

Structure of the retinal chromophore in halorhodopsin

A resonance Raman study

T. Alshuth, M. Stockburger, P. Hegemann* and D. Oesterhelt*

*Max-Planck-Institut für Biophysikalische Chemie, 3400 Göttingen, and *Max-Planck-Institut für Biochemie, 8033 Martinsried, FRG*

Received 9 October 1984

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 HR₅₇₈ were compared with those of two unphotolyzed species of the light-driven proton pump bacteriorhodopsin, BR₅₇₀ and BR₅₄₈, which were obtained from light- and dark-adapted purple membranes. Identical structural components in the retinal chromophores of HR₅₇₈ and BR₅₇₀ 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 (BR₅₇₀, fig.1a) while dark adaptation equilibrates this species with a 13-*cis*, 15-*syn* form (BR₅₄₈, 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.

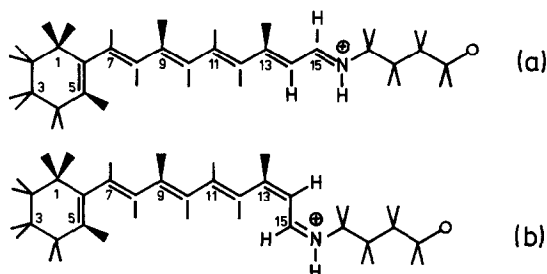


Fig.1. Retinal Schiff base configurations in bacteriorhodopsin: (a) all-*trans*, 15-*anti*; (b) 13-*cis*, 15-*syn*.

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 μ M HR₅₇₈.

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 μ m) 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·s⁻¹ (transit time 10 μ s). 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 HR₅₇₈ 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 ± 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 HR₅₇₈ 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 subtracted 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 BR₅₇₀ and dark-adapted samples containing in addition BR₅₄₈ were recorded as in [16].

3. RESULTS AND DISCUSSION

3.1. Configuration of the Schiff base

The RR spectrum of light-adapted halorhodopsin, HR₅₇₈, is shown in fig.2 together with the spectra of BR₅₇₀ and BR₅₄₈ 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

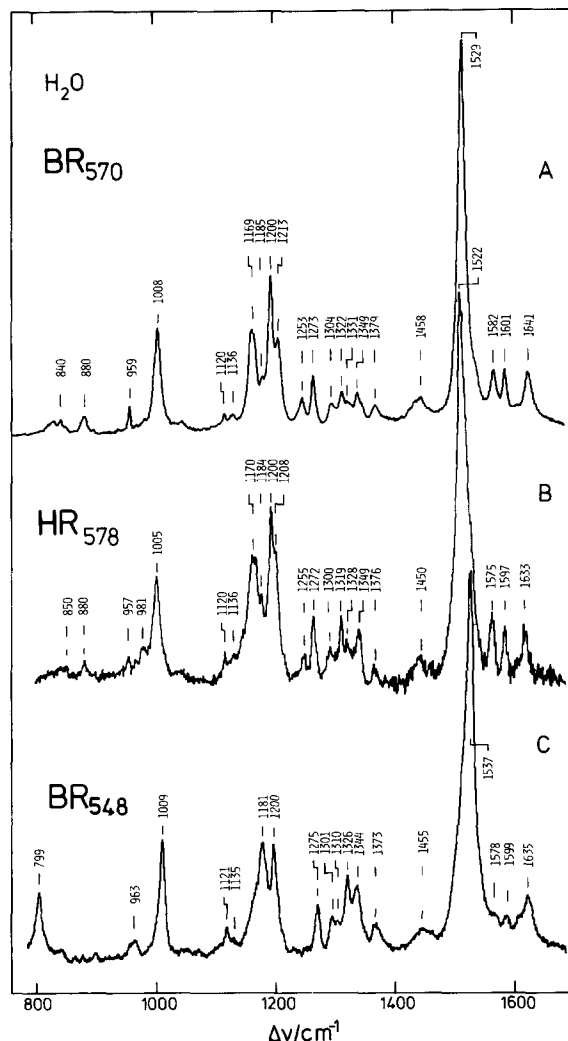


Fig.2. Resonance Raman spectra in H₂O suspension. (A) Bacteriorhodopsin unphotolyzed state, BR₅₇₀, from light-adapted purple membrane suspension, excitation at 514 nm. (B) Halorhodopsin unphotolyzed state HR₅₇₈, excitation at 568 nm. (C) BR₅₄₈ chromophore of bacteriorhodopsin from dark-adapted purple membrane suspension, excitation at 514 nm.

spectrum of HR₅₇₈ closely resembles that of BR₅₇₀ but is different from the spectrum of BR₅₄₈ in its main features. Indeed, each band of HR₅₇₈ corresponds in frequency, intensity and linewidth to a counterpart of BR₅₇₀. Frequency deviations are less than 8 cm⁻¹. Therefore the structure of the chromophore in HR₅₇₈ must be closely related to that in BR₅₇₀.

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 D₂O and the RR spectra compared with those of fig.2. In BR₅₇₀ the bands at 1641 and 1349 cm⁻¹ move to 1624 and 976 cm⁻¹ when the exchangeable SB proton is replaced by a deuterium (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 BR₅₇₀ [14]. Analogous bands can be iden-

tified in the spectrum of HR₅₇₈ 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 HR₅₇₈. Nevertheless, minor differences between BR₅₇₀ and HR₅₇₈ are found. The C=N stretch in HR₅₇₈ is 8 cm⁻¹ lower in frequency and the deuterio shift is only 12 cm⁻¹ compared with 17 cm⁻¹ in BR₅₇₀. 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 HR₅₇₈ than in BR₅₇₀ because the deuterium shift of 12 cm⁻¹ in HR₅₇₈ 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 HR₅₇₈. This conclusion corroborates with the fact, that the N-H/N-D frequency ratio for the hydrogen bend in HR₅₇₈ which is given by 1349/968 = 1.394 is higher than the corresponding quantity in BR₅₇₀ (1349/976 = 1.382), indicating that the N-H bending mode is more localized in HR₅₇₈.

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 HR₅₇₈ are in the 15-*syn* position as in BR₅₄₈, 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 BR₅₇₀ and BR₅₄₈ 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

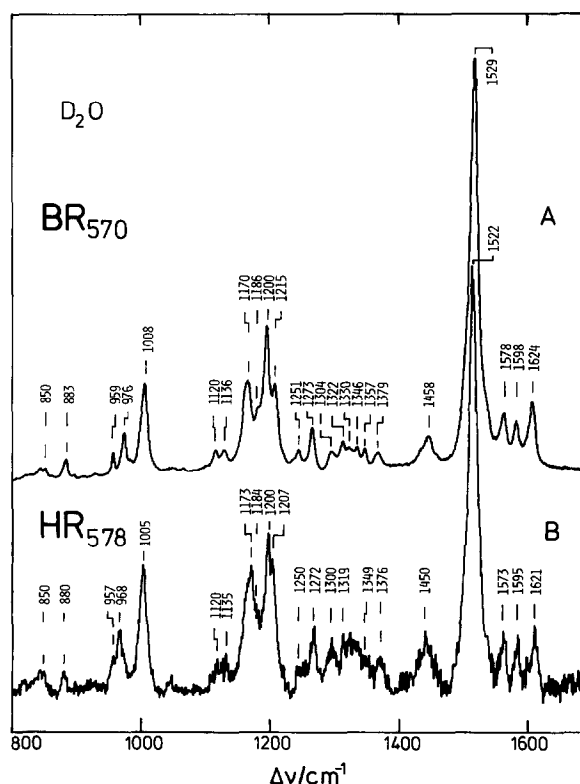


Fig.3. Resonance Raman spectra in D₂O. (A) Same conditions as in fig.2A. (B) Same conditions as in fig.2B.

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 HR₅₇₈ and BR₅₇₀ 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 BR₅₇₀ 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 HC₁₁=C₁₂H 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 C₁₂-D analogue of BR₅₇₀ [23]. The second vibration at 880 cm^{-1} was assigned to the C₁₄H out-of-plane bending mode [20]. The low intensity of these two bands 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 HR₅₇₈ (figs 2,3). This implies that the conformational distortions which are reflected by these bands are identical in HR₅₇₈ and BR₅₇₀. It is interesting to note that a strong band appears in the spectrum of BR₅₄₈ (fig.2C) in the out-of-plane bending region at 799 cm^{-1} . It was assigned to the C₁₄-H out-of-plane mode [20]. On the basis of previous arguments this implies that the retinal skeleton between the C₁₃ and C₁₅ atoms is more distorted in the 13-*cis*, 5-*syn* configuration of BR₅₄₈ than in the all-*trans* form of BR₅₇₀ and HR₅₇₈.

3.3. The C=C stretch modes

The strongest band in the spectra of BR₅₇₀ 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 HR₅₇₈ 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 BR₅₇₀ which refer to more localized C=C stretches appear with identical intensity at 1575 and 1597 cm^{-1} in the spectrum of HR₅₇₈, but are not well defined in the spectra of BR₅₄₈. 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 HR₅₇₈ and BR₅₇₀.

From the shape of the RR spectra it can be concluded that in BR₅₇₀ 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 BR₅₇₀ and HR₅₇₈ 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 BR₅₇₀ and HR₅₇₈ 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].

ACKNOWLEDGEMENT

We thank U. Hegemann for the preparation of the HR samples.

REFERENCES

- [1] Lanyi, J.K. (1984) in: *Bioenergetics* (Ernster, L. ed.) p.315, Elsevier, Amsterdam, New York.
- [2] Oesterhelt, D. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 1554.
- [3] Oesterhelt, D. and Stoekenius, W. (1973) *Proc. Natl. Acad. Sci. USA* 70, 2853.
- [4] Weber, H.-J. and Bogomolni, R.A. (1981) *Photochem. Photobiol.* 33, 601.
- [5] Schobert, B. and Lanyi, J.K. (1982) *J. Biol. Chem.* 257, 10306.
- [6] Bamberg, E., Hegemann, P. and Oesterhelt, D. (1984) *Biochim. Biophys. Acta* 773, 53.
- [7] Steiner, M. and Oesterhelt, D. (1983) *EMBO J.* 2, 1379.
- [8] Taylor, M.E., Bogomolni, R.A. and Weber, H.J. (1983) *Proc. Natl. Acad. Sci. USA* 80, 6172.
- [9] Bamberg, E., Hegemann, P. and Oesterhelt, D. (1984) *Biochemistry*, in press.
- [10] Orlandi, G. and Schulten, K. (1979) *Chem. Phys. Lett.* 64, 370.
- [11] Harbinson, G.S., Smith, S.O., Pardo, J.A., Winkel, C., Lugtenburg, J., Herzfeld, J., Mathies, R. and Griffin, R. (1984) *Proc. Natl. Acad. Sci. USA* 81, 1706.
- [12] Smith, S.O., Myers, A.B., Pardo, J.A., Winkel, C., Mulder, P.P.J., Lugtenburg, J., Herzfeld, J., Mathies, R. and Griffin, R.G. (1984) *Proc. Natl. Acad. Sci. USA* 81, 2055.
- [13] Stockburger, M., Klusmann, W., Gattermann, H., Massig, G. and Peters, R. (1979) *Biochemistry* 18, 4886.
- [14] Massig, G., Stockburger, M., Gärtner, W., Oesterhelt, D. and Towner, P. (1982) *J. Raman Spectrosc.* 12, 287.
- [15] Oesterhelt, D. and Stoekenius, W. (1974) *Methods Enzymol.* 31A, 667.
- [16] Alshuth, T. and Stockburger, M. (1981) *Ber. Bunsenges. Phys. Chem.* 85, 484.
- [17] Aton, B., Doukas, A.G., Narva, D., Callender, R.H., Dinur, U. and Honig, B. (1980) *Biophys. J.* 29, 79.
- [18] Argade, P.V., Rotschild, K.J., Kawamoto, A.H., Herzfeld, J. and Herlihy, W.C. (1981) *Proc. Natl. Acad. Sci. USA* 78, 1643.
- [19] Curry, B., Broek, A., Lugtenburg, J. and Mathies, R. (1982) *J. Am. Chem. Soc.* 104, 5274.
- [20] Curry, B., Palings, I., Broek, A., Pardo, J.A., Mulder, P.P.J., Lugtenburg, J. and Mathies, R. (1983) *J. Phys. Chem.* 88, 688.
- [21] Saito, S. and Tasumi, M. (1983) *J. Raman Spectrosc.* 14, 236.
- [22] Eyring, G., Curry, B., Broek, A., Lugtenburg, J. and Mathies, R. (1982) *Biochemistry* 21, 384.
- [23] Alshuth, T. and Gärtner, W., personal communication.
- [24] Heyde, M.E., Gill, D., Kilponen, R.G. and Rimai, L. (1971) *J. Am. Chem. Soc.* 93, 6776.
- [25] Hildebrand, P. and Stockburger, M. (1984) *Biochemistry* 23, in press.
- [26] Hegemann, P., Oesterhelt, D. and Steiner, M. (1984) *EMBO J.*, submitted.
- [27] Oesterhelt, D., Hegemann, P., Schulten, K. and Tavan, P. (1984) *EMBO J.*, submitted.