Effects of various anions on the Raman spectrum of halorhodopsin

C. Pande, J. K. Lanyi,* and R. H. Callender
Physics Department, City College of New York, New York 10031; and *Department of Physiology and Biophysics, University of California, Irvine, California 92717

ABSTRACT Resonance Raman experiments were conducted to probe and understand the effect of various anions on halorhodopsin. These included monoatomic anions Cl^- and Br^-, which bind to the so-called halorhodopsin binding sites I and II, and polyatomic anions NO_3^- and ClO_4^-, which bind to site I only. The two types of ions clearly show different effects on the vibrational spectrum of the chromophore. The differences are not localized to the Schiff base region of the molecule, but extend to the chromophore structure-sensitive fingerprint region as well. We find that the protonated Schiff base frequency is at 1,633 cm^{-1} for Cl^- and Br^- ions, as reported previously for Cl^-. However, we find that two Schiff base frequencies characterize halorhodopsin upon binding of the polyatomic anions. One frequency lies at the same location as that found for the monoatomic anions and the other is at 1,645 cm^{-1}. Halorhodopsin with bound NO_3^- and ClO_4^- thus may consist of two heterogeneous structures in equlibrium. This heterogeneity does not seem to correlate with a retinal isomeric heterogeneity, which we can also demonstrate in these samples. The results suggest that anions binding to site I do not bind to the Schiff base directly, but can influence chromophore and/or protein conformational states.

INTRODUCTION

One of the retinal-containing photoreactive proteins in the cell membrane of *Halobacterium halobium* is halorhodopsin (HR) (Lanyi, 1986*a*). Like its more extensively studied cousin bacteriorhodopsin (BR), HR contains retinal attached to the opsin by a protonated Schiff base linkage. Recent primary structure elucidation of HR (Blanck and Oesterhelt, 1987) suggests structural similarities with BR. Similar color determinants in these two pigments have been proposed (Lanyi et al., 1988). Despite the obvious similarities, there are some remarkable differences between these two pigments. Unlike BR, which is a proton pump, HR acts as chloride pump upon photoactivation. Further, whereas the pK of bR's protonated Schiff base linkage is ~13, that of HR is ~7.5.

Spectroscopic effects in HR reveal two types of binding sites for anions, so called I and II. Site II binds only Cl^-, Br^-, and, to a lesser degree, I^-, whereas site I is rather nonspecific and binds a variety of anions, including the site II ions. Whereas site I ions cause a 6–7–nm hypsochromic shift in the absorption maximum of the pigment, binding to site II causes a bathochromic shift of about the same magnitude (Schobert et al., 1986). The photochemistry of the pigment in the presence of site I anions is different from that in the presence of site II anions, and only site II anions are pumped upon photoactivation. Two kinds of models of anion transport have been proposed (Lanyi, 1984; Oesterhelt et al., 1986). Although both models involve retinal isomerization from the 13-*trans* to the 13-*cis* form, one of them requires the Cl^- ion to be the counter-ion of the Schiff base (Oesterhelt et al., 1986).

We have performed resonance Raman measurements on HR in the presence of anions that occupy both sites I and II to understand their influence on the chromophore. We have extended the only other resonance Raman study on HR involving both site I and site II ions (Maeda et al., 1985) to include one more anion of each type (namely Br^- as another site II anion and ClO_4^- as a second site I anion) to see whether one ion of each type was a good enough representation of that binding site. We show that, indeed, ions belonging to one class show qualitatively similar effect on the chromophore vibrational pattern which is distinctly different from the effect of the ions belonging to the other type. Not only are the vibrational modes associated with the Schiff base end of the molecule affected differently, the fingerprint region shows marked differences as well. We find that the protonated Schiff base frequency is at 1,633 cm^{-1} for Cl^- and Br^- ions, as reported previously for Cl^- (Smith et al., 1984; Alshuth et al., 1985; Maeda et al., 1985; Diller et al., 1987). However, we find two Schiff base frequencies characterize halorhodopsin upon binding of the polyatomic anions. One frequency lies at the same location as that found for the monomeric anions and the other is at 1,645 cm^{-1}. The data suggest that HR with bound NO_3^- and ClO_4^- consists of two heterogeneous structures in equilibrium, even at concentrations well above the dissociation constants of these ions and site I. Simple explanations based

C. Pande's permanent address is Biophysics Division, Clairol Research Laboratories, 2 Blachley Road, Stamford, CT 06922.

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on direct hydrogen bonding of ions with the Schiff base (extraneous ions as the Schiff base counter-ion) are unlikely to be correct. We also show that the specific effects of a particular anion-type occurs at a very low concentration. For example, 50 mM NO_3^- in the presence of 400 mM SO_4^{-2} had the same effect on the chromophore vibrational modes as, say, 200 mM or higher NO_3^-.

MATERIALS AND METHODS

Halorhodopsin from strain OD2W of *H. halobium* was obtained as described before (Duschl et al., 1988), and octylglucoside in the sample was replaced with 0.5% Lubrol PX (Pierce Chemical Co., Rockford, IL). The Lubrol-solubilized sample was then dialyzed against buffers containing 0.36 M Na_2SO_4, 0.023 M MOPS (pH = 6.0) and 0.2 M of either $NaNO_3$, $NaClO_4$, or NaBr. For studies involving concentration dependence of NO_3^-, the sample was dialyzed against the buffer containing the required concentration of $NaNO_3$, and the ionic strength was maintained constant by appropriately increasing the Na_2SO_4 concentration. In one set of experiments with ClO_4^-, the sample contained 0.1 M $NaClO_4$ instead. Deuteriated samples were prepared by dialyzing HR samples against D_2O containing the required ionic species. Complete exchange of D_2O for water was assured by frequent changes of the dialysate, and by maintaining a large volume ratio between the dialysate and the sample.

A spectrometer (model 1877 triplemate, Spex Industries, Inc., Edison, NJ) and a cooled solid state detector (model 1420-2; EG&G Princeton Applied Research Corp., Princeton, NJ), with a multichannel detector controller (model 1218; EG&G Princeton Applied Research Corp.) were used to obtain the Raman spectra. Spectra were obtained using a 1,200-g/mm grating. This allowed a "viewing window" of 800 cm^{-1} under the experimental conditions described below. Spectral resolution under these conditions was estimated to be 8 cm^{-1}. The spectra were calibrated using toluene as the standard.

Raman measurements were made in a plexiglass spinning cell which could be rotated at variable speeds. The 514.5-nm line from an Ar^+ laser at a nominal power level of 8 mW was used to excite Raman scattering from the sample. Light was focused (1.5 × 0.1 mm) at the sample using a cylindrical lens (f = 5 mm) and the cells (5.5 cm diameter) was spun at frequency of 25 Hz. This frequency allows sufficient time (~40 ms) for the photointermediates to decay before the sample arrives again at the excitation beam for the next pass. Under these conditions, and assuming $\epsilon_{514} = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$ for HR (Lanyi and Weber, 1980), the photoconversion probability, P (Calender et al., 1976), was calculated to be ~0.03 assuming maximum quantum yield ($\Phi = 1$). Measurements were made at room temperature which was 21 ± 1°C. Lowering the probe power to 3–4 mW did not affect the sample spectrum, suggesting that no photochemically induced side reactions were contaminating the pigment spectrum.

A 600-μl sample was taken in the spinning cell and "light adapted" for 10 min with the probe beam, but at a power level of 40 mW, before the measurements. The probe beam power was then reduced to ~8 mW and the spectra collected for the desired time. Longer light adaptation times or higher light intensities before data collection had no measurable effect on the frequencies of the observed peaks, suggesting that complete light adaptation had occurred under our experimental conditions. The sample concentration was typically 100 μM. Except for the sample containing perchlorate (ClO_4^-) which slowly degraded over a period of days, all other samples were stable in the refrigerator even after 2 wk. All samples were used within 72 h of their preparation.

The isomeric composition of the HR was determined by extraction of the retinal and HPLC identification of the components, as described earlier (Zimányi and Lanyi, 1987).

RESULTS

Fig. 1 *c* shows the resonance Raman spectrum of HR in the presence of typical site I anion NO_3^- (HR[NO_3]) while that of Fig. 1 *a* shows the spectrum for Cl^- (HR[Cl]), which binds also to site II. The spectrum of Br^-, which binds to site II as Cl^-, was virtually identical to HR(Cl), and the site I anion ClO_4^- also showed a spectrum the same as that due to HR(NO_3) except for the differences noted below, which appear to be due to vibrations associated with the protonated Schiff base moiety of the chromophore. On the other hand, a careful examination of the data reveals that spectra with Cl^- and

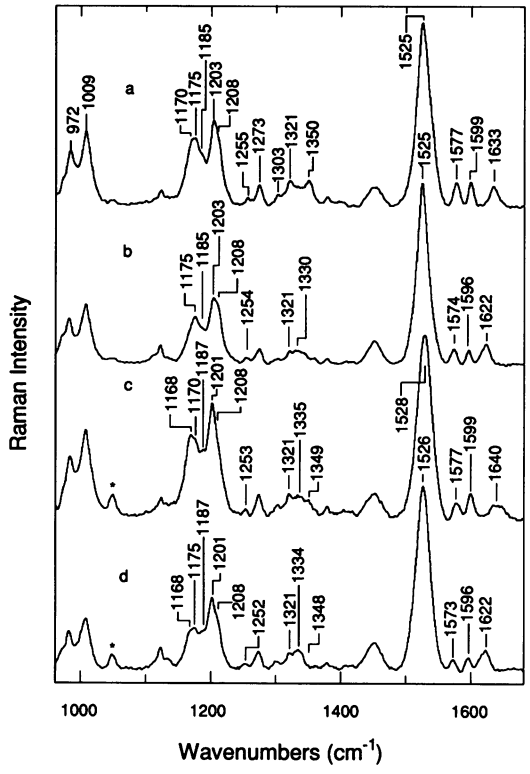

FIGURE 1 Resonance Raman spectra of halorhodopsin solubilized in lubrol. All samples contain 0.36 M Na_2SO_4, 0.023 M MOPS (pH/pD = 6.0), and 0.2 M NaCl (*a* and *b*) or 0.2 M $NaNO_3$ (*c* and *d*). Spectra *a* and *c* correspond to HR(Cl) and HR(NO_3) in H_2O, whereas *b* and *d* represent the respective samples in D_2O. All spectra were measured at ambient temperatures in a plexiglass spinning cell using 514.5 nm line from an Ar^+ laser as described in the test. The asterisks (*) in spectra *c* and *d* represent lines originating from NO_3^-. The bands around 972 cm^{-1} in all spectra contain strong contribution from the presence of 0.2 M SO_4^{-2} in the samples.

Br^- and with NO_3^- and ClO_4^- differ from each other at various vibrational frequencies as will be discussed below. Fig. 1, *a* and *c*, represent the spectra of these samples in H_2O; the effect on these spectra resulting from sample deuteriation is shown in panels *b* and *d*, respectively.

Ethylenic (C=C stretch) region

The main ethylenic band (in phase C=C stretching mode) in HR(Cl) appears at 1,525 cm^{-1} (Fig. 1 *a*) with two smaller ethylenic bands at 1,577 and 1,599 cm^{-1}. The main band is 16 cm^{-1} broad (fwhm), after subtracting the 8 cm^{-1} resolution of the spectrometer. HR(NO_3) has its main ethylenic band at 1,528 cm^{-1}, 19 cm^{-1} wide, with the smaller bands at 1,578 and 1,599 cm^{-1} (Fig. 1 *c*). For comparison, the analogous band in light-adapted BR, an all-*trans* pigment, is ~8 cm^{-1} wide while that for the dark-adapted form of BR, which contains an equimolar mixture of the 13-*cis* and the all-*trans* pigments, is ~16 cm^{-1} wide. This would suggest that HR(Cl) and HR(NO_3) are not isomerically pure species but both contain a mixture. Indeed, chromophore extraction data suggests that HR always contains a mixture of isomers (Lanyi, 1986*a*) and under the conditions used here contains a mixture of the all-*trans* and 13-*cis* isomeric forms in a 70:30 ratio (Maeda et al., 1985; this study). From the well-known linear correlation between the absorption maximum of the pigment and the frequency of the most intense C=C stretching mode for retinal pigments (cf. Aton et al., 1977), the difference in the ethylenic band frequencies between HR(NO_3) and HR(Cl) would predict a bathochromic shift of ~10 nm in the absorption maximum of HR(Cl) compared with HR(NO_3). This is in good agreement with the observed 6–7 nm shift (e.g., Schobert et al., 1986).

Deuteriation of the sample produces changes in the C=C stretching modes. For HR(Cl), the frequency maximum of the most intense mode remains unchanged but the band width decreases by 3 cm^{-1}. The less intense bands at 1,577 and 1,599 cm^{-1} move to 1,574 and 1,596 cm^{-1}, respectively. In the case of HR(NO_3), deuteriation shifts the main ethylenic band to 1,526 cm^{-1} from 1,528 cm^{-1} and the band narrows by 2 cm^{-1}. The other two ethylenic bands at 1,578 and 1,599 cm^{-1} move to 1,573 and 1,596, respectively, as in the case of HR(Cl). It should be noted that these smaller bands also shift by about the same amount upon deuteriation of light-adapted BR.

Schiff base (C=N stretch) region

The vibrational mode at 1,633 cm^{-1} (Fig. 1 *a*) is identified as the C=N stretching mode of retinal in HR(Cl). As has been noted before (Smith et al., 1984; Maeda et al., 1985; Diller et al., 1987), this band downshifts by 11 to 1,622 cm^{-1} in D_2O solution (Fig. 1 *b*), thereby showing that the chromophore is linked to the protein by a protonated Schiff base linkage in HR(Cl). The broad band centered around 1,642 cm^{-1} in HR(NO_3) Raman spectrum (Fig. 1 *c*) also undergoes a shift to 1,622 cm^{-1} upon deuteriation (Fig. 1 *d*), assigning this mode to the protonated Schiff base in HR(NO_3) (Maeda et al., 1985). The remarkable feature of the data is that whereas the protonated Schiff base band in HR(Cl) is relatively narrow, the analogous band in HR(NO_3) is broad and consists of an overlap of two distinct bands of nearly equal intensity, one centered at 1,633 cm^{-1} and the other at 1,645 cm^{-1}. This is shown in the expanded scale of Fig. 2. The two constituent HR(NO_3) bands appear to be almost half in intensity of the HR(Cl) band. This characteristic band shape for HR(NO_3) is unaffected by NO_3^- concentrations between 50 and 400 mM. The K_d for site I is 25 mM for nitrate (Schobert and Lanyi, 1986). The Schiff base band of HR(Br) was identical to HR(Cl) (data not shown). The Schiff base band for HR(ClO_4) is like in HR(NO_3) except that the 1,645 cm^{-1} band is substantially larger than the 1,633 cm^{-1} band, as shown in Fig. 2 *c*.

The frequency of the protonated Schiff base mode has

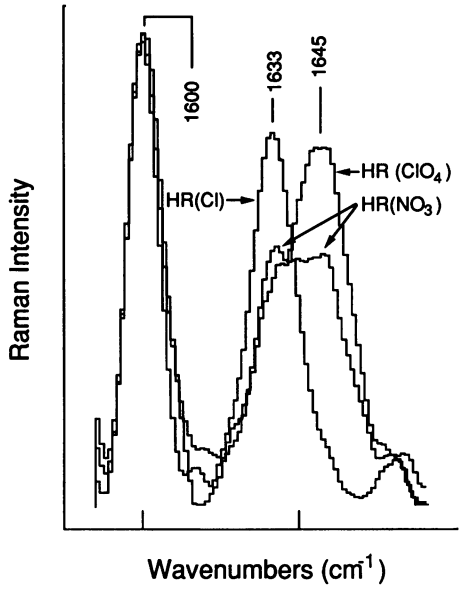

FIGURE 2 Schiff base (C=NH$^+$ stretch) region of HR(Cl), HR(NO_3), and HR(ClO_4) blown up to show the differences between these pigments. The HR(Cl) and HR(NO_3) data are the same as in Fig 1 (spectra *a* and *c*) except they were smoothed with a nine-point smoothing procedure. The three spectra were normalized with respect to the 1,600 cm^{-1} ethylenic bands.

been shown to be affected by its environment, particularly to hydrogen bonding patterns with its counter-ion (see cf. Aton et al., 1980; Bassov et al., 1987; Deng and Callender, 1987; Gilson et al., 1988). Thus, the data strongly suggest that halorhodopsin with bound polyatomic anions, like NO_3^- and ClO_4^-, exists as two heterogeneous conformations in equilibrium. One conformation yields a protonated Schiff base frequency of 1,633 cm^{-1}, like that found for the monomeric anions Cl^- and Br^-. The second conformation, unique to polyatomic anions, yields a Schiff base frequency of 1,645 cm^{-1}. Because both HR(Cl) and HR(NO_3) contain essentially the same isomeric composition (70% *trans* and 30% 13-*cis*; see below) and because both HR(NO_3) and HR(ClO_4) yield essentially the same fingerprint region spectra (a spectral region sensitive to retinal isomer), the two states almost certainly involve two different structures of the protonated Schiff base, fairly localized to the Schiff base moiety of the chromophore. Attempts to obtain other kinds of evidence for this heterogeneity were not successful. Although the absorption band of HR(NO_3) is somewhat broader than that of HR(Cl), the same extent of broadening is seen for HR(ClO_4) which appears to be less heterogeneous for the C=NH^+ stretch.

A very important issue is whether or not the anion is itself the counter-ion to retinal's protonated Schiff base group. This type of structural question is difficult to answer definitively with Raman data. However, for several reasons, the results suggest that the anions are not directly and/or simply bonded with the protonated Schiff base C=NH^+ moiety. In simple Cl^- and Br^- complexes with protonated Schiff bases of retinal in various model solutions (Blatz and Mohler, 1975; Sheves et al., 1983; Baasov et al., 1987), the C=NH^+ stretching frequency is substantially higher (near 1,650 cm^{-1}) than those observed here. Moreover, the models show a 5 cm^{-1} difference between Cl^- and Br^- salts and none is observed for HR(Cl) compared with HR(Br). Replacement of Cl^- by ClO_4^- has been shown to result in a somewhat lower C=NH^+ frequency (Baasov et al., 1987). In HR, the C=NH^+ band of HR(Cl) is replaced by a mixture of the HR(Cl) band and a new band at a higher frequency. The models also show a ~10 nm shift in λ_{max} of the visible absorption band between Cl^- and Br^- which is also not observed in direct measurements of HR(Cl) and HR(Br). It would thus seem that the Schiff base environment involves the anions bound to other protein groups and, perhaps, water forming some sort of complex (see Discussion). As has been pointed out before (Smith et al., 1984), the low frequency of the Schiff base mode in halorhodopsin with Cl^- and Br^- relative to bacteriorhodopsin (at 1,642 cm^{-1}) indeed suggests a weakened counter-ion interaction.

C=N—H Bending region

Resonance Raman studies on BR have shown that the region of 1,300–1,400 cm^{-1} contains contributions from vinyl C—C—H rocks as well as from the C=N—H in-plane bending motion. Based on the *N*-deuteriation studies with isotopically substituted retinals, the mode at 1,350 cm^{-1} has been assigned to the C=N—H bend in the case of BR (Massig et al., 1982; see also Smith et al., 1987*a*). Recent studies on HR have also shown that this mode, or a deuterium sensitive mode, appears at about the same position in HR as well (Smith et al., 1984; Maeda et al., 1985; Diller et al., 1987).

Fig. 3 shows comparative spectra in this region of HR(Cl), HR(NO_3), and HR(ClO_4). The bands at 1,303 and 1,321 cm^{-1} appear at the same position for all three species and are almost equally intense. In BR, these bands have been assigned to normal modes containing predominant contributions from the antisymmetric combination of C_7H and C_8H rock, and to the $C_{12}H$ rock, respectively

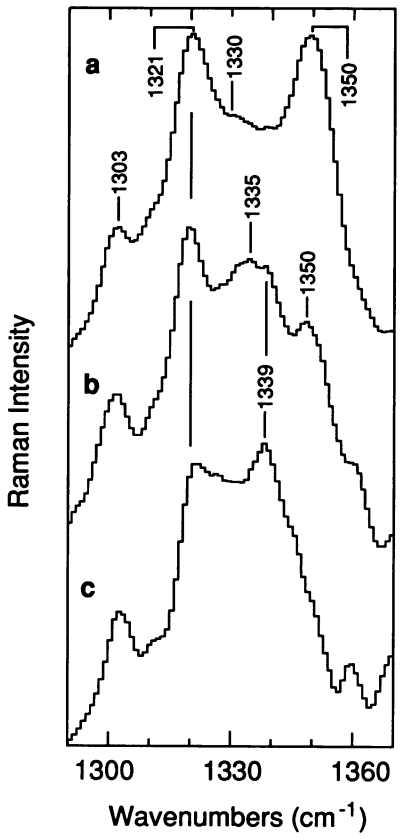

FIGURE 3 C=N—H in plane and vinylic C—C—C hydrogen bending region of (*a*) HR(Cl), (*b*) HR(NO_3), and (*c*) HR(ClO_4) blown up to magnify the differences between the two pigments. The data for HR(Cl) and HR(NO_3) are from the same set as in Fig. 1.

(Smith et al., 1987*a*). All three HR(Cl), HR(NO_3), and HR(ClO_4) spectra show a band at ~1,350 cm^{-1}, but with decreasing intensity. The relative intensity, I_{1350}/I_{1321} for HR(Cl) and for HR(Br) (data not shown) is 1, in HR(NO_3) it is 0.7, and in HR(ClO_4) it is 0.3 or less. A broad band centered at 1,335 cm^{-1} in HR(NO_3) and at 1,339 cm^{-1} with an intensity equal to the 1,321 cm^{-1} band in HR(ClO_4) is absent in HR(Cl) and HR(Br) (data not shown) spectra. Deuteriation results in the disappearance of the ~1,350 cm^{-1} band (see Fig. 1). Thus, the band at 1,350 cm^{-1} appears to contain predominantly C=N—H bending character, consistent with previous suggestions (Smith et al., 1984; Maeda et al., 1985; Alshuth et al., 1985; Diller et al., 1987).

Given the results above, suggesting that there are two environments for the Schiff base for HR(NO_3) and HR(ClO_4) yielding protonated Schiff base frequencies of 1,633 and 1,645 cm^{-1}, it is reasonable to suppose that the 1,339 cm^{-1} band is a second C=N—H bending mode as this band is not found in the HR(Cl) data of Fig. 3 *a*. This is particularly so because the increase in the I_{1645}/I_{1633} ratio comparing HR(ClO_4) to HR(NO_3), suggesting more of the 1,645 cm^{-1} species in HR(ClO_4) compared with HR(NO_3), is matched by an increase in intensity of the I_{1339}/I_{1350} ratio of HR(ClO_4) compared with HR(NO_3). Unfortunately, while it appears upon a careful examination of the data that the 1,339 cm^{-1} band disappears upon deuteriation (compare Fig. 1, *c* and *d*), this is not certain. A new band near 1,330 cm^{-1} appears upon deuteriation of HR which appears to add some intensity at 1,339 cm^{-1} and which masks an intensity decrease at this frequency to some extent. In our samples, strong scattering from the 0.2 M SO_4^{-2}, in the ~975 cm^{-1} region of the spectrum, prevented us from observing the C=N—D bending modes which are expected to appear in this region upon sample deuteriation (see, Diller et al., 1987).

C—C stretch region

The single bond C—C stretching vibrations and C—C—C and C—C—H bending modes in retinal and retinal proteins are observed in the 1,150–1,250 cm^{-1} range. This region of the vibrational spectrum of retinal proteins is very sensitive to the isomeric composition of the chromophore and is, therefore, also called the fingerprint region.

The main bands in HR(Cl) (Fig. 1 *a*) appear at 1,175 cm^{-1} with a relatively intense shoulder at 1,170 cm^{-1} and a weak shoulder at 1,185 cm^{-1}, a prominent band at 1,203 cm^{-1} with a broad shoulder at ~1,208 cm^{-1}. The situation with HR(NO_3) (Fig. 1 *c*) is somewhat different. The main peak at 1,175 cm^{-1} in HR(Cl) becomes a shoulder to the main peak which is located at 1,168 cm^{-1}. There appears a well-defined, although small, band at ~1,187 cm^{-1} in HR(NO_3) which only appeared as a small shoulder in HR(Cl). The strongest line in this region of the HR(NO_3) Raman spectrum is at 1,201 cm^{-1}, compared with 1,203 cm^{-1} in HR(Cl), and the shoulder at 1,208 cm^{-1} is negligible (Fig. 1 *c*). For comparison, bR_{568}, which is an all-*trans* retinal pigment, shows three bands in this region of the spectrum at 1,170 and 1,201 cm^{-1}, and a shoulder at 1,213 cm^{-1} (Smith et al., 1985). On the other hand bR_{548}, a 13-*cis* pigment, shows bands at 1,167, 1,183, and 1,202 cm^{-1} (Smith et al., 1987*b*). Using these bands as markers for retinal isomer, our data would suggest that the chromophore composition in HR(Cl), illuminated with blue light, is predominantly all-*trans* as concluded by the earlier studies (Smith et al., 1984; Maeda et al., 1985; Diller et al., 1987). However, in accordance with the chromophore extraction data, which suggests an equilibrium mixture of 30% *cis* and 70% *trans* isomers for the chromophore, our data suggest the presence of 13-*cis* pigment in the form of shoulders at 1,169 and 1,185 cm^{-1}. In comparison with HR(Cl), the HR(NO_3) spectrum shows a marked reduction in the 1,175 cm^{-1} band with a concomitant increase in the intensities of the 1,167 cm^{-1} and 1,188 cm^{-1} shoulders, as well as a decrease in the 1,208 cm^{-1} shoulder. A simple interpretation of the data would suggest that HR(NO_3) contains ~5–10% higher 13-*cis* isomeric pigment compared with HR(Cl). The HR(Br) pigment yielded an identical fingerprint region to HR(Cl) as did HR(ClO_4) to HR(NO_3).

This region of the spectrum is insensitive to the Schiff base deuteriation in the case of all-*trans* BR. For 13-*cis* BR, however, deuteriation of the Schiff base results in a marked decrease in the 1,168 cm^{-1} band with a simultaneous increase in the 1,215 cm^{-1} shoulder. In contrast to all-*trans* BR, both HR(Cl) and HR(NO_3) show changes in this region upon deuteriation, but the effects, though similar, are not completely identical for the two. The HR(Cl) data show that the Schiff base deuteriation results in a complete loss of the 1,167 cm^{-1} (Fig. 1 *b*) shoulder, with a concomitant increase in intensity at 1,208 cm^{-1}. This results in a decrease in the ratio I_{1167}/I_{1175} and an increase in I_{1208}/I_{1203} (Fig. 1, *a* and *b*). Again these data can be rationalized by using the BR results and considering the HR(Cl) spectrum as containing contributions from both all-*trans* and the 13-*cis* pigment forms. *N*-Deuteriation of such a sample would result in a shift in the 1,167 cm^{-1} band to ~1,208 cm^{-1}. This would result in a loss in the intensity at ~1,167 cm^{-1} along with a concomitant increase at 1,208 cm^{-1}. The all-*trans* component would remain unaffected as a result of this modification. Because HR(Cl) contains only 30% 13-*cis* component, one would expect a small, but notice-

able, effect in the fingerprint region upon *N*-deuteriation. This is exactly what we observe.

It should be noted that HR(Br) spectrum (data not shown) in this region is virtually identical to HR(Cl), whereas HR(ClO_4) spectrum (also not shown) is very similar to HR(NO_3). The main difference between HR(ClO_4) and HR(NO_3) is that in the former, the 1,168 and 1,175 cm^{-1} bands as well as the 1,188 band are better resolved, although their relative ratio appears to be unchanged.

DISCUSSION

By extending our study to include several anions, we have generalized and strengthened the earlier conclusion (Maeda et al., 1985) that the nature of bound anions affect the chromophore vibrational spectrum of HR. Also, our experiments show that these ions exert their specific effects at far lower concentrations than had been previously measured.

The data clearly show that site I is close to and exerts a direct effect on the retinal protonated Schiff base moiety. We, and others, obtain a Schiff base frequency of 1,633 cm^{-1} for simple anions like Cl^- and Br^-. Replacement of these anions by polyatomic anions like NO_3^- or ClO_4^- apparently results in two protein conformations, one where the Schiff base frequency is the same as that found for monoatomic anions at 1,633 cm^{-1} and the other state having a 1,645 cm^{-1} frequency. The 1,633 and 1,645 cm^{-1} states would appear to be in equilibrium with each other, but do not depend on partial saturation of site I by the anion. We find only the 1,633 cm^{-1} state for Cl^- or Br^- and about equal amounts of the two states for NO_3^- while the 1,645 cm^{-1} state predominates for ClO_4^-. As in previous studies (Schobert and Lanyi, 1986; Maeda et al., 1985), our data suggest that the binding site is not in immediate contact with the C=NH^+ group as discussed above. Based on the recent determination of the HR sequence (Blanck and Oesterhelt, 1987) and the modeling of BR by Engelman et al. (1980), Lanyi et al. (1988) have proposed that Arg-108 could extend to retinal binding site as the site I anion binding moiety.

Recent studies using selected dihydro and dehydro retinal derivatives (Lanyi et al., 1988) have suggested that the chromophore electronic structures in HR and BR are quite similar. Based on the primary structure and the proposed secondary structure (Blanck and Oesterhelt, 1987) of HR, residues similar to those that have been implicated as the color determinants in BR have been observed at similar spacial locations with respect to the chromophore in HR as well. It has been suggested that the red shift in the λ_{max} of HR relative to BR may be due a weakened Schiff base counter-ion interaction. This is prompted by the observation that the C=NH^+ stretching frequency is ~7 cm^{-1} lower in HR compared with BR and shows a smaller shift in frequency in deuteriated samples (Smith et al., 1984). Weaker counter-ion or hydrogen bonding interactions lead to such trends (Aton et al., 1980; Sheves et al., 1987; Deng and Callender, 1987; Gilson et al., 1988). Good candidates for the putative counter-ions are both aspartate groups, Asp-238 in HR and Asp-212 in BR, and it has been proposed that the interaction of Asp-238 with Arg-108 weakens the Schiff base counter-ion interaction in HR (Lanyi et al., 1988). Our results provide evidence for these concepts in that they demonstrate lack of an otherwise expected effect of the size and electronegativity of the bound anion on the Schiff base frequency. Instead, replacement of Cl^- and Br^- by NO_3^- or ClO_4^- results in the appearance of a single larger Schiff base frequency mode species, whose origin is probably a different protein conformational state. Moreover, our results show that more of this 1,645 cm^{-1} species is present in ClO_4^- compared with NO_3^-, and this is consistent with somewhat larger blue shift observed HR(ClO_4) compared with HR(NO_3) (Schobert et al., 1986; this study).

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