Chromophore−Anion Interactions in Halorhodopsin from *Natronobacterium pharaonis* Probed by Time-Resolved Resonance Raman Spectroscopy[†]

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ABSTRACT: Halorhodopsin of *Natronobacterium pharaonis* which acts as a light-driven chloride pump is studied by time-resolved resonance Raman spectroscopy. In single-beam experiments, resonance Raman spectra were obtained of the parent state HR_{578} and the first thermal intermediate HR_{520}. The parent state is structural heterogeneous including ca. 80% *all-trans* and 20% *13-cis* isomers. The resonance Raman spectra indicate that the *all-trans* conformer exhibits essentially the same chromophoric structure as in the parent states of bacteriorhodopsin or halorhodopsin from *Halobacterium salinarium*. Special emphasis of the resonance Raman spectroscopic analysis was laid on the C═C and C═N stretching region in order to probe the interactions between the protonated Schiff base and various bound anions (chloride, bromide, iodide). These investigations were paralleled by spectroscopic studies of retinal Schiff base model complexes in different solvents in an attempt to determine the various parameters which control the C═C and C═N stretching frequencies. From these data, it was concluded that in the parent state the anion is not involved in hydrogen bonding interactions with the Schiff base proton but is presumably bound to a nearby (positively charged) amino acid residue. On the other hand, the anion still exerts an appreciable effect on the chromophore structure which is, for instance, reflected by the variation of the isomer composition in the presence of different anions and in the anion-depleted form. In contrast to the parent state, the intermediate HR_{520} reveals frequency shifts of the C═N stretching in the presence of different anions. These findings indicate a closer proximity of the bound anion to the Schiff base proton which is sufficient for hydrogen bonding interactions. These changes of the anion−chromophore interaction upon transition from HR_{578} to HR_{520} may be related to the coupling of the chromophore movement with the anion translocation.

A variety of halophilic archaebacteria contain retinal proteins which act as light-driven ion pumps (Lanyi, 1990; Oesterhelt et al., 1992a,b; Oesterhelt, 1995). The best characterized representative of this class of proteins is bacteriorhodopsin (BR)[1] which is the main membrane pigment in *Halobacterium salinarium* (Oesterhelt & Stoeckenius, 1973). Upon irradiation with visible light, the retinal which is covalently attached to the polypeptide chain via a Schiff base linkage to a lysine residue undergoes an *all-trans*→13-*cis* photoisomerization which is followed by a sequence of thermal reactions including conformational and configurational changes of the retinal chain as well as a de- and reprotonation of the Schiff base. The structural changes of the chromophore are linked to the translocation of a proton through the protein eventually leading to a proton gradient across the membrane which in turn is used to drive ATP synthesis.

The bacterial retinal protein of *H. salinarium*, halorhodopsin (HR), functions as a light-driven chloride pump. The photocycle of HR is coupled to the electrogenic transport of chloride across the membrane into the cell (Schobert & Lanyi, 1982; Bamberg et al., 1984; Oesterhelt, 1995). The different functions of BR and HR are striking in view of their structural similarity including a ∼30% amino acid identity and the same *all-trans*-retinal structure in the parent states (Lanyi et al., 1990). Moreover, the primary photochemical process is as in BR an *all-trans*→13-*cis* isomerization. Thus, it has been suggested that both photocycles exhibit common mechanistic features which trigger the ion translocation (Oesterhelt et al., 1992b; Haupts et al., 1997). On the other hand, the reaction sequence following the photochemical process is different as compared to BR. In particular, evidently no de- and reprotonation of the Schiff base occur. Clearly, a deeper understanding of the similarities and differences in the structure−function relationships of both proteins requires a more comprehensive analysis of the structural and dynamic aspects of HR's photocycle.

Among the techniques which have successfully been employed for studying BR, resonance Raman (RR) spectroscopy is of particular importance since it exclusively probes the vibrational band pattern of the retinal chro-

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[1] Abbreviations: BR, bacteriorhodopsin; HR, halorhodopsin; NRB, *all-trans-N*-retinylidene-*n*-butylamine; RR, resonance Raman; FT, Fourier-transform.

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mophore, i.e., the photoactive center [for reviews, see Curry, et al. (1985), Smith et al. (1985b), Stockburger et al. (1986), Mathies et al. (1987), and Althaus et al. (1995)]. In addition, time-resolved experiments can sensitively monitor the temporal evolution of the intermediates, thereby providing simultaneously structural and kinetic data of the photocycle.

In a few studies, RR spectroscopy has also been applied to HR from *H. salinarium* (Smith et al., 1984; Alshuth et al., 1985; Maeda et al., 1985; Diller et al., 1987; Fodor et al., 1987; Pande et al., 1989; Ames et al., 1992); however, such experiments were hampered by the instability of the protein and interference by the fluorescence background. Thus, high-quality spectra which are a prerequisite for structural and kinetic studies of the photocycle are difficult to obtain, and, so far, only two intermediates have been probed by this technique. In this respect, HR from *Natronobacterium pharaonis* is more appropriate for RR spectroscopic studies. This protein has been found homologous to HR of *H. salinarium* as indicated by a ~65% amino acid identity (Lanyi et al., 1990). In addition, HR of *N. pharaonis* offers the advantage that complexes with various anions as well as the anion-depleted state can be prepared in a well-defined manner, allowing for the investigation of specific anion interactions with the retinal binding site (Scharf & Engelhard, 1994). This is of particular importance as previous studies on HR from *H. salinarium* have provided conflicting results (Pande et al., 1989; Walter & Braiman, 1994).

In the present work, we report a RR spectroscopic study of HR from *N. pharaonis* loaded by different anions. In addition, we have extended the spectroscopic investigations to retinal Schiff base model compounds. In this way, it is possible to gain more insight into the specific anion−chromophore interactions in HR which in turn may contribute to a better understanding of the anion pump mechanism in HR and, moreover, of the (light-driven) ion translocation processes in general.

MATERIALS AND METHODS

Sample Purification and Preparation. HR from *N. pharaonis* was isolated and purified as described elsewhere (Scharf & Engelhard, 1994). For RR spectroscopic measurements, the protein (30 μM), solubilized in 0.1% dodecyl maltoside, contained 10 mM citrate/phosphate buffer (pH 6.1). H/D exchange was achieved by centrifuging the sample and resuspending the pellets in D_2O-containing buffer. Preparation of the anion-depleted form and preparation of the complexes loaded with specific anions followed the procedure described by Scharf and Engelhard (1994). The complex obtained upon rebinding chloride to the anion-depleted form exhibits the same spectral properties as the native protein. Chromophore extraction and subsequent determination of the isomeric composition were carried out as described by Scherrer et al. (1989).

all-trans-Retinal was purchased from Sigma. *all-trans*-*N*-Retinylidene-*n*-butylamine (NRB) and its tetrafluoroborate salt (NRB-HBF_4) were synthesized according to Favrot et al. (1979). The chloride salt (NRB-HCl) was prepared in an analogous way by bubbling gaseous HCl through the NRB solution. The solid NRB-DCl was obtained by H/D exchange in CH_3OD solution and subsequent removal of the solvent under vacuum. Since the addition of H_2O from 0.02 to 0.1 M in the organic solutions of NRB-HCl and NRB-HBF_4 did not affect the infrared and Raman bands, the deuterated NRB-DCl and NRB-DBF_4 complexes in solution were directly prepared by adding D_2O (up to 0.1 M) to the organic solvents initially dried on molecular sieves. There was no spectroscopic indication for iminium hydrolysis in such samples. All solvents used were of spectroscopic grade.

Spectroscopic Measurements. RR spectra of HR were measured with the 514-nm line of an Ar^+ laser using a scanning double monochromator equipped with a photon counting system. The spectral resolution was 2.2 cm^{-1} (with 0.2-cm^{-1} increments) or 3.0 cm^{-1} (with 1.0-cm^{-1} increments). The signal-to-noise ratio was improved upon repetitive scanning so that the total accumulation time was between 10 and 20 s per data point. The resetability of the monochromator during the experiments was $\pm$0.1 cm^{-1} as repeatedly checked by calibration against the position of the laser line. The sample was deposited in a rotating cell with variable rotational frequencies. All measurements were carried out at ambient temperature. Further details of the experimental setup are described elsewhere (Hildebrandt et al., 1993).

The single-scan spectra of each experiment were carefully compared and only combined if no spectral differences were noted. The spectra obtained in this way included a structureless background which was removed by polynomial subtraction. The spectra were analyzed by a fitting program as described elsewhere (Hildebrandt et al., 1993; Döpner et al., 1996).

Fourier-transform (FT) Raman spectra (1064-nm excitation), FT-IR, and IR-spectra of the model compounds were measured with Perkin Elmer 2000, 1720, and 983 instruments, respectively. UV−Vis absorption spectra were obtained by a Cary 3 spectrophotometer. The mulls of the solid samples for the infrared study were made either in Fluorolube or in Nujol. The concentrations of the NRB solutions were 2.2×10^{-4} M for UV−Vis and 2.2×10^{-2} M for infrared and Raman experiments. All manipulations of the samples were carried out in the dark using a glovebox under an argon atmosphere.

RESULTS AND DISCUSSION

Resonance Raman Spectroscopy of Halorhodopsin. In order to maintain a defined composition of the various states of HR in the exciting laser beam, the experimental conditions of the RR measurements had to be adapted to the specific kinetic and spectral properties of HR. In a RR experiment, the photocycle is initiated by the laser line of the wavelength (λ) according to a photochemical rate constant (l_0) as defined by eq 1:

$$l_0 = \frac{\ln 10}{\sqrt{2\pi} N_A h c} \gamma \epsilon(\lambda) \frac{P_0}{r^2} \qquad (1)$$

where γ is the quantum yield of the primary photoprocess, $\epsilon(\lambda)$ the extinction coefficient of HR_{578} at λ, P_0 the laser power at the sample, and r the radius of the spherically focused laser beam in the sample (Althaus et al., 1995). N_A, h, and c denote the Avogadro number, the Planck constant, and the velocity of light, respectively. The residence time Δt of the sample in the laser beam is defined by eq 2:

$$\Delta t = \frac{r}{\pi \nu_0 R} \quad (2)$$

where R and ν_0 are the radius and the rotational frequency of the cell. As the product of both parameters, Δt and l_0, controls the degree of photoconversion of HR_{578}, the appropriate choice of P_0 and ν_0 in a single-beam time-resolved RR experiment allows for probing either the parent state ($l_0\Delta t \ll 1$) or a mixture of the parent and intermediate states ($l_0\Delta t > 1$) (Stockburger et al., 1979). In any case, ν_0 has to be adjusted in such a way that the thermal back-conversion to the parent state HR_{578} is essentially complete before the photolyzed sample reenters the laser beam; i.e., the rotational period of the cell ($1/\nu_0$) must be larger than the time constant of the rate-limiting step of the photocycle ("fresh-sample" condition).[2]

RR Spectra of the Parent State HR_{578}. Figure 1 shows the RR spectra of the chloride-bound HR in H_2O and D_2O measured with $P_0 = 4.5$ mW and $\nu_0 = 60$ s^{-1}. Assuming the same quantum yield for the primary photochemical process as in BR, i.e., 0.65 (Schneider et al., 1989), l_0 and Δt are calculated according to eqs 1 and 2, yielding a value of 0.1 for $l_0\Delta t$. Under these conditions, one can estimate that these RR spectra should reflect about 95% the parent state.

The RR spectra in Figure 1 display the region between 1480 and 1680 cm^{-1} which is dominated by the C═C stretching vibrations of the retinal chain. These modes have been shown to be particularly useful to distinguish between the various states of the retinal chromophore and for probing their time dependence (Smith et al., 1985b; Althaus et al., 1995). It can clearly be seen that in H_2O (Figure 1A) the dominant peak at 1525 cm^{-1} ($\nu_{C=C}$) is asymmetric and, in fact, the band-fitting analysis reveals two components at 1525.0 and 1538.4 cm^{-1}. According to the empirical correlations between the wavelength of the absorption maximum (λ_{max}) and the $\nu_{C=C}$ frequency (Heyde et al., 1971; Rothschild et al., 1984), the 1525-cm^{-1} band corresponds to the HR_{578} state. On the other hand, a stretching frequency at 1536 cm^{-1} would be related to a chromophore absorbing at 555 nm which does not fit either to the primary photoproduct or to the first thermal intermediate (Varo et al., 1995). In addition, upon using a laser power between 4.5 and 1.5 mW, the RR spectra are indistinguishable in the entire spectral range, and, in particular, the intensity ratio of the 1525- and 1538-cm^{-1} bands remains at a constant value of ca. 5.5. This implies that, in a good approximation, the contribution of any photocycle intermediate to the RR spectrum measured with 4.5 mW power can be neglected so that it is regarded as an essentially pure spectrum of the parent state HR_{578}.[3]

On the other hand, it may be that the parent state is heterogeneous as found for HR from *H. salinarium* (Maeda et al., 1985). This would imply that the two bands at 1525 and 1538 cm^{-1} originate from two structurally different chromophores. In fact, we have determined the isomer compositions of the chromophores extracted from halorhodopsin yielding 80% *all-trans* and 20% *13-cis* isomer. This finding implies that the RR spectrum of HR_{578} includes both isomers in a ratio of 4. This value compares very well with that of the RR band intensities (5.5) taking into account that the RR scattering cross sections of the 1525- and 1538-cm^{-1} bands may be slightly different. Most remarkably, the latter band nearly disappears in D_2O which would indicate a lower content of the *13-cis* isomer. In fact, extraction experiments from light-adapted samples in D_2O yield 84% *all-trans* and 16% *13-cis*, corresponding to an isomeric ratio of 5.25. Assuming the ratios of the RR cross sections of the C═C stretching modes of both isomers are the same in protonated and in the deuterated forms, one would expect a band intensity ratio of 7.2 for HR_{578} in D_2O. Indeed, the lower intensity of the C═C stretching mode of the *13-cis* form in D_2O relative to that of the *all-trans* isomer (and a potential stronger overlap brought about by opposite frequency shifts of both bands) may make it impossible to identify the 1538-cm^{-1} band in the RR spectrum by band fitting. The overlap of both bands would also account for the increased intensity of the 1525-cm^{-1} peak relative to the other bands in this region, e.g., at 1576 and 1597 cm^{-1}.

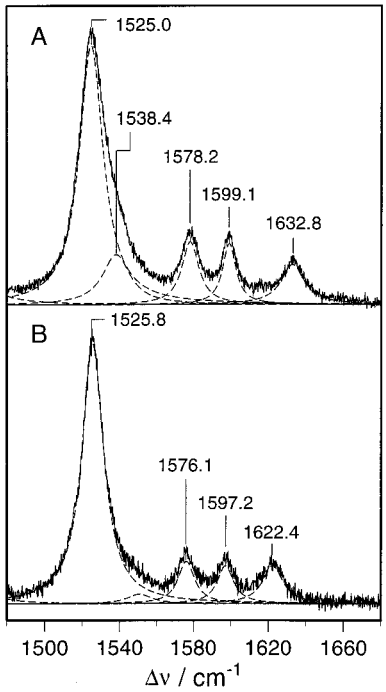

FIGURE 1: RR spectra in the C═C stretching region of the chloride-bound HR_{578} solubilized in H_2O (A) and D_2O (B). The laser power was 4.5 mW and the rotational frequency of the cell 60 s^{-1}. The dashed lines represent fitted Lorentzian bands.

The distribution between two retinal isomers in HR_{578} is not a unique feature of halorhodopsins, but it is well-known also for the parent state of BR which exhibits an *all-trans*/*13-cis* ratio of about 1:1 in the dark-adapted form. Upon illumination (light-adaptation), BR is completely converted to the *all-trans* form due to an efficient *13-cis*→*all-trans* photoisomerization. An analogous process in HR must be associated with a much smaller quantum yield. In extraction experiments, we have found that the same illumination conditions, which in BR provide a complete conversion to

[2] In a good approximation, the "fresh sample" condition is fulfilled for rotational frequencies between 60 and 20 s^{-1} as the accumulation of long-lived intermediate HR_{640} with a decay time of 1.4 ms is calculated to be less than 0.5% (Varo et al., 1995; Chizhov and Engelhard, unpublished results). A long-lived species with a very low amplitude which was inferred from flash photolysis studies is ascribed to the independent photocycle of the parent *13-cis* component of HR and is not enriched during the RR experiments.

[3] A more detailed spectral analysis reveals a very weak contribution from the HR_{520} state reflected by a band at 1550 cm^{-1} with an intensity of 1.5% of that of the parent state. In D_2O, this band is slightly higher.

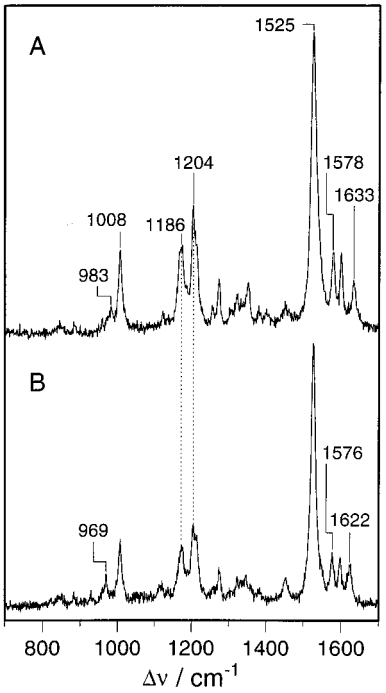

FIGURE 2: Overview RR spectra of the chloride-bound HR_{578} solubilized in H_2O (A) and D_2O (B). The laser power was 4.5 mW and the rotational frequency of the cell 60 s^{-1}.

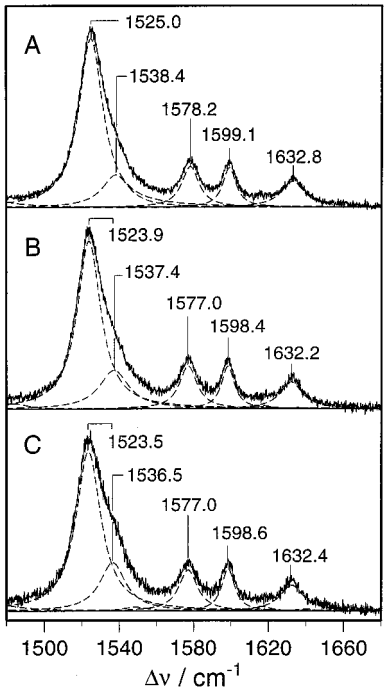

FIGURE 3: RR spectra in the C═C stretching region of the chloride-bound (A), bromide-bound (B), and iodide-bound (C) HR_{578} in H_2O. The laser power was 4.5 mW and the rotational frequency of the cell 60 s^{-1}. The dashed lines represent fitted Lorentzian bands.

the *all-trans* form, yield only a slightly higher *all-trans* content (i.e., 80%) in HR compared to samples which have been kept in the dark (77%). These results are in good agreement with recent findings by Varo et al. (1995) and Zimanyi and Lanyi (1997). In addition, we have found that increasing the laser power in the RR experiments by a factor of 10 does not lead to a decrease of the *13-cis* contribution.

The overview RR spectra of $HR_{578}(Cl^-)$ in both H_2O and D_2O are shown in Figure 2. The so-called fingerprint region between 1150 and 1250 cm^{-1} is of particular interest because the modes involved are dominated by C−C stretches which are sensitive markers for the configuration and conformation of the retinal (Mathies et al., 1987; Althaus et al., 1995). In $HR_{578}(Cl^-)$, two prominent bands are observed at 1186 and 1204 cm^{-1} while weaker bands may contribute to the shoulders at 1172 and 1212 cm^{-1}. In D_2O, these bands vary only slightly. Both the frequencies and the relative intensities as well as their isotopic shifts are very similar to those observed for the parent states of bacteriorhodopsin and halorhodopsin of *H. salinarium*, BR_{570} and HR_{578} (Smith et al., 1984; Alshuth et al., 1985; Maeda et al., 1985). This comparison strongly suggests that not only the configuration (*all-trans*) but also the conformation of the retinal Schiff base is essentially the same in all three species, i.e., an *anti* conformation (Smith et al., 1987).

Deviations from planarity of the polyene structure should be reflected by the C−H o.o.p. modes which are expected in the region between 1000 and 800 cm^{-1} (Eyring et al., 1982). The RR activity in this part of the spectrum of $HR_{578}(Cl^-)$ in H_2O is very low, indicating a largely planar structure of the chromophore. The only clearly identifiable band is the one at 983 cm^{-1} which appears to be present also in the RR spectra of HR_{578} and BR_{570} of *H. salinarium* but with a much weaker intensity (Alshuth et al., 1985; Althaus et al., 1995). Conversely, the RR spectrum of BR_{570} displays a sharp peak at 959 cm^{-1} which in HR_{578} cannot be detected unambiguously (FIgure 2).

The 983-cm^{-1} band of $HR_{578}(Cl^-)$ is sensitive toward H/D exchange at the Schiff base although the signal-to-noise ratios of the spectra do not allow for a decision whether in D_2O this band is shifted or just has lost intensity. Thus, at this point, one may suggest that in H_2O the 983-cm^{-1} mode may include contributions of the N−H o.o.p. and/or internal vibrations of the lysine residue in order to account for the H/D sensitivity.

On the other hand, the 969-cm^{-1} band of $HR_{578}(Cl^-)$ in D_2O is readily assigned to a mode dominated by N−D i.p. bending (Smith et al., 1985b). Its counterpart in H_2O is assigned to the 1351-cm^{-1} band. This band in fact disappears in D_2O while the weak band close to this position (1345 cm^{-1}) is attributed to a mode involving C(15)−H i.p. bending in analogy to BR_{570}.

The stretching coordinate of the Schiff base provides the main contribution to the mode at 1632.8 cm^{-1} ($\nu_{C=N}$; Figure 1). In fact, upon H/D exchange, this band shows the strongest frequency downshift in this region, i.e., 10.4 cm^{-1}, whereas the nearby bands at 1599.1 and 1578.2 cm^{-1} reveal substantially smaller downshifts (1.9 and 2.1 cm^{-1}, respectively). The frequency downshift of the C═N stretching mode at 1632 cm^{-1} is accompanied by a band-narrowing of 2.0 cm^{-1} which is slightly more than the average variations of the half-widths of the other bands below 1600 cm^{-1} but clearly less than previously observed for the C═N stretching in BR_{570} (Hildebrandt & Stockburger, 1984).

When in HR Cl^- is quantitatively replaced by Br^- or I^-, the RR spectra of HR_{578}, measured under the same conditions as described above, reveal only minor changes indicating that the overall chromophore structure remains largely the same as in the Cl^--bound complex (Figure 3; Table 1). In particular, the relative intensity and the frequency of the $C{=}NH^+$ stretching mode at 1632 cm^{-1} do not appear to be affected by the anion replacement since the frequency variations of the fitted Lorentzian bands are within the

Table 1: Frequencies of the C═C and C═N Stretching Modes of Various States of Halorhodopsin[a]

state	anion	$\nu_{C=C}$	$\nu_{C=N}$
HR_{578}	Cl^-	1525.0	1632.8
	Br^-	1523.9	1632.2
	I^-	1523.5	1632.4
HR_{520}	Cl^-	1550.3	1651.5
	Br^-	1549.4	1649.4
	I^-	1548.7	1648.9

[a] Frequencies are given in cm^{-1}. The accuracy is ca. ± 0.3 cm^{-1} and ± 0.5 cm^{-1} for the $\nu_{C=C}$ and $\nu_{C=N}$ modes, respectively.

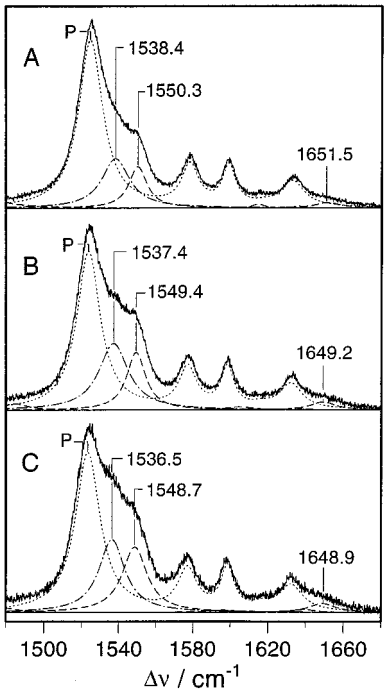

FIGURE 4: RR spectra in the C═C stretching region of the HR in H_2O loaded with different anions (A, chloride; B, bromide; C, iodide), measured with 25 mW laser power and 20 s^{-1} rotational frequency of the cell. The component spectra of the parent *all-trans* (denoted by "P") and *13-cis* states are represented by the dotted (▪▪▪▪) and dashed-dotted (-▪-▪-) lines, respectively. The dashed lines (----) represent the fitted Lorentzian bandshapes attributable to the intermediate HR_{520}.

accuracy of the fits. The only detectable alterations refer to the C═C stretching mode. In the Br^-- and I^--bound complex, this mode shifts down by ca. 1.1 and 1.5 cm^{-1}, respectively, as compared to the Cl^--bound form. A similar tendency seems to hold for the C═C stretching of the *13-cis* form although the frequency determination of this component is much less accurate due to its weaker intensity and stronger overlap with the band of the *all-trans* form. However, the intensity of this mode relative to that of the *all-trans* state increases in the order of $Cl^- < Br^- < I^-$.

RR Spectra of Intermediates. Upon increasing the laser power ($P = 25$ mW) and lowering the rotational frequency of the cell ($\nu_0 = 20$ s^{-1}), the photoconversion parameter $l_0\Delta t$ is raised to 1.7, and the dwell time is increased to 140 μs. Under these conditions, a substantial population of the first thermal intermediate HR_{520} (Varo et al., 1995; Chizhov and Engelhard, unpublished results) in the RR probe beam is expected. In fact, drastic changes are noted in the RR spectra of the various anion-loaded HR complexes (Figure 4). All three spectra display a new band at ca. 1550 cm^{-1} which is unequivocally attributed to the intermediate HR_{520}. Moreover, there is a distinct intensity decrease of the 1525-cm^{-1} band (denoted by "P") relative to that at 1540 cm^{-1}. This finding implies that only the *all-trans* chromophore runs through a photocycle or, at least, a phototransformation of the *13-cis* state is much less efficient. As discussed above, an alternative assignment of the 1540-cm^{-1} bands in these RR spectra to an additional intermediate of HR's photocycle is not very likely since this frequency would correspond to a species absorbing at ca. 550 nm which, however, was not detected in transient absorption experiments (Varo et al., 1995; Chizhov and Engelhard, unpublished results).

In the C═N stretching region, the RR spectra reveal shoulders at ca. 1649 cm^{-1} in all three complexes (Figure 4) which have grown-in concomitant to the 1550-cm^{-1} band and, hence, are also attributed to the intermediate HR_{520}. This finding is in good agreement with previous results on *H. salinarium* by Fodor et al. (1987) and Diller et al. (1987), who also observed a substantial upshift of the $\nu_{C=N}$ mode from the parent state to the first thermal intermediate. However, the $\nu_{C=N}$ frequencies of the latter are still lower than in HR_{520} of *N. pharaonis*. This may be taken as an indication for subtle structural differences of the retinal and anion binding sites in HR_{520} between both proteins.

An additional weaker band in this region at ca. 1636 cm^{-1} (at similar frequency in all three spectra of Figure 3) is identified by the band-fitting analysis and attributed to the C═N stretching of *13-cis* isomer. Following these assignments, one can obtain RR spectra of the intermediate HR_{520} by subtracting the spectrum of the parent state (*all-trans*) as well as the 1636- and 1540-cm^{-1} bands attributable to the *13-cis* isomer. The underlying assumption is that the 1540-cm^{-1} band *exclusively* originates from the *13-cis* isomer. However, it cannot be ruled out that the C═C stretching in HR_{520} is split into two components similar to the corresponding intermediate of HR from *H. salinarium* which exhibits a dominant band at 1552 cm^{-1} and a shoulder at ca. 1542 cm^{-1} (Diller et al., 1987). Then, the 1540-cm^{-1} peaks in the spectra of Figure 3 would also include minor contributions from HR_{520}. In this respect, the resultant spectra, which are displayed in Figure 5, must regarded as approximate. The $\nu_{C=C}$ and $\nu_{C=N}$ frequencies of HR_{520} complexed with different anions are listed in Table 1.

Anion-Depleted HR. Removal of the anion from HR leads to a significant red-shift of the absorption maximum to ca. 600 nm. Also the anion-depleted HR undergoes a photoinduced reaction cycle (Scharf & Engelhard, 1994); however, kinetic data and spectral properties of the intermediates involved are not known in detail. Thus, the RR spectra of the anion-depleted form were measured under the same conditions as in the case of the anion-bound species (Figure 6A). However, even at a laser power as low as 4.5 mW, the RR spectrum does not reflect a uniform state rather than a mixture involving various intermediates. This conclusion is drawn from a careful inspection of the C═C stretching region which reveals a broad peak including, at least, four components. In particular, the shoulder at ca. 1534 cm^{-1} increases upon raising the laser power and lowering the rotational frequency (Figure 6B), implying that it results from species formed during the photoinduced reaction cycle. In order to obtain crude spectra of the parent and the intermediate states, we have subtracted both spectra from each other. The spectrum in Figure 6C, hence, should reflect largely the parent state which stills reveals two bands in the C═C

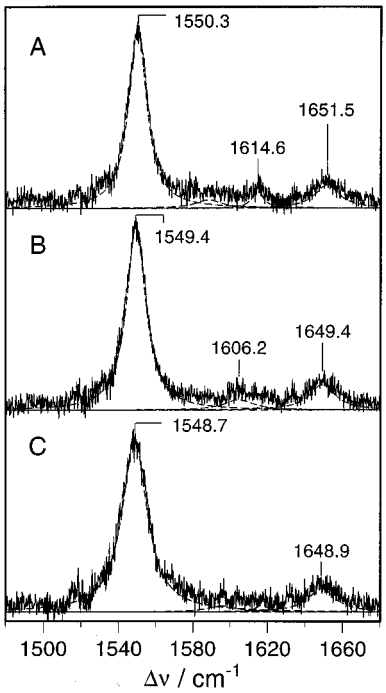

FIGURE 5: RR spectra of the HR_{520} intermediates of the chloride-bound (A), bromide-bound (B), and iodide-bound (C) HR in H_2O by subtracting the contribution of parent states (*all-trans* and *13-cis*) from the RR spectra measured at high laser power (Figure 4).

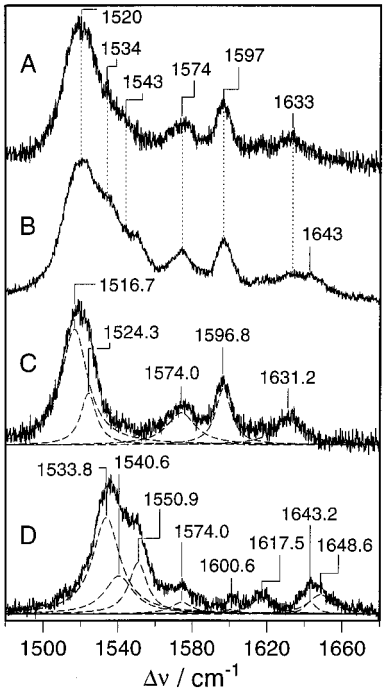

FIGURE 6: RR spectra in the C═C stretching region of the anion-depleted HR in H_2O. A, spectrum measured with 4.5 mW laser power and 60 s^{-1} rotational frequency of the cell; B, spectrum measured with 25 mW laser power and 20 s^{-1} rotational frequency of the cell. The pure spectra of the parent (C) and the intermediate (D) states were obtained after subtracting the component spectra of the intermediates from the spectrum A and that of the parent state from spectrum B, respectively. The dashed lines represent the fitted Lorentzian bands.

stretching region. In analogy to the anion-bound complexes, we assign the bands at 1516.7 and 1524.3 cm^{-1} to the C═C stretching mode of the *all-trans* and *13-cis* component of the parent state, respectively. The corresponding C═N stretching vibration is found at a frequency not very different from those of the anion-bound complexes (1631.2 cm^{-1}). On the other hand, the difference spectrum reflecting the photocycle intermediates (Figure 6D) includes three bands in the C═C stretching region, implying that there are at least three different states involved. According to the frequencies at 1533.9, 1540.6, and 1550.9 cm^{-1}, these species should exhibit blue-shifted absorption maxima relative to the parent state. In the C═N stretching region, there are two bands at 1643 and 1649 cm^{-1}, i.e., at much higher frequencies than in the parent state but again similar to those of HR_{520} of the anion-bound HR.

Table 2: Spectral Parameters of Protonated Retinal Schiff Bases[a]

anion	solvent	λ_{max}/-nm	$\nu_{C=C}$/-cm^{-1}	$\nu_{C=N}$/-cm^{-1}	ν_{N-H}/-cm^{-1}
Cl^-	$CDCl_3$	440	1554 (0)	1652 (−20.5)	2570 (−580)
BF_4^-	$CDCl_3$	480	1552 (0)	1651 (−20)	3240 (−840)
Cl^-	CD_3CN	440	1559 (1)	1655.5 (−21.5)	2580 (−520)
BF_4^-	CD_3CN	450	1558 (0)	1651.5 (−19)	3240 (−840)
Cl^-	solid (IR)		1568 (−1)	1657 (−26)	2580 (−562)
Cl^-	solid (Raman)		1562 (+1)	1652 (−20)	
BF_4^-	solid (IR)		1543	1643	3240
BF_4^-	solid (Raman)		1543	1643	

[a] Vibrational frequencies ($\nu_{C=C}$, $\nu_{C=N}$, and ν_{N-H}) were determined from the IR spectra unless indicated otherwise. Frequency shifts upon H/D exchange are given in parentheses. The accuracy of the frequency determination is ca. ±0.5 cm^{-1} for the $\nu_{C=C}$ and $\nu_{C=N}$ modes. The frequencies for the N−H stretchings refer to the center of gravity of the bands.

Model Compounds. The IR and FT-Raman spectra of NRB model compounds, complexed by HBF_4 or HCl, were measured in various solvents or in the solid state. The frequencies of the C═C, C═N, and N−H stretching modes as well as the H/D isotopic shifts are listed in Table 2 along with the absorption maxima of the retinal $\pi \rightarrow \pi^*$ transition. In all cases, the spectral data, in particular the red-shifted λ_{max} (compared to λ_{max} = 363 nm of the unprotonated NRB), indicate that the retinal Schiff bases are protonated. On the other hand, both λ_{max} as well as the vibrational frequencies vary in a considerable range depending on the kind of the counterion and the molecular environment (e.g., solvent).

In order to sort out the various parameters which influence the spectral properties of the protonated NRB, it is instructive to consider the N−H stretching frequency which is the most sensitive marker for hydrogen bonding interactions (Joesten & Schaad, 1974). For NRB-HBF_4, the spectral region between 3500 and 2500 cm^{-1} reveals a complex vibrational band pattern (Figure 7A) involving C−H and N−H stretching fundamentals as well as overtones and combination modes. This spectrum has been previously analyzed in detail by Baran et al. (1995) so that the N−H stretching can readily be assigned to the 3240-cm^{-1} band. The position of this band is similar in CD_3CN and $CDCl_3$ and in the solid state. In contrast, the spectrum of the corresponding chloride complex does not show any strong bands above 3000 cm^{-1} but a broad and poorly resolved hump of considerable intensity which is centered at ca. 2580 and 2570 cm^{-1} in CD_3CN and $CDCl_3$, respectively (Figure 7B). In the solid state, this peak is not so broad, but its frequency is very similar to that of the dissolved species (2580 cm^{-1}; spectrum not shown). Comparable downshifts are also noted in the deuterated complexes NRB-HBF_4 and NRB-HCl. While the low frequencies indicate significant hydrogen bonding interactions between the protonated Schiff base and the

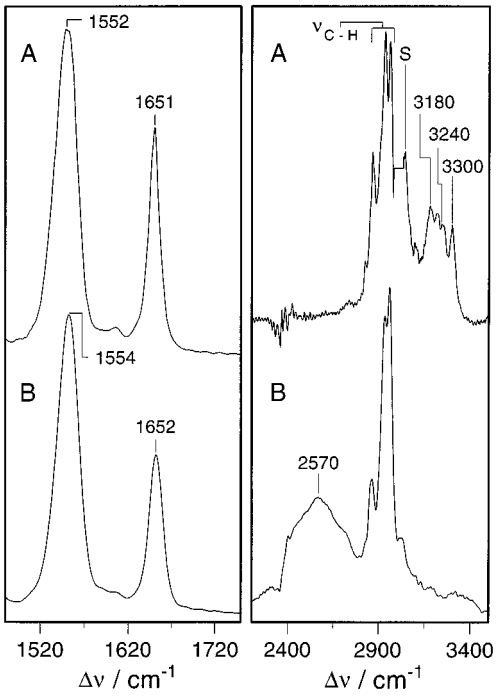

FIGURE 7: IR spectra of NRB-HBF_4 (A) and NRB-HCl (B) in $CDCl_3$ in the C═C/C═N stretching (left panel) and N-H/C-H stretching region (right panel).

chloride counterion (Joesten & Schaad, 1974), such effects can be regarded to be relatively small for NRB-HBF_4.

Previous studies have shown that the sensitivity of $\nu_{C=N}$ toward hydrogen bonding is related to a small contribution of the N−H i.p. bending which in turn shifts up with increasing hydrogen bond strength (Baasov et al., 1987; Lussier et al., 1987; Masuda et al., 1996). Thus, increasing hydrogen bond strength in the order HI < HBr < HCl, as reflected by a downshift of the N−H stretching from 2930 cm^{-1} by more than 300 cm^{-1}, leads to an upshift of the $\nu_{C=N}$ from 1633 (I^-) by only 13 cm^{-1} (Lussier et al., 1987). Consequently, one would expect that the C═N stretching in NRB-HBF_4 should show up at a lower frequency than in the corresponding HCl complex, i.e., below 1640 cm^{-1}. Instead, this mode is found at ca. 1651 cm^{-1} in $CDCl_3$ and CD_3CN while that of the HCl complex is nearly at the same position in $CDCl_3$ and only slightly higher in CD_3CN (Figure 7). The tetrafluoroborate anion has a tetrahedral geometry, and the overall size is significantly larger even than that of iodide. Thus, it is not capable of forming even a weak hydrogen bond, implying that positive charge on the retinal Schiff base is not reduced.

As shown by López-Garriga et al. (1986a,b), the formation of an adduct between a Schiff base and the strong Lewis acid BF_3 leads to an even higher $\nu_{C=N}$ frequency than found for strongly hydrogen-bonded protonated Schiff bases. This observation was attributed to the electron withdrawing capability of BF_3 which leads to a rehybridization at the nitrogen so that the C═N bond order is increased. Consequently, an increase of the C═N stretching force constant is responsible for the frequency upshift. Extending this explanation to the NRB complexes, a decreasing hydrogen bond strength has a 2-fold but opposing effect on the $\nu_{C=N}$ mode. The C═N/N−H coupling is weaker because of the lower energy of the N−H i.p. bending (downshift) whereas the higher positive partial charge on the nitrogen increases the C═N bond order (upshift). The second effect appears to dominate in NRB-HBF_4 but the first one in NRB-HCl. Such effects of chloride and tetrafluoroborate interactions were already observed for nonconjugated iminium compounds (Zine et al., 1995).

In conjugated iminium compounds, any alterations of the charge density on the nitrogen should not only affect the Schiff base but also affect the entire conjugated retinal chain. In fact, the frequencies of the $\nu_{C=C}$ modes vary in the same direction as those of $\nu_{C=N}$ (Table 2). Moreover, changing the polarity of the solvent ($CDCl_3$→CD_3CN) has a more pronounced effect on the $\nu_{C=C}$ than on the $\nu_{C=N}$ mode. This is particularly true in the non-hydrogen-bonded NRB-HBF_4 system for which an upshift of 7 cm^{-1} (0.5 cm^{-1}) is noted for the $\nu_{C=C}$ ($\nu_{C=N}$) mode as compared to the hydrogen-bonded NRB-HCl (5 cm^{-1} vs 3.5 cm^{-1} for $\nu_{C=C}$ and $\nu_{C=N}$, respectively).

The variations of the $\nu_{C=C}$ frequency are related to changes of the sensitivity of the π-electron delocalization in the retinal chain not only upon hydogen bonding interactions but also on the polarity of the environment. In the non-hydrogen-bonded NRB-HBF_4 complex, the change from a electron donor (CD_3CN) to an electron acceptor solvent ($CDCl_3$) increases the conjugation in the retinal chain which is reflected by a 7-cm^{-1} downshift of $\nu_{C=C}$ and a concomitant red-shift of the absorption band. In the case of a strongly hydrogen-bonded system (NRB-HCl) with a reduced positive partial charge on the nitrogen, a similar strong effect of the solvent on the conjugation is not expected (Elia et al., 1996). In fact, the absorption maximum remains unchanged, which, however, is in contrast to the 5-cm^{-1} downshift of the $\nu_{C=C}$ mode. Evidently, the $\lambda_{max}-\nu_{C=C}$ relationship (Heyde et al., 1971; Kakitani et al., 1983; Rothschild et al., 1984) does not hold strictly for NRB complexes upon varying the polarity of the environment since the solvent may exert a different influence on the π and π^* orbital energies.

In the solid state of NRB-HBF_4 and NRB-HCl, the strengths of hydrogen bonding interactions are the same as in solution as reflected by the N−H stretching frequencies (Table 2). However, the C═C and C═N stretching modes reveal significant frequency shifts which are attributed to direct interactions of the anions with the delocalized π-electrons and between adjacent chromophores. In addition, there may be conformational differences between the solid and the dissolved state since the fingerprint regions of the FT-Raman and IR spectra display distinct changes between the solid NRB-HCl and NRB-BF_4 as well as the dissolved species [Figure 8; cf. Mathies et al. (1977)]. In particular, we note that the broad band at 1158 cm^{-1} of NRB-HCl is narrowed and upshifted to 1165 cm^{-1} in the NRB-HBF_4 complex. In addition, the spectrum of the latter complex displays an extra band at 1173 cm^{-1} which may be assigned to a mode calculated at 1178 cm^{-1} but not yet observed in NRB model complexes (Smith et al., 1995). Also the nearby C−C stretching modes at ca. 1190 and 1200 cm^{-1} differ with respect to frequencies and band widths in both species.

Anion−Chromophore Interactions in HR. The results discussed in the previous section have shown that the $\nu_{C=N}$ frequency *per se* is not a reliable indicator for hydrogen bonding interactions since it also depends on those parameters which directly affect the C═N stretching force constant. Such parameters (e.g., the polarity of the chromophore environment) should be largely unchanged in HR when

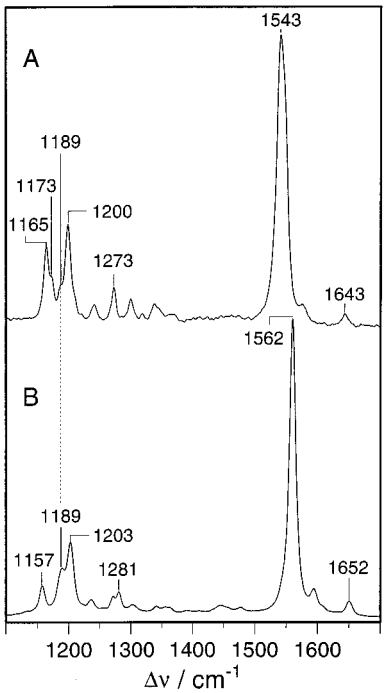

FIGURE 8: FT-Raman spectra of solid NRB-HBF_4 (A) and NRB-HCl (B).

loaded with Cl^-, Br^-, or I^-, or in the corresponding NRB halide complexes (in the same solvent). In the latter case, the substantial frequency variations by up to 13 cm^{-1} (from Cl^- to I^-) of the $\nu_{C=N}$ mode and the much smaller shifts (7 cm^{-1}) of the $\nu_{C=C}$ mode (Lussier et al., 1987) can be attributed to the effect of different hydrogen bond strengths. Conversely, the lack of any frequency differences for the $\nu_{C=N}$ mode in $HR_{578}(Cl^-)$, $HR_{578}(Br^-)$, and $HR_{578}(I^-)$ rules out any hydrogen-bonding interactions between the protonated Schiff base and the counterion. These findings imply that the halide ions are bound to an amino acid residue somewhat remote from the Schiff base. As discussed in detail previously (Braiman et al., 1994; Rüdiger et al., 1995; Oesterhelt, 1995), the anion binding site may be an arginine (Arg-108 in *H. salinarium* corresponding to Arg-123 in *N. pharaonis*). On the other hand, the anion is located close enough to the chromophore to exert a small but detectable influence on the electron density distribution of the retinal (Table 1). These effects are specially reflected by the shifts of the $\nu_{C=C}$ which decreases in the order $Cl^- > Br^- > I^-$ (i.e., decreasing charge density), resulting in a weakening of the electrostatic interactions. In the absence of complexed anions, the downshift of $\nu_{C=C}$ is even more pronounced. Since the concomitant frequency lowering of the $\nu_{C=N}$ mode is very small (1 cm^{-1}), also for this species hydrogen-bonding interactions can be ruled out. Instead, it is very likely that the drastically altered electrostatic interactions in the retinal binding pocket upon removal of the counterion also induce structural changes in the chromophore as suggested by an increase of the RR activity in the C−H out-of-plane region (spectrum not shown).

Evidently, the bound anion plays an important role in stabilizing the structure of the chromophoric site. Further support for this conclusion comes from the anion dependence of the *all-trans/13-cis* equilibrium. Based on the RR intensity ratios of the $\nu_{C=C}$ modes of both species (cf. Figures 3 and 6) and the isomeric ratio determined for the light-adapted chloride complex, one can estimate a *13-cis* content of 20%, 24%, and 29% for the complexes with Cl^-, Br^-, and I^-, respectively, and even 38% for the anion-depleted form.

Previous studies on the binding of different anions in HR_{578} referred to the protein from *H. salinarium*. While Walter and Braiman (1994) reported a downshift of the $\nu_{C=N}$ mode by 1 and 3 cm^{-1} when Cl^- is replaced by Br^- and I^-, respectively, Pande et al. (1989) could not detect any frequency difference for this mode between the Cl^-- and Br^--bound complex. Taking into account the far-reaching similarities between the HR from *H. salinarium* and *N. pharaonis*, as far as the parent state is concerned, the present results which are obtained from the present highly resolved RR spectra confirm the conclusion by Pande et al. (1989). It may be that the accuracy of the experimental approach by Walter and Braiman (1994), i.e., deriving absolute band frequencies by a band fitting of IR difference spectra, is not sufficient to determine potential shifts as small as 3 cm^{-1} unambiguously. On the other hand, the previously reported 7-cm^{-1} upshift of $\nu_{C=N}$ of HR_{578} upon replacement of Cl^- by nitrate (Maeda et al., 1985) may largely be due to a conformational change of the Schiff base group (Pande et al., 1989).

It is interesting to note that the BR mutant D85T of *H. salinarium* reveals a different picture. The underlying substitution of the Schiff base counterion provides an anion binding site and, moreover, transforms BR's function to a photoinduced anion pump (Sasaki et al., 1995). In this variant protein, however, the $\nu_{C=N}$ stretching displays anion dependence with a 4-cm^{-1} downshift from Cl^- to Br^- reflecting a weakening of the hydrogen bonding interactions (Chon et al., 1996). Most likely, in this case the anion is directly bound to the protonated Schiff base in D85T.

The first thermal intermediate of HR, HR_{520}, exhibits similar anion-dependent downshifts for the $\nu_{C=C}$ mode as the parent state. However, the $\nu_{C=N}$ mode frequency decreases from Cl^- to Br^- and I^-, i.e., in the order of decreasing hydrogen bonding capabilities (Table 1). The overall downshift is 2.6 cm^{-1} which has to be compared with ca. 13 cm^{-1} in model systems (Lussier et al., 1987). Evidently, in HR_{520}, the hydrogen bonding interaction between the protonated Schiff base and the bound anion is stronger than in the parent state albeit still weak in comparison with model compounds in solution. These findings imply that the *all-trans*→13-*cis* isomerization which precedes the formation of HR_{520} brings the Schiff base in closer proximity to the bound anion (Haupts et al., 1997). It may well be that subsequent relaxation processes of the chromophore may displace the Schiff base−halide ion pair toward the channel which provides access to the cytoplasm. Further time-resolved RR studies of the anion dependence of HR's photocycle which focus on the late intermediates of the photocycle are required to gain more insight into the coupling between chromophore movement and anion translocation in HR.

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