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Resonance Raman study of intermediates of the halorhodopsin photocycle

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The resonance Raman (RR) study of the retinal protein halorhodopsin (HR_{578}) was extended to two of its photoproducts: HR_{520} and HR^{L}_{410} RR spectra of both species were recorded in H_2O and D_2O and compared with the RR spectra of the intermediates L_{550} and M_{412} from the bacteriorhodopsin photocycle. HR_{520} was found to be a protonated Schiff base in the 13-*cis* configuration and HR^{L}_{410} a deprotonated Schiff base in the 13-*cis* configuration.

Resonance Raman spectroscopy; Photocycle; Light-driven chloride pump; Retinal isomerization; Retinal protein

1. INTRODUCTION

Halorhodopsin (HR) and bacteriorhodopsin (BR) are two retinal proteins in the cell membrane of halobacteria, both acting as light-driven ion pumps (review [1]). BR translocates H^+ to the medium [2,3] and mediates phototrophic growth of the cells, whereas HR translocates Cl^- into the cell and serves an accessory function for net salt uptake during growth [4–6]. In both proteins ion translocation is connected to a thermoreversible photochemical reaction of the light-adapted chromophore occurring as a protonated Schiff base (SB) of all-*trans*-retinal and an ϵ-amino group of Lys 216 (BR) and Lys 242 (HR) in the respective polypeptide chains [7–10]. This cyclic reaction involves several spectroscopically distinct intermediates and is called the photocycle of the chromoproteins. Some important similarities between the photocycles of BR and HR are obvious from the schemes in fig.1 concerning the lifetimes of various intermediates and their absorption characteristics. The light-adapted parent chromophores BR_{570} and HR_{578} are photoconverted within a few picoseconds into the red-shifted intermediates K_{590} and HR_{600}, respectively [11–13]. On the microsecond time scale the slightly blue-shifted intermediates L_{550} and HR_{520} appear. After this stage the two molecules behave differently. In the case of BR the most strongly blue-shifted species M_{412} is formed within 60 μs, whereas HR_{520} is converted into HR_{640} within 15 ms. An analogue of M_{412}, HR^{L}_{410}, occurs to a significant extent only in the presence of NaN_3 [14] and is not an intermediate of the photocycle but a side product of HR_{640}. Under photostationary conditions its concentration reaches an azide-independent but pH-dependent level.

In BR the frequency of the cycle is controlled by the reconstitution time of BR_{570} which under normal conditions is about 5 ms (cf. fig.1). In HR, on the other hand, this period is determined by the 15 ms lifetimes of HR_{520} (in the absence of azide; cf. fig.1) since the lifetime of the consecutive species HR_{640} is about 10-times shorter [14].

For both photocycles reversible *trans*-to-*cis* isomerizations have been demonstrated. All intermediates in BR have been assigned to one or the other configuration by resonance Raman (RR)

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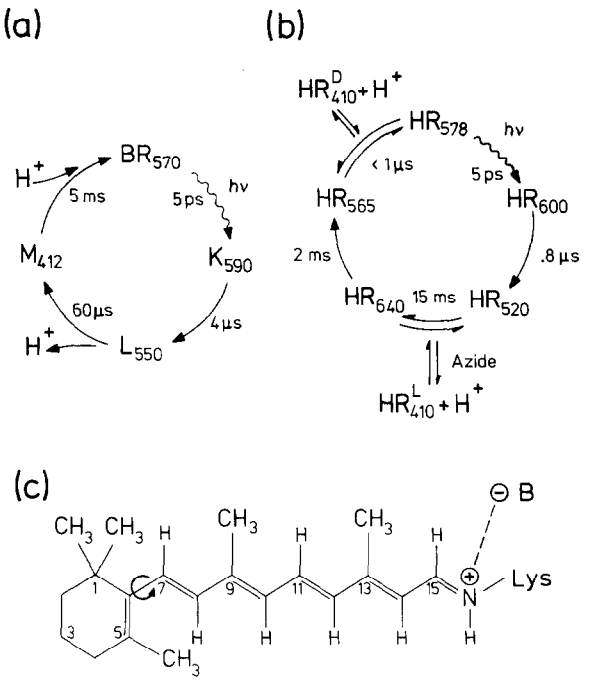

Fig.1. (a) Simplified model of the photocycle of BR [30]: the decay times refer to room temperature and pH 7.4 [21]. (b) Simplified model of the photocycle of HR [14]: the decay times refer to room temperature, pH 7.0 and 1 M NaCl. (c) Retinylidene chromophore in BR or HR in all-*trans* configuration. B^- is a counterion.

spectroscopy [15], but only in a more indirect way by UV spectroscopy [16] and chemical reactions in HR [17]. The major difference between the photocycles of HR and BR are steps at which either chloride influences equilibria of intermediates (in HR: HR_{520}/HR_{640} and HR_{565}/HR_{578}) or deprotonation and reprotonation reactions occur (in BR: L_{550}/M_{412} and M_{412}/BR_{570}). A common model for the photochemical *trans*-to-*cis* isomerization of retinal in both molecules with thermal reisomerization in the millisecond range has been suggested on the basis of these apparent differences [17]. It requires a 13-*cis* protonated SB in HR_{520} and the experiments described here establish this postulate.

Vibrational RR spectra can provide detailed information on the molecular structure of retinylidene chromophores (reviews [18,19]). In particular, this refers to the configuration of the retinylidene moiety (*cis* or *trans*), to the structure and protonation state of the SB group by which the retinylidene moiety is linked to the protein, and to electrostatic interactions with charged side groups.

In a previous study we presented RR spectra of the parent species HR_{578} [10]. Here we report on the spectra of the intermediates HR_{520} and HR^L_{410} which have significance for the interpretation of structural changes during the HR photocycle.

2. MATERIALS AND METHODS

HR was isolated according to [20] and mixed with halobacterial lipids in a molar ratio of about 1:20 before extensive dialysis (5 days) against 1 M NaCl to remove detergents. The present RR studies showed that this type of sample is much more stable against laser irradiation than preparations still containing detergent which had been used in previous RR experiments [10]. Lipid-containing samples were bleached irreversibly only after 10–20 h of laser irradiation under the conditions of our experiments. All HR samples were prepared in buffers (10 mM Mops, pH 7.2 or 8, 1 M NaCl) at a concentration of 20 μM. Deuterated HR was prepared by dialysing the sample against the same buffer prepared in D_2O. For recording the RR spectrum of HR^L_{410}, NaN_3 (50 mM) was added to the sample.

The noise in the spectra is mainly due to a fluorescent background which was 10-times stronger than the most intense RR band. For presentation of spectra this background was subtracted. All measurements were carried out at 23°C.

The RR spectra were recorded by conventional flow techniques [21]. Single- and double-beam experiments were performed using a rotating cell (quartz). Lines of a krypton ion laser at 413 and 647 nm (Coherent 2000 k) and of an argon ion laser at 476 and 514 nm (Coherent CR 15) were used as photolysis and Raman probe beams. The spectra were recorded with a double monochromator (Spex 14018) at a spectral band width of 4.5 cm^{-1} for 476 nm and of 2.8 cm^{-1} for 413 nm excitation. The accuracy of the given Raman shifts is ± 2 cm^{-1}.

The experimental parameters for the RR studies on the HR photocycle are given in table 1. The various time delays, δ, between pump and probe events were optimized on the basis of kinetic data [14]. The diameter and power of the probe beam

Table 1

Experimental parameters for recording the RR spectra of HR

	HR_{578}		HR_{520}[a]		HR^{L}_{410}[b]
	H_2O	D_2O	H_2O	D_2O	H_2O
Pump beam					
λ (nm)	–	–	647	647	514
d (μm)	–	–	160	160	200–400
P (mW)	–	–	100	200	7– 19
Probe beam					
λ (nm)	476	476	476	476	413
d (μm)	80	90	70	80	70
P (mW)	6	4	2	5	1.2
ν_{rot} (s^{-1})	50	50	10	50	30
δ (ms)	–	–	0.2	0.1	33
pH	7.2	7.2	7.2	7.2	8.0

[a] The recorded spectrum consists of signals from HR_{520} and HR_{578}

[b] 50 mM $NaNO_3$ was added to the buffer

Buffer: 10 mM Mops, 1 M NaCl. λ, laser wavelength; d, $1/e^2$ – diameter of the focused beam; P, laser power; ν_{rot}, frequency of the rotating cell (diameter 40 mm); δ, time delay between photolysis (pump) and RR excitation (probe)

were selected for minimization of additional photoproducts. The RR spectrum of the parent species HR_{578} was obtained by irradiation of the sample with the probe beam only. For studying photointermediates an additional pump beam was used in which 30–40% of the parent species were converted into intermediates. The sample therefore still contained more than 60% of the parent species when it was excited in the probe beam. Since the absorption spectra of HR_{578} and HR_{520} strongly overlap, RR spectroscopic separation by selective resonance excitation is therefore not possible. The contribution of the parent species HR_{578} had to be subtracted from the recorded spectrum to obtain a pure spectrum of HR_{520}. The delay time in this experiment was 200 μs (table 1) which guarantees temporal separation from other intermediates.

The RR spectrum of HR^{L}_{410} (HR^{L}_{410}, HR_{410} observed in the light) can be completely separated from other species by selective resonance excitation in the violet region at 413 nm. 50 mM NaN_3 was used to favour the formation of this deprotonated species [22] and to increase its rate of formation to a value of 44 s^{-1} [14]. HR_{578} equilibrates with a pK of 8.9 with a second species absorbing at 410 nm, HR^{D}_{410} (HR^{D}_{410}, HR_{410} observed in the dark), which could contribute to the recorded spectrum to about 10–20% at a pH value around 8 in the dark. However, the specific RR signal increased by a factor of 5 by switching on the pump laser which is only due to formation of HR^{L}_{410}.

3. RESULTS AND DISCUSSION

Fig.2 compares the RR spectra of BR_{570} and HR_{578}. The three main groups of vibrations, C=C stretch vibrations, fingerprint region and SB group, are listed in table 2 and document the general similarity of the chromophores in the two proteins.

In addition to the three main features of vibrations, bands below 1000 cm^{-1} occur and are weak but characteristic. The feature at 880 cm^{-1} can be assigned to the ($C_{14}H$) wag, an out-of-plane bending motion of the C_{14} hydrogen, while the fairly sharp band at 959 cm^{-1} refers to a coupled out-of-plane motion of the two hydrogen atoms at C_{11} and C_{12}. The main intensity of the strong feature at 1008 cm^{-1} refers to a bending vibration of the hydrogen atoms of the two methyl groups at C_9 and C_{13}. Later it will be shown that in BR^{L}_{550} and HR_{520} this band also involves a large contribution from an SB mode (see below).

Related spectra of BR and HR and of their photoproducts upon light excitation shall be compared mainly with respect to the three most conspicuous groups of bands: the C=C stretches, the fingerprint bands and SB bands. If the vibrational spectra are close to identity in all three groups we conclude that the geometries of the retinyl chain (*trans* or *cis* configuration) and of the SB group (*syn, anti*) are very similar and that the electrostatic interaction of the chromophore with its environment is of similar quality and magnitude. Deviations from identity, on the other hand, indicate specific differences in conformation.

3.1. *HR_{578}*

The spectra obtained for HR_{578} reconstituted in-

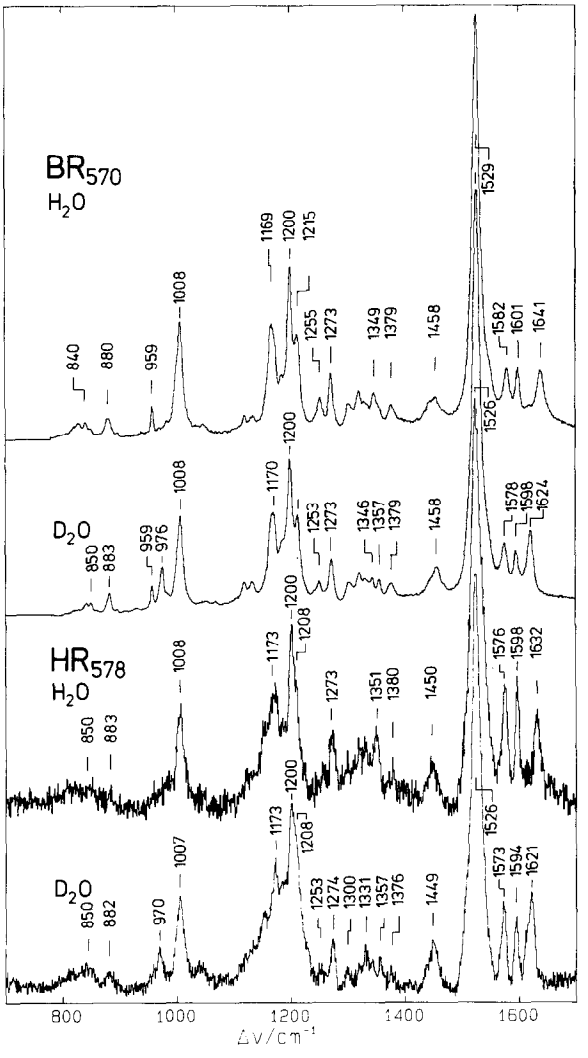

Fig.2. RR spectra of BR_{570} [21], in H_2O and in D_2O, excitation at 514 nm; and of HR_{578} in H_2O and in D_2O (cf. table 1).

to a lipid matrix (fig.2) are identical with those obtained earlier from isolated HR molecules in detergent solution (HR [12,18]) and also confirm that in the lipid matrix the retinylidene chromophore occurs in the all-*trans*,15-*anti* configuration as in BR_{570}. The striking similarity of the spectra of HR_{578} and BR_{570} is immediately obvious for the three C=C stretching modes. The slight downshift of the strong band from 1529 (BR) to 1526 (HR) is in accordance with the red-shifted absorption maximum of HR.

Additionally, in the fingerprint region between 1169 and 1215 cm^{-1} analogous features occur in the spectra of BR_{570} and HR_{578}. It must be noted that in HR the two bands around 1200 cm^{-1} are not clearly resolved. The asymmetric shape of this vibrational feature, however, indicates that a second band at 1208 cm^{-1} is involved. This is consistent with previously published spectra where the two bands were more clearly separated [10].

Of basic interest are the vibrational bands of the SB group which again are similar for BR_{570} and HR_{578}. The H_2O/D_2O couples at 1641/1624 and 1349/976 cm^{-1} in the spectra of BR have their counterparts at 1632/1621 and 1351/970 cm^{-1} in the spectra of HR. Quantitative differences, however, exist. The ($C=NH^+$) stretch frequency at 1632 cm^{-1} in HR_{578} is 9 cm^{-1} lower than in BR_{570}. Also the deuterium shift in HR of 11 cm^{-1} is significantly lower than that in BR of 17 cm^{-1}. Another difference concerns the (ND) rock which in BR is found at 976 cm^{-1}, while in HR this band occurs at 970 cm^{-1}. These differences trace back to the electronic structure of the SB group which is apparently modified by the protein environment in HR compared to BR_{570}. Here, it is accepted that the positively charged SB group interacts with an intrinsic negative counterion, B^- [24,25]. From RR spectroscopic experiments it was concluded that this ion-pair complex, SBH^+----B^-, is stabilized by water molecules which are located in the vicinity of the SB group [26].

Of much interest in this context are further RR spectroscopic studies on HR reported by Maeda et al. [27]. These authors showed that the frequency of the ($C=NH^+$) stretch at 1632 cm^{-1} (1635 cm^{-1} in [27]) is raised by 7 cm^{-1} when Cl^- is replaced by NO_3^-. This argues that at least one binding site for Cl^- in HR must be in the nearest neighbourhood of the chromophore and further raises the question as to whether Cl^- in HR plays the role of the counterion B^- in BR. Experiments with BR reported in [24] might help to provide an answer. Native BR_{570} can be converted to a red-shifted species BR_{605} (blue membrane) with a pK_a value of 2.9 [28]. If Cl^- is added to BR_{605} a different chromophore is formed with an equilibrium constant of about 1 M whose absorption maximum is shifted back to 565 nm. A similar effect is observed for F^- (0.1 M). This can be described formally by the reaction scheme:

Table 2

Comparison of the main features of the RR spectra of BR and HR

Group of bands	Wave number (cm^{-1})			Interpretation
	BR_{570}	HR (octyl-glucoside)	HR (lipid)	
(1) C=C stretch vibrations	1529	1522	1526	in-phase-stretching vibration along the central C=C bands (C_9 to C_{14})
	1582	1575	1576	out-of-phase combination of $C_{13}=C_{14}$ and $C_9=C_{10}$ stretch motion
	1601	1597	1598	$C_5=C_6$ stretch vibration
(2) Fingerprint region[a]	1200	1200	1200	mainly governed by C-C stretch
(a) Region 1170–1215	1169	1169	1173	vibrations; frequency and intensity are
	1215	1208	1208	characteristic of the geometry of the side chain C_7-C_{15}
(b) Region 1250–1360				normal modes of in-plane-bending motion of a single hydrogen atom
(3) Schiff base group[b]	1641→1624	1633→1621	1632→1621	$C=NH^+$ stretch vibration
	1349→ 976	1349→ 968	1351→ 970	NH rock (in-plane H bending)

[a] Many of the bands in the fingerprint region are governed by strong C-C/CH coupling, therefore the assignment is still under discussion

[b] The shift of the frequency upon deuteration of the Schiff base is indicated by the arrow

$$BR_{570} \underset{}{\overset{H^+,pK\ 2.9}{\rightleftharpoons}} BR_{605} \overset{Cl^-,F^-}{\rightleftharpoons} BR_{565}(Cl^-),BR_{545}(F^-)$$

$$SBH^+\text{--}B^- \qquad SBH^+,BH \qquad SBH^+\text{--}Cl^-,SBH^+\text{--}F^-$$

in which the second row gives the molecular interpretation suggested in [24]. In this mode B^- is neutralized in a first step to BH giving the red-shifted form BR_{605}. When the ionic strength is further increased, Cl^- or F^- occupies a binding site in the vicinity of the SB group and simulates the role of the counterion shifting λ_{max} back to the range of the original values. This interpretation could be supported by RR spectroscopic studies [29]. Thus, in the RR spectrum of BR_{605} the bands of the SB group are degenerated to broad features indicating that in this species the SB group is fairly mobile and therefore occurs in a variety of different conformational states. This picture changes completely in the RR spectra of BR_{565} (Cl^-) and BR_{545} (F^-). The characteristic bands of the SB group are narrow, suggesting that this group is fixed to the counterions. Such spectra closely resemble those of BR_{570} indicating that the structure of the chromophore with Cl^- or F^- as a counterion is essentially the same as that of the native species. In summary, it is confirmed by RR spectroscopic evidence that under conditions where the native counterion B^- is decoupled from the SB group it can be replaced by Cl^- or F^- to form a chromophore whose structure is very similar to that of BR_{570}.

If in HR_{578} Cl^- were to act as a counterion the effects on the SB bands would be similar to those in BR_{565} (Cl^-). The data in table 3 confirm this expectation. Thus, with respect to BR_{570} the frequency of the ($C=NH^+$) stretch of both species decreases by several cm^{-1} and also the deuterium shift is significantly lower. A similar tendency occurs for the ND rock in the 970 cm^{-1} region. Although the effects in BR_{565} (Cl^-) are smaller than in HR_{578} we consider these data as an argument that in HR_{578} the Cl^- acts as a counterion of the SB group, or at least, is largely involved in the ionic structure.

3.2. *HR_{520}*

In fig.3 the RR spectra of the intermediate HR_{520} and its BR analogue L_{550} are shown. The similarities between the two spectra are obvious. It

Table 3

	Energy $\tilde{\nu}$ (cm^{-1})			
	$\nu(C=NH^+)$	$\nu(C=ND^+)$	$\gamma(NH)$	$\gamma(ND)$
BR_{570}	1641	1624	1349	976
HR_{578}	1632	1621	1351	970
BR_{565} (Cl^-)	1636	1622	1347	973
BR_{545} (F^-)	1639	1622	1348	974

Frequencies of the ($C=NH^+$) stretch, (NH) rock and their deuterium analogues in HR_{578} (this study); BR_{570} and the two species BR_{565} (Cl^-) and BR_{545} (F^-), which were obtained at low pH and high ionic strength [29]. The frequencies of the two latter species were determined on a relative scale with respect to the RR bands of BR_{570} and therefore are slightly different from those given in [29]

is generally accepted that in the primary photochemical event of BR at least a rotation around the $C_{13}=C_{14}$ double bond is involved and L_{550}, which is formed from the primary photoproduct in a thermal relaxation process, also has the 13-*cis* configuration [15]. This is expressed in the vibrational spectra which in all details are different from those of BR_{570} (cf. spectra in figs 2,3). On the other hand, the frequencies of the C=C and C-C stretches are very similar in L_{550} and HR_{520}. We therefore interpret HR_{520} as a 13-*cis* configuration of the retinylidene chromophore.

Compared to BR_{570} significant changes also occur in the vibrational features of the SB group of L_{550}. The deuterium shift of the ($C=NH^+$) stretch at 1642 cm^{-1} is 24 cm^{-1}, which is 1.5-times larger than in BR_{570}. The (NH) rock at 1350 cm^{-1} in BR_{570} is not seen in L_{550}. Instead, there is a weak shoulder at 1398 cm^{-1} in the H_2O spectrum which disappears in D_2O. Obviously the N-H bending motion is involved in this band. An interesting observation can be made in the 1000 cm^{-1} region. In D_2O the relatively strong band at 1009 cm^{-1} loses intensity while a new band arises at 985 cm^{-1}. This is explained in the following way. In the native compound (H_2O) the two hydrogen atoms of the SB group carry out a coupled out-of-plane vibration at 1009 cm^{-1}. This band coincides accidentally with the well-known 'methyl-rock' vibration. In D_2O the ND out-of-plane component of this vibration shifts down below 800 cm^{-1} and

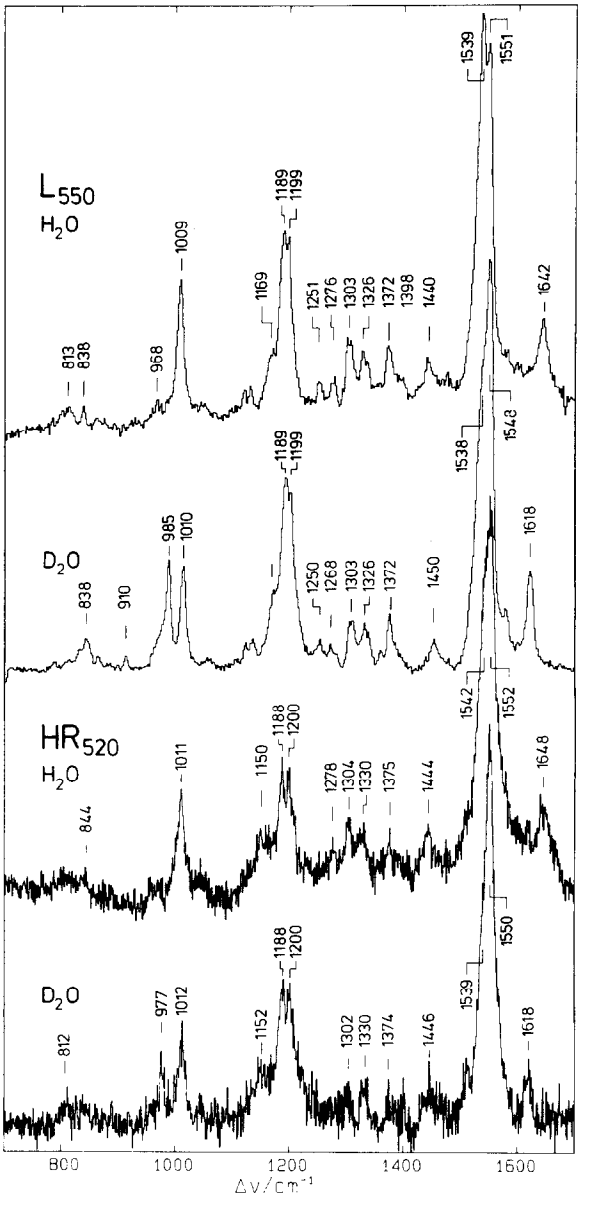

Fig.3. RR spectra of L_{550} [31], in H_2O and in D_2O, λ_{pump} = 647 nm, λ_{probe} = 514 nm; and of HR_{520}, in H_2O and in D_2O (cf. table 1).

the remaining C_{15}-H out-of-plane component gives rise to the band at 985 cm^{-1}. The methyl-rock vibration accounts for the residual intensity at 1009 cm^{-1} in D_2O. The fact that the mode at 985 cm^{-1} attains so much RR intensity reveals that in L_{550} the SB group is distorted with respect to the C_{14}-C_{15} and/or the C=N bond. The spectroscopic

behaviour in the 1000 cm^{-1} region, therefore, is quite characteristic of the SB group in L_{550}.

It is immediately seen from fig.3 that the main vibrational features in the spectra of HR_{520} are closely related to those of L_{550}. Indeed, in the C=C stretching region again a broad feature with a peak at 1552 and a shoulder at 1542 cm^{-1} are observed, indicating that two bands are involved. The isotope shift of the ($C=NH^+$) stretch again is very high (30 cm^{-1}), the fingerprint region is governed by two peaks at 1188 and 1200 cm^{-1}, and the typical behaviour of the SB hydrogen modes in the 1000 cm^{-1} region is also observed. Differences between the two spectra concern the slightly different intensity ratio of the two C=C stretch components at 1552 and 1542 cm^{-1}, the somewhat higher isotope shift of the ($C=NH^+$) stretch, and finally the down-shift of the C_{15}-H out-of-plane mode from 985 to 977 cm^{-1}.

Considering the resemblance of the RR spectra of L_{550} and HR_{520}, it seems obvious that HR_{520} is a protonated species in the 13-*cis* configuration. However, the small spectral differences must indicate the different interaction of retinal with the environment in the two proteins which favour the reaction path to the red-absorbing HR_{640} and suppress that to the deprotonated HR^L_{410}, whereas in BR an inverse behaviour, favouring deprotonation, occurs (fig.1).

3.3. HR^L_{410}

An inspection of the RR spectra of HR^L_{410} and M_{412} in fig.4 reveals great similarities. Both spectra are dominated by a single strong band in the C=C stretching region which in M_{412} occurs at 1566 cm^{-1} and in HR^L_{410} at 1568 cm^{-1}. The near-coincidence of these bands is consistent with the coincidence of the absorption maxima of the two species.

H_2O-D_2O exchange has no effect in the RR spectrum of M_{412}, indicating that this intermediate has a deprotonated SB. The weak band at 1621 cm^{-1} in M_{412} was assigned as the (C=C) stretching mode of the SB group. An analogous feature occurs at 1622 cm^{-1} in the RR spectrum of HR^L_{410}.

The (C-C) stretching vibrations in the fingerprint region (M_{412}: 1180, 1198, 1228 cm^{-1}) appear in a corresponding way in the spectrum of HR^L_{412}. On the other hand, some obvious differences occur in the upper fingerprint region between 1270 and

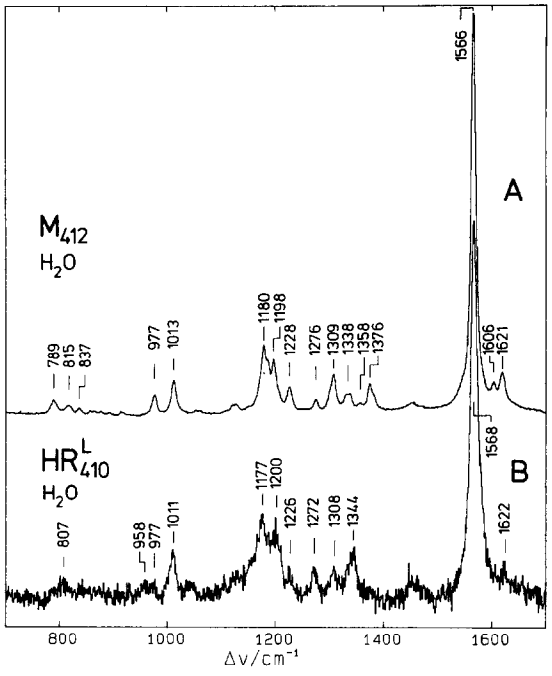

Fig.4. RR spectra of M_{412} [32] in H_2O, λ_{pump} = 514 nm, λ_{probe} = 413 nm and of HR^L_{410} in H_2O; for experimental parameters, see table 1.

1380 cm^{-1}. In particular, this concerns the band at 1376 cm^{-1} in M_{412} which is partially due to an ($C_{15}H$) in-plane bending mode and which does not occur in HR^L_{410}.

In view of the RR spectroscopic analogy to M_{412} we conclude that HR^L_{410} is formed as a deprotonated species in HR. The nearly identical vibrational pattern in the lower fingerprint region is an indication of the 13-*cis* configuration in HR^L_{410} as well. The residual differences between the two spectra in fig.4 reveal minor conformational differences of the two chromophores.

4. CONCLUSIONS

The RR spectra of the three species HR_{578}, HR_{520} and HR^L_{410} exhibit obvious similarities to those of the analogous RR spectra of BR_{570}, L_{550} and M_{412}. Our data suggest that retinal bound via an SB to different proteins serving as light-driven, charge-separating, unidirectional pumps occurs in very similar environment not only in the parent species but also in several intermediates.

Nevertheless, differences between the RR spectra of the HR species and those of the corresponding BR species, which are not negligible, also merit further investigation. Especially, the study of the dependence between the concentration of various anions and the structure of the chromophore intermediates should yield important information about the binding sites of anions, their influence on chromophore stabilisation and their role in chloride transport activity.

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