Fourier Transform Raman Study of Retinal Isomeric Composition and Equilibration in Halorhodopsin

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Earlier results suggested that the description of the photoreaction of halorhodopsin depends on our being able to distinguish and dissect the photoproducts of the all-trans chromophore from those of the 13-cis,15-syn chromophore. We have used FT Raman spectroscopy, a nonperturbing method, to analyze the isomeric states of the retinal under various conditions in halorhodopsins from *Halobacterium salinarium* and *Natronobacterium pharaonis*. The results indicate that light adaptation occurs in the former protein, and the amounts of the two isomers in the light-adapted and dark-adapted forms are consistent with the weights given to them, from spectroscopic and mathematical criteria, in the earlier calculations. No light adaptation occurs in the latter protein, which contains mostly, but not entirely, *all-trans*-retinal. There is no light adaptation in either protein in the absence of chloride, the transported substrate of these anion pumps, and in spite of a 13-cis-like photocycle, they contain both *all-trans*- and 13-*cis*,15-*syn*-retinal under these conditions. These findings emphasize the need to consider the photoreactions of both isomers when studying halorhodopsin and provide a reliable method for determining the isomeric mixtures in this protein.

1. Introduction

Bacteriorhodopsin and halorhodopsin are retinal-containing light-driven transport systems for protons and chloride ions, respectively, in membranes of halobacteria.[1−5] The study of their photoreactions identified transient states and their interconversions on the femtosecond-to-millisecond time scale (the "photocycle"), which reveal the individual steps of the retinal and the protein and, thus, the ion-translocation process. Bacteriorhodopsin is a mixture of all-trans and 13-cis,15-syn chromophores ("dark-adapted" state) but becomes converted to 100% all-trans upon illumination, and this "light-adapted" state relaxes over a few hours to the original thermal mixture.[6−8] Under most conditions, only the photocycle of the all-trans species transports protons. Studies of the photochemical reactions of halorhodopsin have been complicated by the fact that, unlike in bacteriorhodopsin, the isomeric composition of its retinal is a mixture of all-trans and 13-cis,15-syn even after light adaptation.[9−12] We had suggested that in halorhodopsin from *Halobacterium salinarium*, the spectral changes observed contain contributions from the photoreaction of both isomeric species.[13] In particular, according to our analysis, which dissected time-resolved difference spectra from dark-adapted and light-adapted samples into mixtures of two photocycles, the red-shifted intermediate that arises on the millisecond time scale[14−16] and contains 13-*cis* retinal[17,18] is to be placed into the photocycle of the 13-cis state rather than the all-trans state. If this analysis is correct, the earlier derived photocycle schemes for the transport-active all-trans *H. salinarium* halorhodopsin, on which the chloride-transport mechanism was based, should be revised.

The validity of the spectral decomposition was supported by a similar analysis of the photoproducts of halorhodopsin from *Natronobacterium pharaonis*.[19,20] In this protein, the difference spectra that describe the photocycle were the same in dark-adapted and light-adapted samples and at high NaCl concentrations corresponded to those calculated for the all-trans cycle in *H. salinarium* halorhodopsin. Importantly, however, at low chloride concentrations, a red-shifted state appeared at the end of the *N. pharaonis* halorhodopsin photocycle also, but it was one that contained *all-trans*-retinal and whose decay rate constant was dependent on the chloride concentration. Its kinetics suggested a mechanistic model for chloride translocation.[20] This state had no counterpart in *H. salinarium* halorhodopsin, but we assumed that the reason for this is that the rates of its rise and decay are such that in this protein, it does not accumulate in observable amounts.

A crucial piece of evidence for these conclusions was the actual isomeric composition of the retinal in the two kinds of halorhodopsins under the various conditions in question. This information was provided by extraction of the retinal and its analysis by HPLC. The results[19] seemed to support the assumptions made: (a) the proportions of 13-*cis*- and *all-trans*-retinal agreed with the weights of the 13-cis and all-trans photocycles of *H. salinarium* halorhodopsin in the calculations, based on spectral and mathematical criteria, and (b) the *N. pharaonis* halorhodopsin did not dark-adapt (although the samples contained some 13-cis chromophore).

In view of the possibility of problems with determining the isomeric composition through extraction (preferential release of one of the isomeric forms, isomerization during the extraction, etc.) and the importance of the conclusions made for the halorhodopsin photocycle and chloride-transport mechanism, we have reexamined the isomeric states of the retinal with the nonperturbing method of FT Raman. Unlike retinal extraction which denatures the protein and resonance Raman which can lead to photostationary equilibria, the FT Raman method which utilizes near-infrared excitation[21] provides reliable information not only about the in situ configuration of the C13−C14 double bond but also the C15−N bond. FT Raman has been used in the study of the pressure dependence of dark adaptation in bacteriorhodopsin.[22]

2. Methods

H. salinarium with cloned genes either for its homologous halorhodopsin or for *N. pharaonis* halorhodopsin in a nonin-

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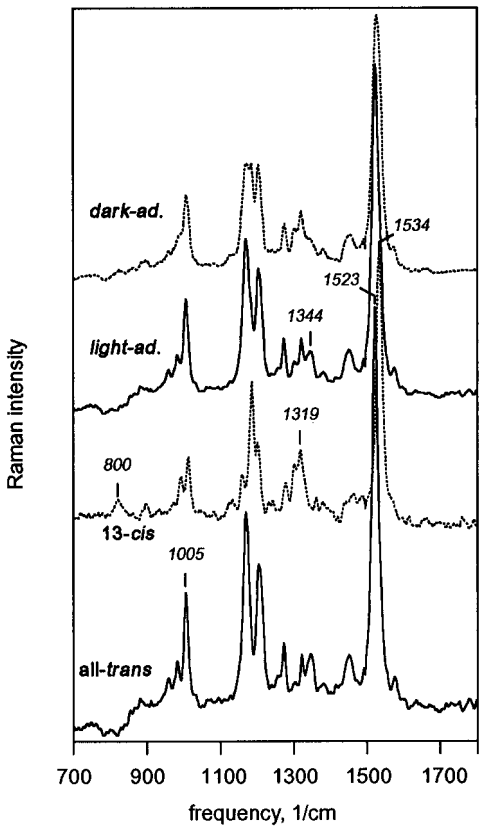

Figure 1. FT Raman spectra of dark-adapted and light-adapted *H. salinarium* halorhodopsin and the calculated spectra for the 13-cis and all-trans states.

tegrating vector that also contained a bacteriorhodopsin promoter was used as before.[13,19] Membranes highly enriched in the halorhodopsin were prepared as purple membranes[23] and encased in poly(acrylamide) gels at optical densities at 580 nm of 21 (*H. salinarium* samples) or 30 (*N. pharaonis* samples) in a quartz cuvet. The buffer contained either 2 M NaCl or 1 M Na_2SO_4, as indicated, and 50 mM MES at pH 6.0. In light adaptation, the illumination was with white light for 20 min blue light (390−490 nm) for 20 min, or red light (>610 nm) for 5 min, when indicated.

FT Raman spectra were measured in a Bruker IFS66/S-FRA106/S spectrometer at 22 °C. The spectral resolution was 4 cm^{-1}. The energy of the Nd-Yag excitation source was 300 mW, and for dark-adapted samples or samples that did not show light adaptation, 18−27 spectra were averaged. The dark adaptation process was measured by averaging 1200 scans within numerous sequential 20-min intervals, and the midpoints of 39 of these intervals were taken as the times of the resulting spectra. Singular value decomposition[24] (SVD) was used to recover two significant components from such data. The spectra and their weights were used to reconstruct the data with much reduced noise. The respective columns of the **v** matrix gave the kinetics of the changes that allowed extrapolation to zero time to calculate the spectrum for the light-adapted state.

3. Isomeric Composition of the Retinal in *H. salinarium* Halorhodopsin

FT Raman spectra indicated that illumination of a dark-adapted sample with white light causes changes that in bacteriorhodopsin would be characteristic of an increase in all-trans content at the expense of 13-cis. It relaxed on a 2−3-h time scale in the dark (cf. below). The dark-adapted spectrum and the light-adapted spectrum obtained by extrapolation to zero time after illumination are shown in Figure 1. The main differences are in the frequencies of the C═C stretch band at around 1520−1540 cm^{-1} (which correlate reciprocally with the absorption maximum in the visible[25]) and in the C−C stretch region at 1150−1200 cm^{-1}. Close examination of the shapes of the ethylenic stretch bands indicated that they contain two bands; i.e., both dark-adapted and light-adapted samples appeared to be heterogeneous. The spectra of the all-trans and 13-cis components that make up the mixtures, and their relative concentrations, were calculated by iterative spectral decomposition until both C═C stretch bands were approximately symmetrical. The resulting spectra are included in Figure 1. Table 1 gives the isomeric compositions. Illumination with white and blue light produced light-adapted states with similar all-trans/13-cis mixtures, but in red light the photostationary state contained less all-trans isomer. This is as expected from the photochromic behavior of *H. salinarium* halorhodopsin.[10]

TABLE 1: All-Trans/13-Cis Isomeric Compositions of the Retinal in *H. salinarium* and *N. pharaonis* Halorhodopsins under Various Conditions

sample	salt	adaptation	% all-trans	% 13-cis
H. salinarium	chloride	LA (white)	75	25
		LA (blue)	70	30
		LA (red)	60	40
		DA	45	55
	sulfate		67	33
N. pharaonis	chloride	LA (white)	84	16
		DA	86	14
	sulfate		85	15

The FT Raman measures resonance-enhanced Raman scattering,[21] and although the amplitudes of some bands differ, their frequencies are very similar in the two methods. Thus, comparisons of the spectra for 13-cis and all-trans *H. salinarium* halorhodopsin in Figure 1 with resonance Raman spectra for bacteriorhodopsin are justified. The ethylenic stretch bands at 1534 and 1523 cm^{-1} for 13-cis and all-trans are consistent with the absorption maxima at 558 and 585, calculated from the spectra in the visible,[13] respectively. The main difference between 13-*cis*- and *all-trans*-bacteriorhodopsin is in the amplitudes of the fingerprint bands at 1167, 1183, and 1202 cm^{-1}. In a thorough study of the Raman bands of bacteriorhodopsin with 13-*cis*,15-*syn*-retinal,[26] it was found that the 1167- and 1202-cm^{-1} bands contain contributions from the C14−C15, C12−C13, and C8−C9 stretching intensities and have higher amplitudes in all-trans, while the band at 1183 cm^{-1}, which is also mixed, has increased amplitude in 13-cis. Further, the coupled CH_3 rocking mode at 1008 cm^{-1} is significantly increased in amplitude in 13-cis, as is the antisymmetric combination of N−H rock with C15−H rock at 1344 cm^{-1}. In the halorhodopsin spectra (Figure 1), these changes are by and large reproduced, but there are some differences. If the band at 1319 cm^{-1} with increased intensity in the 13-cis spectrum is in fact the combined N−H rock, it is shifted about 25 cm^{-1} from the frequency in bacteriorhodopsin. While closer examination of these differences with deuterium-substituted retinal is outside the scope of the present work, we note that there must be significant differences in the retinal binding pocket between halorhodopsin and bacteriorhodopsin.

Figure 2 shows the spectral changes in the fingerprint region during dark adaptation. The signal/noise ratio allows us to follow the increase of the 13-cis content on the tens of minutes time scale. The **v** matrix in the SVD analysis yielded a single time constant, consistent with what appears to be a single isosbestic point, at 1182 cm^{-1}. It was 165 ± 5 min under these conditions.

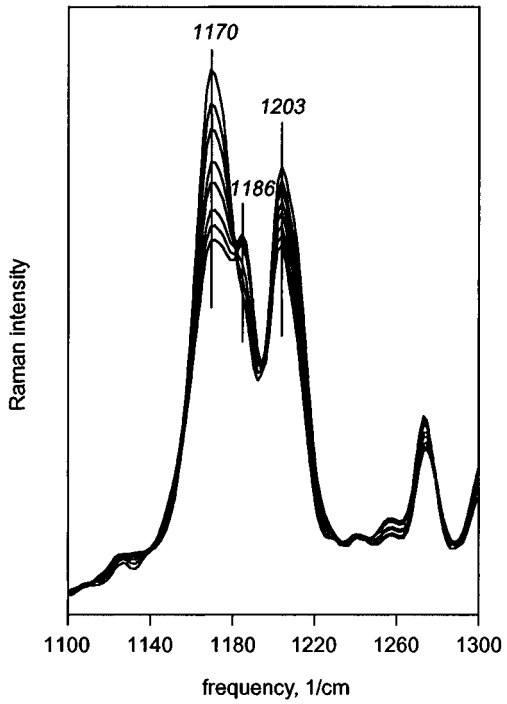

Figure 2. Dark adaptation of *H. salinarium* halorhodopsin, as followed by changes in the fingerprint region. The spectra shown are averages measured over 20-min intervals of incubation centered at 15, 55, 95, 165, 225, 305, 485, and 585 min, in the direction of decreasing amplitude at 1170 and 1203 cm^{-1}.

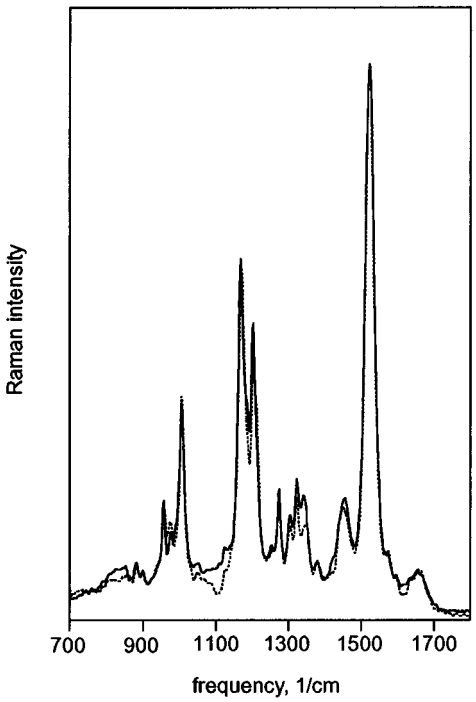

Figure 3. FT Raman spectra of halorhodopsin in the absence of chloride. The measurements were in 1 M Na_2SO_4. Solid line, *H. salinarium* halorhodopsin; dashed line, *N. pharaonis* halorhodopsin. Unlike in Figure 1, the dark-adapted and light-adapted spectra were essentially the same (not shown).

In Figure 3, the solid line shows the spectrum measured in sulfate. The light-adapted and dark-adapted spectra were virtually identical (not shown). The isomeric composition was estimated roughly by fitting the fingerprint region (between 1150 and 1250 cm^{-1}) with the spectra for all-trans and 13-cis in the presence of chloride (Figure 1). The all-trans content was high, comparable to what would be obtained after light adaptation when in the presence of chloride (Table 1).

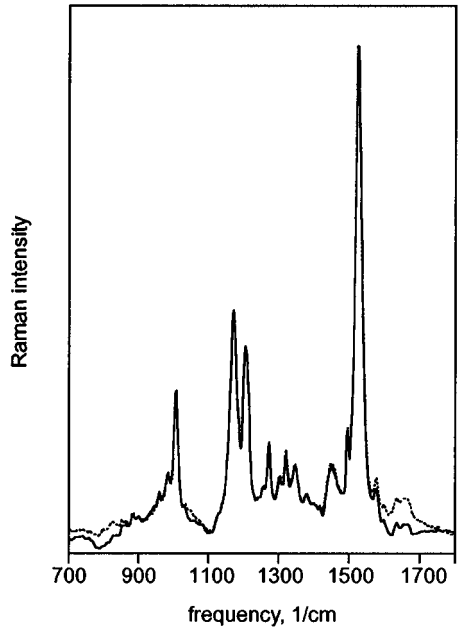

Figure 4. Lack of light adaptation in *N. pharaonis* halorhodopsin. The measurement was in 2 M NaCl. No significant difference between the dark-adapted (solid line) and light-adapted (dashed line) samples is seen.

4. Isomeric Composition of the Retinal in *N. pharaonis* Halorhodopsin

Halorhodopsin from *N. pharaonis* exhibited no spectral changes upon illumination in chloride, as shown in Figure 4. Its isomeric composition, estimated approximately by fitting the fingerprint region of the spectra to mixtures of the all-trans and 13-cis spectra from the *H. salinarium* chromophore in Figure 2, yielded about 85% all-trans content (Table 1). Likewise, no light adaptation was seen in sulfate. The spectrum in sulfate is shown with a dashed line in Figure 3. The all-trans content in sulfate was also about 85% (Table 1).

5. Discussion

Halorhodopsin, like many site-specific mutants of bacteriorhodopsin,[27,28] is unavoidably a mixture of species that contain either *all-trans*- or 13-*cis*,15-*syn*-retinal. The description of the photochemical reactions of these proteins, with the aim of understanding chloride and proton transport, has to take into account the fact that both of these species can contribute to the observed light-dependent changes. Dissection of the two sets of intermediates had lead to the suggestion[13] that the red-shifted 13-cis photointermediate of *H. salinarium* halorhodopsin previously thought to be in the photocycle of the all-trans chromophore is in fact the photoproduct of the 13-cis,15-syn species. Reliable information on the isomeric composition of these proteins, such as we provide here by measuring FT Raman spectra, is essential in making such distinctions. This is particularly so in the case of halorhodopsin, since it was a further complication that a red-shifted intermediate that arises on the same time scale (millisecond) but contains *all-trans*-retinal rather than 13-*cis*-retinal appeared in fact to be a genuine photointermediate in the photocycle of all-trans *N. pharaonis* halorhodopsin.[19,20,29] This state turned out to be the key intermediate in understanding chloride transport since its decay is dependent on chloride concentration, suggesting that (a) the red shift of its maximum reflects the absence of chloride near the retinal

Schiff base and (b) it initiates chloride uptake from the bulk to replace the chloride that is transported.

The isomeric compositions of dark-adapted halorhodopsins from both bacterial species reported earlier from retinal extraction are now confirmed: *H. salinarium* halorhodopsin contains 48% all-trans by the chemical method and 45% by FT Raman and *N. pharaonis* halorhodopsin 83 and 86%, respectively (ref 19 and Table 1). The results for the light-adapted *N. pharaonis* halorhodopsin are likewise similar, with 83 and 84% all-trans by the two methods. The results for light-adapted *H. salinarium* halorhodopsin are somewhat less in agreement, with 83 and 75% all-trans contents. This difference may reflect the photochromic behavior of this protein[10] that will respond differently to different illumination conditions, or it may be an error in the retinal analysis.

Does this discrepancy jeopardize the method for dissecting the absorbance changes into two photocycles? If the 13-cis content of the light-adapted state had been less than estimated, some of the red-shifted state would have to be assigned to the all-trans photocycle after all. However, we now find that the 13-cis content was somewhat underestimated in the earlier study.[13] Correction for this will change the shape of the calculated spectra of the N and O intermediates of the all-trans photocycle (by adding more extinction at the red edge) but not change the conclusion. The resolution of the photocycles of the all-trans and the 13-cis forms is therefore justified.

The same kind of analysis of the photoproducts of *N. pharaonis* halorhodopsin, as well as the absence of a spectral shift after illumination, suggested that this protein does not light-adapt. However, the latter result may have had the explanation that the absorption maxima of the 13-cis and all-trans chromophores were not sufficiently different to be detectable. The absence of light adaptation is now confirmed (Figure 4). The absorption changes measured in the photocycle of *N. pharaonis* halorhodopsin resembled those of the *H. salinarium* protein after subtraction of the contribution of the 13-cis species. Since there is about 15% 13-cis content, a value now confirmed, the 13-cis species in this protein, unlike the *H. salinarium* protein, seems not to accumulate photoproducts measurable in the 100-ns to hundreds of milliseconds time-range examined.[13] It is not at all clear where the origin of the difference in the halorhodopsins from the two bacterial species lies since the protein residues in the retinal binding pocket are largely conserved.[30]

Another undecided question concerned the absorption changes in the absence of chloride, as measured, for example, in sulfate, which is not transported (or poorly transported in the case of *N. pharaonis* halorhodopsin). Since the spectra and their kinetics resemble those calculated for the 13-cis species, we had considered the possibility that when the transported anion is not present, the retinal of at least the *H. salinarium* protein will assume the 13-cis configuration. The composition of the *H. salinarium* chromophore from retinal extraction in sulfate[10] did not support this, but it was possible that under this highly nonphysiological condition, the extraction results are not reliable. This alternative is now eliminated. In this, as well as in the other protein, the all-trans isomer dominates in the absence of chloride. Light adaptation is absent in both halorhodopsins in sulfate. Additionally, in *N. pharaonis* halorhodopsin, the anion makes no detectable difference for the isomeric composition (Table 1).

The results we report here put the interpretation of spectroscopic measurements with halorhodopsin on firmer ground. By confirming the earlier analysis, they establish the need to determine the isomeric composition of the retinal in order to resolve the photoreactions of the two isomers before the interpretation of light-induced spectral changes for *H. salinarium* halorhodopsin, or its mutants, is undertaken. This may be true for some bacteriorhodopsin mutants as well.

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