Structure of the retinal chromophore in halorhodopsin

A resonance Raman study

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Resonance Raman (RR) spectra of the light-driven chloride pump halorhodopsin (HR) were recorded after isolation of the protein from cell membranes of *Halobacterium halobium*. The spectra of the unphotolyzed state HR_{578} were compared with those of two unphotolyzed species of the light-driven proton pump bacteriorhodopsin, BR_{570} and BR_{548}, which were obtained from light- and dark-adapted purple membranes. Identical structural components in the retinal chromophores of HR_{578} and BR_{570} are found, both having the all-*trans* configuration of the retinal chain and a protonated Schiff base linkage to the protein with *anti*-position of the two hydrogens. Only minor conformational differences between the chromophoric structures in the two proteins could be inferred from the vibrational structure of the RR spectra. The function of the two chromophores as triggers of light-induced chloride or proton transport emphasizes the role of the protein part in the ion specificity of the pumps.

Halorhodopsin *Light-driven chloride pump* *Resonance Raman spectroscopy* *Protonated Schiff base*

1. INTRODUCTION

The cell membrane of *Halobacterium halobium* contains at least 3 different retinal-binding proteins (review [1]). The predominant bacteriorhodopsin (BR) forms two-dimensional regular arrays called purple membranes (PM) and acts as a light-driven proton pump [2,3]. The second chromoprotein, halorhodopsin (HR), occurs at much lower concentrations and in contrast to BR does not enrich into a specific membrane fraction of the cells. Spectroscopic experiments with intact cells and cell envelope vesicles established a photochemical cycle for HR different from that of BR [4]. HR was proposed to act as a light-driven chloride pump [5] and photoelectric experiments with HR-containing cell membranes on black lipid films proved the function of a halide (Cl^-, Br^-, I^-) pump [6].

Recently, HR was isolated in its native state as a chromoprotein of apparent M_r 20000 [7,8]. Furthermore, it was demonstrated by reconstitution experiments with a liposomal-black lipid membrane system that the isolated chromoprotein carries the halide transport activity [9]. Therefore, in both, BR and HR, retinal mediates directly ion translocation and the ion specificities are introduced by the protein part.

The absorption maxima of HR and BR at 578 and 570 nm are close to each other, indicating a similar retinal protein interaction. Bacteriorhodopsin contains retinal as a protonated Schiff base (SB) of its lysine residue 216. Upon light adaptation the all-*trans*, 15-*anti* form of the Schiff base dominates (BR_{570}, fig.1a) while dark adaptation equilibrates this species with a 13-*cis*, 15-*syn* form (BR_{548}, fig.1b) [10–12]. RR spectroscopy is an especially suitable method to analyze and compare the chromophoric structures in BR and HR. The results of these experiments are presented here.

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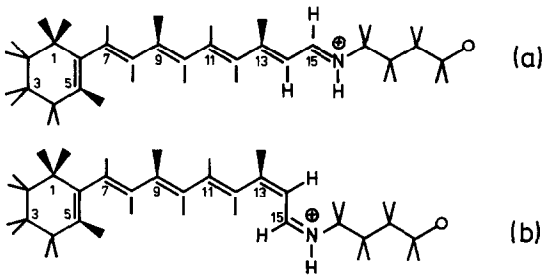

Fig.1. Retinal Schiff base configurations in bacteriorhodopsin: (a) all-*trans*, 15-*anti*; (b) 13-*cis*, 15-*syn*.

2. MATERIALS AND METHODS

Halorhodopsin was isolated according to [7] with the modifications described in [9]. All samples were in 1 M NaCl containing 1% octylglucoside, 10 mM Mops, pH 7. All RR spectra were recorded at 20 μM HR_{578}.

The 568 nm line of a krypton laser (coherent 2000 K) was used for excitation of RR scattering. The Raman probe beam was focussed (60 μm) onto the sample (0.3 ml) placed in a rotating cell (50 s^{-1}) and the sample passed the beam at a velocity of 10 $m \cdot s^{-1}$ (transit time 10 μs). The laser power was kept below 10 mW [13] and a second laser beam of blue light was focussed on the sample before passing the probe beam. Under such conditions photoproducts in the laser beam can be neglected and only the unphotolysed species HR_{578} was probed. The spectra were recorded by conventional monochromatic scanning equipment as in [14] at a spectral bandwidth of 3.7 cm^{-1}. The accuracy of the peak positions on a relative scale is $\pm$ 2 cm^{-1}. The samples in the rotating cell were irreversibly bleached after illumination for 2 h.

The signal-to-noise ratio in the spectra of HR_{578} could be improved by multiple scanning. A limit for the spectral quality was set by a fluorescence background which was 10-times stronger than the most intense RR band. This background was substracted by computational methods.

Purple membranes were isolated from *H. halobium* S9 cells in the conventional way [15]. RR spectra of the light-adapted PM containing BR_{570} and dark-adapted samples containing in addition BR_{548} were recorded as in [16].

3. RESULTS AND DISCUSSION

3.1. *Configuration of the Schiff base*

The RR spectrum of light-adapted halorhodopsin, HR_{578}, is shown in fig.2 together with the spectra of BR_{570} and BR_{548} containing the retinal Schiff base in an all-*trans*, 15-*anti* and a 13-*cis*, 15-*syn* configuration, respectively (fig.1) [10–12]. Inspection of fig.2 immediately reveals that the

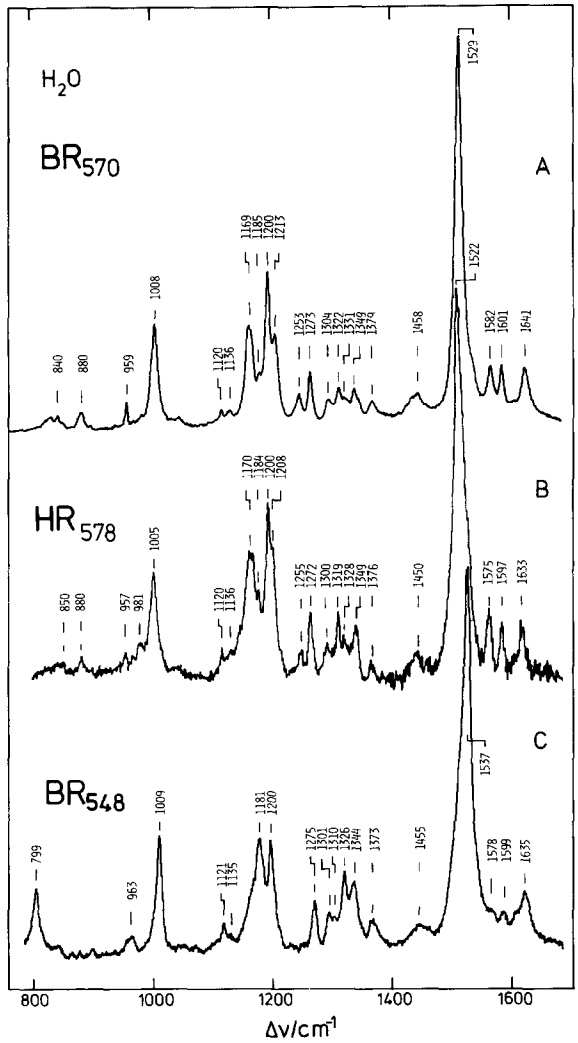

Fig.2. Resonance Raman spectra in H_2O suspension. (A) Bacteriorhodopsin unphotolyzed state, BR_{570}, from light-adapted purple membrane suspension, excitation at 514 nm. (B) Halorhodopsin unphotolyzed state HR_{578}, excitation at 568 nm. (C) BR_{548} chromophore of bacteriorhodopsin from dark-adapted purple membrane suspension, excitation at 514 nm.

spectrum of HR_{578} closely resembles that of BR_{570} but is different from the spectrum of BR_{548} in its main features. Indeed, each band of HR_{578} corresponds in frequency, intensity and linewidth to a counterpart of BR_{570}. Frequency deviations are less than 8 cm^{-1}. Therefore the structure of the chromophore in HR_{578} must be closely related to that in BR_{570}.

The spectroscopic data and their structural implications merit a more detailed discussion. First the Schiff base group of both proteins is considered. Samples were dialysed against D_2O and the RR spectra compared with those of fig.2. In BR_{570} the bands at 1641 and 1349 cm^{-1} move to 1624 and 976 cm^{-1} when the exchangeable SB proton is replaced by a deuteron (figs 2A and 3A). These bands were assigned as C=N stretching and N-H in-plane hydrogen bending modes, respectively, and are characteristic of the structure of the SB group in BR_{570} [14]. Analogous bands can be identified in the spectrum of HR_{578} at 1633 and 1349 cm^{-1} (fig.2B). When the nitrogen of the SB group is deuterated by dialysis the bands move to 1621 and 968 cm^{-1}, respectively (fig.3B). These findings provide convincing evidence for a protonated SB group in HR_{578}. Nevertheless, minor differences between BR_{570} and HR_{578} are found. The C=N stretch in HR_{578} is 8 cm^{-1} lower in frequency and the deuterio shift is only 12 cm^{-1} compared with 17 cm^{-1} in BR_{570}. This difference can be explained if one takes into account that the C=N stretch can be coupled to the N-H in-plane hydrogen bending motion at (1349 $cm^{-}1$) but not to the N-D in-plane bending motion (976 cm^{-1}) [17]. The stronger this coupling the more the C=N stretch is shifted to higher frequency. The deuterium shift of the C=N stretch therefore has, besides the constant mass effect, an additional component which depends on the strength of C=N/N-H coupling. The experimental data imply that this coupling is much smaller in HR_{578} than in BR_{570} because the deuterium shift of 12 cm^{-1} in HR_{578} is largely due to the constant mass effect which for H/D exchange is estimated to be about 10 cm^{-1} [17,18]. This means that C=N/N-H coupling nearly vanishes in HR_{578}. This conclusion corroborates with the fact, that the N-H/N-D frequency ratio for the hydrogen bend in HR_{578} which is given by 1349/968 = 1.394 is higher than the corresponding quantity in BR_{570} (1349/976 = 1.382), indicating that the N-H bending mode is more localized in HR_{578}.

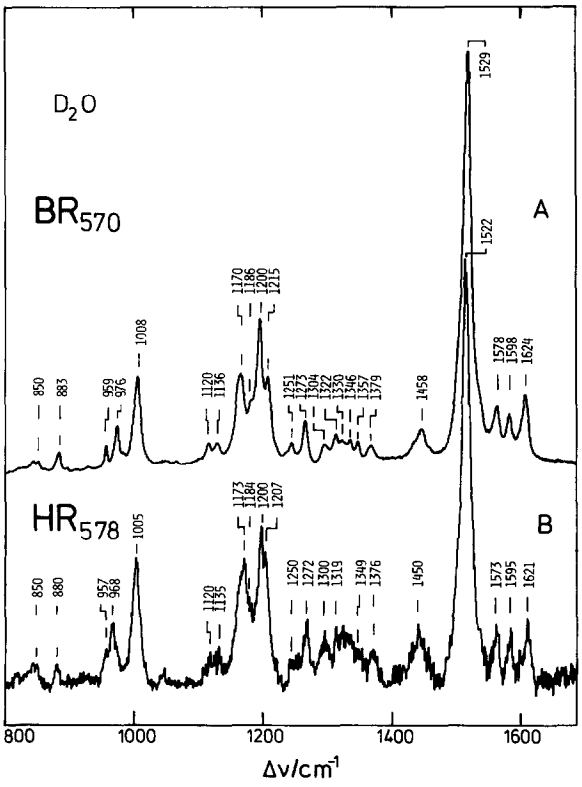

Fig.3. Resonance Raman spectra in D_2O. (A) Same conditions as in fig.2A. (B) Same conditions as in fig.2B.

The difference in C=N/N-H coupling we had invoked probably has its origin in slightly different local interactions of the protein with the terminal SB group which modify the electronic configuration and hence the vibrational modes. On the other hand, it can be rigorously ruled out that the SB hydrogens in HR_{578} are in the 15-*syn* position as in BR_{548}, since the characteristic bands of this configuration behave in a quite different manner [16].

3.2. *Configuration of the retinal chain*

Looking at the characteristic bands of the retinal chain it turns out from the comparison of the spectra of BR_{570} and BR_{548} in fig.2 that the different configuration of these two species is reflected by characteristic differences in the vibrational pattern in the 'fingerprint region' between 1150 and 1380 cm^{-1}. Thus the two forms can be easily

distinguished by their frequency and intensity distribution in this region. Vibrational analysis has shown that the corresponding normal modes predominantly involve C-C single bond stretching as well as C-H in-plane bending components which are coupled to various extents and more or less delocalized along the chain [19–21]. Such modes are expected to depend sensitively in frequency and RR intensity even on minor conformational distortions which are imposed on the retinal chain by interaction with the protein. In other words, the vibrational pattern in the region between 1150 and 1380 cm^{-1} should be characteristic of the geometry which a retinal moiety exhibits in the binding site. The spectra in fig.2A and B document that HR_{578} and BR_{570} have only minor differences in this fingerprint region between 1150 and 1380 cm^{-1}.

The appearance of strong RR bands between 800 and 1000 cm^{-1}, which can be assigned to hydrogen out-of-plane modes of the retinal chain, has been interpreted as a sign of considerable conformational distortion of the chain [22]. In the spectra of BR_{570} two bands of this type occur. The first one at 959 cm^{-1} can be assigned to a coupled out-of-plane bending motion of the hydrogens in the $HC_{11}=C_{12}H$ group. This assignment is based on the vibrational analysis of all-*trans* retinal [19] and on the fact that the 959 cm^{-1} band vanishes in the spectrum of the C_{12}-D analogue of BR_{570} [23]. The second vibration at 880 cm^{-1} was assigned to the $C_{14}H$ out-of-plane bending mode [20]. The low intensity of these two bonds indicates that large deviations from the planar structure do not occur. It is important to note that corresponding weak features can also be identified in the spectra of HR_{578} (figs 2,3). This implies that the conformational distortions which are reflected by these bands are identical in HR_{578} and BR_{570}. It is interesting to note that a strong band appears in the spectrum of BR_{548} (fig.2C) in the out-of-plane bending region at 799 cm^{-1}. It was assigned to the C_{14}-H out-of-plane mode [20]. On the basis of previous arguments this implies that the retinal skeleton between the C_{13} and C_{15} atoms is more distorted in the 13-*cis*, 5-*syn* configuration of BR_{548} than in the all-*trans* form of BR_{570} and HR_{578}.

3.3. *The C=C stretch modes*

The strongest band in the spectra of BR_{570} at 1529 cm^{-1} was assigned to the in-phase stretching motion along the various C=C bonds of the chain [19–21]. The slight frequency down-shift to 1522 cm^{-1} in HR_{578} can be ascribed to a corresponding slight decrease of π-electron density in the C=C double bonds of HR which is also reflected by the red-shift of the maximum absorption to 578 nm [13,14]. The weak satellites at 1582 and 1601 cm^{-1} in the spectrum of BR_{570} which refer to more localized C=C stretches appear with identical intensity at 1575 and 1597 cm^{-1} in the spectrum of HR_{578}, but are not well defined in the spectra of BR_{548}. Thus the C=C stretching region in the RR spectra which provides an additional characteristic fingerprint for the chromophoric structure also points to the great similarity between HR_{578} and BR_{570}.

From the shape of the RR spectra it can be concluded that in BR_{570} the retinal chain is rather rigid in the binding site. Thus it could be demonstrated that RR bands are considerably broadened when the PM is dehydrated [24]. This was interpreted in terms of conformational broadening which means that the chromophore acquires a variety of different conformational states which then contribute to the spectrum. No difference in the half-width of the RR bands is found between BR_{570} and HR_{578} which indicates that the chromophore in HR has also a rigid conformation.

4. CONCLUSIONS

On the basis of RR spectroscopic evidence we conclude that retinal in BR_{570} and HR_{578} has a nearly identical conformation which is all-*trans* and fairly planar. It is bound to the protein via a protonated Schiff base linkage with the two hydrogens in the *anti*-position. Minor structural deviations were only indicated for the terminal Schiff base group. The nearly identical configuration of retinal suggests that also the protein environment at the binding sites is similar. Considering the photocycle of the two molecules, however, a clear difference is found in protonation/deprotonation of BR and unchanged protonation state of the HR chromophore [25]. This difference might help to explain how the two chromophoric structures can trigger such different functions as anion and cation transport [26].

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