15—H contributions to the 1348 cm⁻¹ vibrational mode, bR was prepared with C15—2H labeling in the retinal. In this enriched sample, a remaining absorption at 1348 cm⁻¹ continued to be detected.

Fig. 2. FTIR difference spectra for the photoreaction bR568 → M412 at 250 K. Negative and positive peaks are due to bR568 and M412, respectively. Spectra: A, native bR; B, [Ca–2H]lysine-labeled bR.

Fig. 3. Comparison of the 1300- to 1400-cm⁻¹ region of the FTIR difference spectra of bR568 → M412 at 250 K. Spectra: A, native bR in H2O; B, [Ca–2H]lysine-labeled bR in H2O; C, native bR in 2H2O; D, [Ca–2H]lysine-labeled bR in 2H2O.
These results, which are also similar to the RR data (18, 19), show that the bands in the RR and FTIR spectra arise from the same chromophore vibrational modes. Similar results on bR enriched with deuterium at retinyl C15 have also recently been described by Maeda et al. (20).

With the above identification of the 1348-cm\(^{-1}\) (FTIR) vibrational mode, we can interpret the results of the \(\text{[Ca}^{2}\text{H]lysine labeling seen in Fig. 3. In Fig. 3, spectra A and B, the FTIR difference spectra of native and \([\text{Ca}^{2}\text{H}]\)lysine-labeled bR are compared. These spectra show that \(\text{Ca}^{2}\text{H}\) labeling produces a split in the 1348-cm\(^{-1}\) band. One of the two bands arising from this splitting occurs at 1349 cm\(^{-1}\) and is due to the N-H rock, since resuspension of this labeled membrane in \(^2\text{H}_2\text{O}\) shifts this band (observed in Fig. 3, spectrum B) out of the addressed region, leaving the second component of the split band at 1339 cm\(^{-1}\) unaffected (see Fig. 3, spectrum D). Thus, it is concluded that the 1348 cm\(^{-1}\) band, which has a contribution from the C15-H rocking vibration of bR\(_{568}\), is strongly affected by \(\text{Ca}^{2}\text{H}\) labeling and is shifted by this substitution of \(^2\text{H}\) for H from 1348 cm\(^{-1}\) to 1339 cm\(^{-1}\). The observation is indicative for a coupling of C15-H and Ca-H modes in the native system. In contrast, the N-H rock is weakly affected by the \(^2\text{H}\) labeling—i.e., only weakly coupled to a Ca-H mode.
Other bands have also been observed with N—H and C15—H character. One such band is the peak in br at 1253 cm⁻¹. This band is also affected by the backbone. Further characterization will have to await additional isotopic substitution data.

K/bR. The K/bR difference spectra for native br and Ca—²H-enriched br is seen in Fig. 4. It is apparent from these spectra (see boxed regions) that certain bands disappear as a result of this labeling, while other bands are altered in their structure and position. With regard to the retinal lysine coupling, the two spectral regions between 1300 and 1400 cm⁻¹ and between 900 and 1000 cm⁻¹ are of interest.

The 1348 cm⁻¹ band, which is associated with the K intermediate, moves 5 cm⁻¹ to 1343 cm⁻¹ in the deuterated sample (Fig. 5, spectra A and B). [¹⁵N]Lysine br FTIR data we have obtained (data not shown) support the RR assignment of this band to the N—H rock vibration.

Resuspension of the membranes in ²H₂O significantly reduces the intensity of the 1348 cm⁻¹ band (e.g., compare the relative intensities of the 1348 cm⁻¹ and 1326 cm⁻¹ bands in Fig. 5, spectra A and C). Two bands are observed after the N—H contribution is removed from this spectral region by suspending the membranes in ²H₂O. One of these bands is at 1349 cm⁻¹, and this is unaffected by backbone labeling, while another band, observed at 1355 cm⁻¹, shifts to 1343 cm⁻¹ upon Ca—H labeling. Assignment of these two bands needs further investigation. Nonetheless, the observation that the K N—H rocking vibration at 1348 cm⁻¹ shifts with the backbone labeling indicates that in K intermediate there is a coupling between the N—H rock and the Ca—H rock. This is in contrast to the brøw state in which the Ca—²H coupling is to the C15—H rock and not to the N—H rock. Thus, the photochemical transformation to K intermediate alters the lysine—retinal coupling.

In the 800- to 1000 cm⁻¹ region (see Fig. 6), an intense band is observed at 957 cm⁻¹, which has been assigned by RR spectroscopy to a hydrogen out-of-plane wag of the retinal C15—H. In the FTIR spectra of the native membrane in H₂O (see Fig. 6, spectrum A), an intense band is also seen at this frequency. Maeda et al. (20) confirm the similar origin of the band in RR and FTIR spectra, since C15—²H enrichment causes a dramatic intensity loss at 957 cm⁻¹. Our data indicate that resuspension of the native membrane in ²H₂O (Fig. 6, spectrum B) shifts the frequency of this mode from 957 to 952 cm⁻¹, indicating a possible coupling of the C15—H wag and N—H wag vibrations. Furthermore, Ca—²H labeling of lysine (Fig. 6, spectrum C) shifts the 957 cm⁻¹ band by 4 cm⁻¹ to 953 cm⁻¹. This is most interesting in view of the fact that full deuteration of the lysine without Ca deuteration has no effect on the frequency of this mode (7). Thus, the band at 957 cm⁻¹, which has been assigned to retinal by previous studies, is also coupled to the Ca of the lysine residue.

An interesting feature of this band is its large intensity in both RR and FTIR spectra. To appreciate similarities in the intensity of this mode in the respective spectroscopic analyses, the basis of band intensities in infrared and Raman spectra will be considered. In the RR spectra, the basis for the intensity of the 957 cm⁻¹ mode is the large Franck–Condon factors associated with this mode in K. In other words, the associated motions undergo large excursions when going from the ground state to the vertically excited state, and these large excursions are the basis for the intensity of the 957 cm⁻¹ vibration. Unlike RR, FTIR spectroscopy provides information solely on the ground state. The infrared spectrum is
intense, assuming no symmetry restrictions, when a large dipole is present in the ground state. The intense band probably results from an accumulation of charge at C15 produced by the photochemical transformation. Accumulation of charge may be explained by one of two possible mechanisms. Charge accumulation in K at C15 could occur as a result of delocalization of the positive charge at the Schiff-base linkage. Alternatively, a charge could be localized on C15 if there were an out-of-plane twist of the C=NN bond. In terms of the delocalization mechanism, $^{13}$C nuclear magnetic resonance data obtained on the protonated Schiff base of all-trans-retinal as a function of the counter-ion–Schiff base interaction indicate a reduction rather than an increase in charge density on the C15 as a result of increased $\pi$ electron delocalization (21). In addition, the absorption of bR shifts to 605 nm when the membrane is suspended at pH 2, and this red shift is similar to the absorption shift observed in K. Thus, in bR$_{K}$, the positive charge of the Schiff base should be delocalized into the retinylidene chromophore more than in bR$_{R}$, but no intense 957-cm$^{-1}$ mode appears (refs. 22 and 23; F. Siebert, personal communication). Therefore, it appears that the delocalization mechanism is not operative, and the possibility remains that a C=NN out-of-plane twist in K causes a large dipole associated with the C15–H out-of-plane motion. It is interesting to note that such a C=NN twist could also explain the large Franck-Condon factor, which gives rise to an intense hydrogen out-of-plane vibration in the RR spectrum of K. Other suggestions in the literature which we cannot exclude for explaining intense hydrogen out-of-plane vibrations in the RR (24) and FTIR (25) include the possibility that single-bond twists may also produce such vibrations.

Assigning the Responsible Lysine. To pinpoint the lysine residue responsible for the changes described in this paper, two experimental lines have been followed. First, the procedure of Longstaff and Rando (12) was used to methylate all six lysine residues except lysine-216. The FTIR spectrum of this sample was identical to that of the native membrane. This experiment certainly suggested that the changes described above arose from the active-site lysine. To further ascertain this conclusion, the protein was proteolytically cleaved into two fragments (10). One fragment includes lysine-172 and lysine-216, while the other fragment includes the remaining five lysine residues. Protease V8 cleavage procedure was carried out with the native and purple membrane that contained [Ca$^{2+}$-H]lysine. From the fragments produced by this procedure, two hybrid structures were regenerated. One sample contained unlabeled lysine-172 and -216, while the remaining lysines were Ca-deuterated. We call this sample D-V$_1$/V$_2$. The second hybrid contained labeled lysine-172 and -216, and the other five lysine residues were unlabeled (sample V$_1$/D-V$_2$). Finally, a third sample was prepared in which the native fragments were utilized to regenerate a refolded bR structure (sample V$_1$/V$_2$). FTIR experiments were performed with each of these samples. In Fig. 7, the M/bR difference spectrum in the region of the 1348-cm$^{-1}$ band shows the characteristic splitting due to Ca$^{2+}$-H bonding. In addition, the 1253-cm$^{-1}$ band is missing only in the V$_1$/D-V$_2$ sample. Fig. 8 Left reports on the same region for the K/bR difference spectrum. Again only the middle spectrum (B) shows the 1348-cm$^{-1}$ band lowered in frequency. Finally, in Fig. 8 Right the K/bR difference spectra of these samples between 900 and 1050 cm$^{-1}$ are documented. The downshift of the 957-cm$^{-1}$ K band and the disappearance of the 941-cm$^{-1}$ K band obviously occur in the middle spectrum (B) only. Therefore, in view of the results on both methylelated purple membrane and regenerated bR, we feel confident that the changes discussed in this paper are caused by the deuteration of the Ca$^{2+}$ of lysine-216.

In summary, this paper has shown a coupling between the lysine Ca$^{2+}$-H and retinal vibrational modes with C15–H and N–H character. The light-induced transition from bR $\rightarrow$ K causes a change in this coupling with the C15–H rock affected by the lysine Ca$^{2+}$-H in bR and the N–H rock affected only by the lysine Ca$^{2+}$-H in K. In addition, the effect of lysine Ca$^{2+}$-H on the C15–H hydrogen out-of-plane wag in K is interesting in view of the fact that deuteration of all lysine hydrogens except Ca$^{2+}$-H had no effect on this vibration. Furthermore, we believe that the C15–H and N–H are mutually coupled. The presence of an intense hydrogen out-of-plane wag associated with C15–H may indicate an out-of-plane twist at the C=NN bond.

We thank Prof. A. Maeda for providing us with his manuscript prior to publication. M.G. was supported by the Minerva Foundation. This work was supported by the Fund for Basic Research (administered by the Israel Academy of Sciences and Humanities), the U.S.-Israel Binational Science Foundation, the U.S. Naval Air Warfare Center, the Rockefeller–Weizmann Collaboration Trust Fund, and the Swiss National Science Foundation.