Structure of the Retinal Chromophore in the hR_L Intermediate of Halorhodopsin from Resonance Raman Spectroscopy†

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ABSTRACT: Time-resolved resonance Raman spectra of the hR_L intermediate of halorhodopsin have been obtained. The structurally sensitive fingerprint region of the hR_L spectrum is very similar to that of bacteriorhodopsin's L_{550} intermediate, which is known to have a 13-cis configuration. This indicates that hR_L contains a 13-cis chromophore and that an all-trans → 13-cis isomerization occurs in the halorhodopsin photocycle. hR_L exhibits a Schiff base stretching mode at 1644 cm^{-1}, which shifts to 1620 cm^{-1} in D_2O. This demonstrates that the Schiff base linkage to the protein is protonated. The insensitivity of the C–C stretching mode frequencies to N-deuteriation suggests that the Schiff base configuration is anti. The 24 cm^{-1} shift of the Schiff base mode in D_2O indicates that the Schiff base proton in hR_L has a stronger hydrogen-bonding interaction with the protein than does hR_{578}.

Halorhodopsin is one of several photoreactive retinal-containing membrane proteins found in the halophilic bacterium *Halobacterium halobium*. These light-harvesting proteins, bacteriorhodopsin (bR),[1] halorhodopsin (hR), and two sensory rhodopsins, undergo cyclic photoreactions effecting transmembrane proton translocation, chloride transport, and phototaxis, respectively (Stoeckenius & Bogomolni, 1982; Lanyi, 1986; Bogomolni & Spudich, 1982; Takahashi et al., 1985; Wolff et al., 1986; Scherrer et al., 1987).

The photocycle of hR has been studied by using flash absorption techniques, and complex salt-dependent pathways have been described (Weber & Bogomolni, 1981; Tsuda et al., 1982; Schobert et al., 1983, 1986; Steiner et al., 1984; Hazemoto et al., 1984; Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986). Similar to the well-characterized photocycle of bR, the halide-dependent photocycle of hR_{578} begins with a red-shifted intermediate (hR_{610}) which decays into a green-absorbing intermediate which we will denote as hR_L. After this point, the photocycles differ considerably, as no M-like intermediate is observed in the hR chloride transport photocycle. Additional intermediates in the latter part of the hR photocycle have been proposed and may include a red-absorbing hR_{640} and a blue-shifted hR_{565} (Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986).

A central problem is to determine the structural changes in the retinal chromophore and the surrounding protein that drive chloride pumping in halorhodopsin. Recently, the primary structure of halorhodopsin has been determined (Blanck & Oesterhelt, 1987). Chromophore extraction studies and absorption difference spectra have suggested that an all-trans to 13-cis isomerization occurs during the photocycle of hR (Ogurusu et al., 1981; Lanyi, 1984). However, direct evidence on the in situ structure of the retinal chromophore in the photointermediates of hR has been lacking.

Resonance Raman spectroscopy is a very effective technique for studying chromophore structure in retinal pigments. The vibrational modes are sensitive to changes in molecular geometry, and the relationship between the normal mode pattern and chromophore structure has been elucidated (Smith et al., 1985, 1987a,b). Previously reported resonance Raman data have shown that the structure of the chromophore in hR_{578} is very similar to the all-trans chromophore in bR_{568} (Smith et al., 1984a; Maeda et al., 1985; Alshuth et al., 1985). In this paper, we present resonance Raman spectra of the long-lived hR_L intermediate of halorhodopsin. The spectrum of hR_L is strikingly similar to that of bacteriorhodopsin's 13-cis L_{550} intermediate, suggesting that they have similar structures. This argues that an all-trans → 13-cis isomerization has occurred during the phototransformation of hR_{578} to hR_L.

Experimental Procedures

Halorhodopsin was isolated according to previously published procedures (Taylor et al., 1983). Briefly, isolated membranes from *H. halobium* strain JW-12 (containing hR but not bR) were solubilized in octyl glucoside and subjected to hydroxylapatite chromatography (Bio-Rad DNA grade) followed by chromatography over octyl-Sepharose C1-4B (Pharmacia). The pooled fractions were extensively dialyzed (48 h) to remove octyl glucoside. Samples with $A_{280}/A_{578} \leq 2.2$ were used for Raman spectroscopy.

The hR samples were contained in a closed-loop recirculating flow system consisting of silicon tubing attached to a 1.5-mm-diameter capillary. The samples were pumped with a Cole–Parmer Model 7553-20 peristaltic pump, yielding flow rates from 40 to 500 cm/s. The optical density was kept at approximately 1.5 ODU/cm, and typical sample volumes were 1–10 mL. The temperature was maintained between 15 and 20 °C by using a stream of cold nitrogen directed on the sample reservoir.

The hR_L and L_{550} data were obtained by using a Kr^+ pump laser at 568.2 nm to initiate the photocycle and an Ar^+ probe beam at 514.5 or 501.7 nm which was spatially displaced downstream from the pump. The amount of photointermediate produced depends upon the photoalteration parameter, $F = (3.824 \times 10^{-21})P\epsilon\phi/vd$ (Mathies et al., 1976), where P is the laser power (photons/s), ϵ is the extinction coefficient (M^{-1} cm^{-1}), ϕ is the quantum yield for the photochemical reaction,

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bR, bacteriorhodopsin; hR, halorhodopsin.

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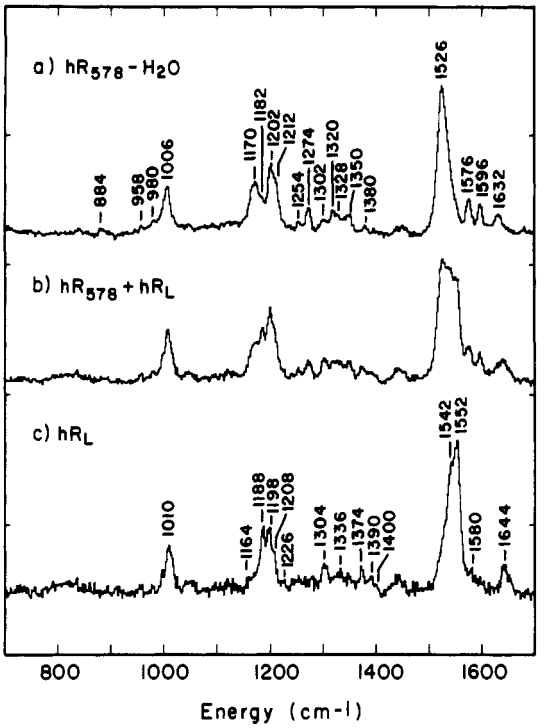

FIGURE 1: Resonance Raman spectra of halorhodopsin in 3 M NaCl/50 mM HEPES, pH 7.3. (a) hR_{578} spectrum excited at 514.5 nm; (b) pump–probe spectrum of hR_{578} + hR_L (568-nm pump and 514.5-nm probe); (c) pure hR_L spectrum obtained by subtracting (a) from (b).

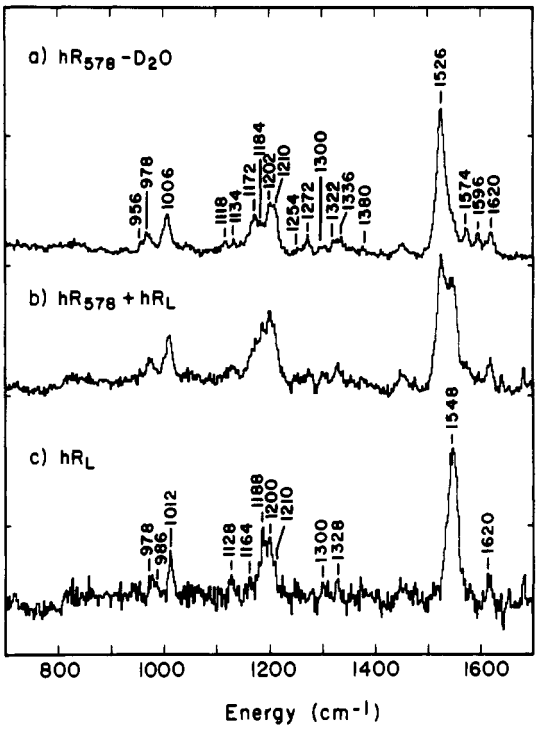

FIGURE 2: Resonance Raman spectra of deuteriated halorhodopsin (3 M NaCl/50 mM HEPES, pD 7.3). (a) hR_{578}; (b) pump–probe spectrum of hR_{578} + hR_L; (c) pure hR_L spectrum resulting from subtraction of (a) from (b).

v is the flow velocity (cm/s), and d is the focused beam diameter (cm). To obtain hR_L spectra, an ~15 mW 568.2-nm pump beam was cylindrically focused on the 0.15-cm-diameter capillary, giving a photoalteration parameter of ~1.0 (flow rate 40 cm/s). The low photoalteration probe beam (<2 mW at 514.5 nm) was also cylindrically focused to give $F \leq 0.1$. The greatest concentration of hR_L was produced with a pump–probe spatial separation of ~0.18 mm, corresponding to a time delay of ~450 μs. For L_{550} spectra, the flow rate was increased to ~400 cm/s, and a time delay of ~25 μs was used. The cylindrically focused pump (80 mW, 568 nm) and probe (5 mW, 514.5 nm) beams had photoalteration values of ~0.8 and <0.1, respectively. The possible presence of unwanted photolysis products of hR_L was tested by lowering the pump or probe powers. No significant changes in the ethylenic band frequencies or relative intensities were observed. The absence of changes in the absorption spectrum even after prolonged irradiation showed that no significant sample degradation had occurred. Spectra of unphotolyzed hR_{578} and bR_{568} were obtained with the same apparatus by blocking the pump beam.

Raman data were acquired with a Spex 1401 double monochromator with photon counting detection interfaced to a PDP 11/23 computer. The monochromator step size was 2 cm^{-1} with a 2-s dwell time, and the spectral band-pass was 5 cm^{-1}. A typical spectrum is an average of 5–15 scans with no smoothing. Band deconvolution of the ethylenic frequencies was used to determine the relative concentration of hR_L at various time delays. The deconvolution gave best fits when three ethylenic bands were used at 1526, 1542, and 1552 cm^{-1} with band widths constrained to 13.5 cm^{-1}.

RESULTS

Resonance Raman spectra of halorhodopsin in H_2O are presented in Figure 1. Under low photoalteration conditions, the spectrum of all-trans hR_{578} is obtained (Figure 1a) as has been shown previously (Smith et al., 1984a; Maeda et al., 1985; Alshuth et al., 1985). Upon addition of a pump beam, a composite spectrum of hR_{578} and hR_L is produced as illustrated in Figure 1b. The 450-μs time delay chosen for these spectra gave the largest amplitude of the hR_L ethylenic stretches. Subtraction of spectrum a from spectrum b yields the pure hR_L spectrum in Figure 1c. The subtraction parameter was determined by examining the intensities in the 1500–1526 cm^{-1} ethylenic region. A correctly subtracted spectrum was obtained when no negative or positive peaks appeared in this region as would result from over- or undersubtraction of the hR_{578} ethylenic line at 1526 cm^{-1}. The corresponding data for hR_L in D_2O are given in Figure 2.

To ensure that we are observing spectra of hR_L, experiments were performed with variable time delays between the pump and probe lasers ranging from 0.45 to 40 ms. The relative intensity of the sum of the 1542 and 1552 cm^{-1} integrated peak areas was plotted vs time (Figure 3). These data were best fit to biexponential decay kinetics and gave $t_{1/2}$ values of ~0.4 and ~21 ms. These kinetic values are in good agreement with decay constants of 0.45 and 18 ms obtained from transient absorption studies on identical preparations (Xie et al., 1986).

The hR_L spectrum is compared with that of bacteriorhodopsin's L_{550} intermediate in Figure 4. The most intense feature in the hR_L spectrum is the ethylenic doublet at 1542/1552 cm^{-1}, which is similar to the ethylenic doublet of L_{550} (Figure 4b). The ethylenic doublet may originate either from the presence of two similar L species or from a pair of intense C=C stretching modes in a single L form (Alshuth & Stockburger, 1986: Argade & Rothschild, 1983). The signal-to-noise ratio in our data was insufficient to determine whether the two ethylenic components in hR_L decay independently or in parallel.

A linear correlation exists between the absorption maximum of retinal compounds and the ethylenic stretching frequencies (Rimai et al., 1973; Aton et al., 1977). For hR_L, the 1552 and 1542 cm^{-1} ethylenic bands correlate with an absorption

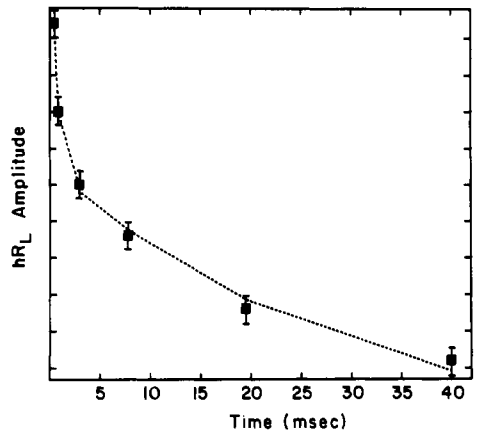

FIGURE 3: Plot of hR_L concentration vs time. The concentration of hR_L was determined from deconvoluted ethylenic band areas as described in the text. The data were fit to a biexponential decay expression yielding $t_{1/2}$ values of ~0.4 and ~21 ms and respective amplitudes of 4.6 and 3.7.

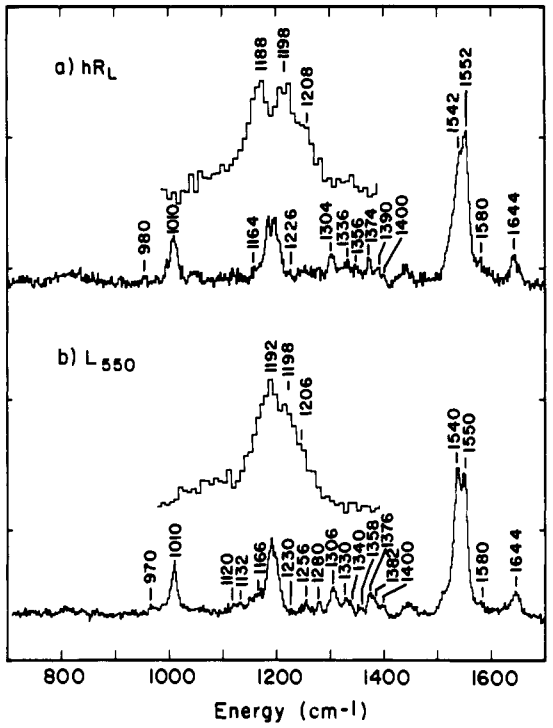

FIGURE 4: Comparison of the resonance Raman spectrum of hR_L (a) with that of bacteriorhodopsin's L_{550} intermediate (b).

maximum of ~495 and ~540 nm, respectively, agreeing satisfactorily with published reports of absorption maxima for hR_L ranging between 500 and 520 nm. This result, in conjunction with the Raman decay experiments of Figure 3, argues that we have obtained spectra of the same hR_L species seen in previous transient absorption experiments.[2]

The Schiff base stretching mode in hR_L (1644 cm^{-1}) has the same frequency as the Schiff base mode in L_{550}. A characteristic downshift of the C=N stretch in D_2O is diagnostic for a protonated Schiff base. The 1644 cm^{-1} C=N stretch in hR_L (Figure 1c) shifts to 1620 cm^{-1} in D_2O (Figure 2c), providing strong evidence for a protonated Schiff base linkage to the protein in hR_L. This shift is identical with the 24 cm^{-1} deuterium shift observed in the Schiff base mode of L_{550} (Alshuth & Stockburger, 1986).

In retinals and retinal-containing proteins, the C–C skeletal stretching modes in the 1100–1300 cm^{-1} fingerprint region provide sensitive markers for chromophore geometry. Characteristic frequency differences are observed between *all-trans*- and 13-*cis*-retinal (Curry et al., 1982, 1984), between their protonated Schiff base derivatives in vitro (Smith et al., 1985, 1987b), and between bR_{568} and bR_{548} (Smith et al., 1987a,b). In L_{550}, the C_{14}–C_{15} stretching mode has been assigned at 1155 cm^{-1} in the infrared (Gerwert & Siebert, 1986) and at 1172 cm^{-1} in the Raman spectrum (Smith et al., 1986). In the present work, a broad, weak band is observed with a maximum at ~1166 cm^{-1}, which we attribute to the C_{14}–C_{15} mode following the earlier assignments. The 1192 cm^{-1} mode is assigned as the "C_{10}–C_{11} stretch", and the shoulder at 1198 cm^{-1} is the C_8–C_9 mode (Smith, 1985). Compared to all-trans bR_{568}, the characteristic feature of the L_{550} spectrum is the reduced frequency of the C_{14}–C_{15} mode (1201 cm^{-1} → ~1166 cm^{-1}) which pushes the C_{10}–C_{11} stretch up from ~1170 cm^{-1} in bR_{568} to 1192 cm^{-1} in L_{550} (Smith et al., 1985). The very close correspondence between the ~1166, 1192, and 1198 cm^{-1} modes in L_{550} with the 1164, 1188, and 1198 cm^{-1} modes in hR_L suggests that similar assignments apply and that hR_L is also 13-cis.

The configuration about the C=N bond can be determined from the deuterium sensitivity of the C_{14}–C_{15} stretch in protonated Schiff bases (Smith et al., 1984b). In C=N syn compounds, C_{14}–C_{15} stretch and N–H rock are strongly coupled so that deuteriation results in a 40–60 cm^{-1} upshift of the C_{14}–C_{15} stretch. In C=N anti compounds, there is little coupling of the C_{14}–C_{15} stretch with the N–H rock, and therefore, deuteriation has no effect on the C_{14}–C_{15} stretch frequency. The lack of a significant shift in any of the 1100–1400 cm^{-1} fingerprint modes including the 1164 cm^{-1} band upon deuteriation suggests that hR_L has an anti C=N configuration.

Discussion

The remarkable similarity between the hR_L and L_{550} resonance Raman spectra (Figure 4) argues strongly that the chromophore in hR_L is 13-cis and that the chromophores in both species experience nearly identical protein perturbations. The C–C stretching modes in hR_L have been assigned by comparison with previous assignments for L_{550} and bR_{548} (Smith, 1985; Smith et al., 1987b). The key diagnostics for 13-trans to 13-cis isomerization are the upshift of the intense C_{10}–C_{11} stretching mode (from ~1170 to ~1190 cm^{-1}) and the drop of the C_{14}–C_{15} stretch due to a decrease in C_{14}–C_{15} stretch–skeletal bend interaction in the 13-cis geometry (Curry et al., 1984; Smith et al., 1985, 1987b). This is precisely the pattern observed for the C–C modes in hR_L. The frequency of the C_{14}–C_{15} stretch can also be used to examine the conformation about this bond. Previous calculations have shown that the C_{14}–C_{15} stretch in s-cis conformers experiences a dramatic (~70 cm^{-1}) downshift as compared to the C_{14}–C_{15} s-trans isomer. The ~1164 cm^{-1} value for the C_{14}–C_{15} stretch in hR_L is consistent with a C_{14}–C_{15} s-trans molecule. This result does not support a recently proposed model for chloride transport in halorhodopsin invoking a 14-s-cis structure (Oesterhelt et al., 1986).

In light of the correspondences between the hR_L and L_{550} spectra, it is of interest to recall the similarities and differences between hR_{578} and bR_{568}. Both chromophores are all-trans-

[2] Several studies have suggested the existence of an $hR_{520} \rightleftharpoons hR_{640}$ equilibrium in the salt-dependent hR photocycle (Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986). No evidence for this equilibrium was found in the present study as judged by the lack of an ethylenic frequency corresponding to a 640-nm form. In addition, transient absorption work on our samples has shown no evidence for a red-shifted intermediate in the latter part of the hR photocycle (Xie et al., 1986).

protonated Schiff bases; however, the Schiff base stretching frequency in hR_{578} (1632 cm^{-1}) is considerably lower than the corresponding frequency in bR_{568} (1641 cm^{-1}) (Smith et. al., 1987a), and the resulting deuterium shift is less (12 cm^{-1} vs 18 cm^{-1}). This decrease in C=N stretch/N-H rock coupling has been attributed to a decreased hydrogen-bonding interaction between the Schiff base proton and its anion in hR_{578} (Smith et al., 1984a; Alshuth et al., 1985; Maeda et al., 1985). Also, there is an increase in the C=N stretching frequency of hR_{578} upon substitution of nitrate for chloride (Maeda et al., 1985; S.P.A. Fodor and R. A. Mathies, unpublished results). This suggests that the reduced hydrogen-bonding interaction in hR_{578} is caused by some chloride-dependent alteration of the Schiff base environment. Recent work on the mechanism of the opsin shift in bacteriorhodopsin has suggested that a large part of the opsin shift ($\sim$3500 cm^{-1}) is due to a decrease in the hydrogen-bonding interaction between the Schiff base proton and an anion in the protein environment (Lugtenburg et al., 1986; Spudich et al., 1986; Baasov & Sheves, 1986). The decreased deuterium effect on the Schiff base mode of hR_{578} suggests that the extra $\sim$300 cm^{-1} red shift for hR (as compared to bR) may be due to a further decrease in the hydrogen-bonding interaction. This provides strong evidence that the mechanism of the opsin shift in hR is also due primarily to changes in Schiff base hydrogen bonding.

When hR_{578} is converted to hR_L, the Schiff base mode shifts from 1632 to 1644 cm^{-1}, and we observe a large 24 cm^{-1} downshift of the C=N stretch upon deuteriation. Thus, the hydrogen-bonding environment has gone from a relatively weak one in hR_{578} to a stronger one in hR_L. Furthermore, the D_2O-induced shift in hR_L is the same as that observed in L_{550}, suggesting that the perturbation responsible for the decrease in hydrogen bonding in hR_{578} has been removed in hR_L. If the weak hydrogen bonding of the Schiff base in hR_{578} is due to some direct or indirect interaction with chloride, then the stronger hydrogen bond in hR_L suggests that this interaction has been lost.

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