Resonance Raman Studies of the HOOP Modes in Octopus Bathorhodopsin with Deuterium-Labeled Retinal Chromophores†

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ABSTRACT: Resonance Raman spectra of the hydrogen out-of-plane (HOOP) vibrational modes in the retinal chromophore of octopus bathorhodopsin with deuterium label(s) along the polyene chain have been obtained. In clear contrast with bovine bathorhodopsin's HOOP modes, there are only two major HOOP bands at 887 and 940 cm^{-1} for octopus bathorhodopsin. On the basis of their isotopic shifts upon deuterium labeling, we have assigned the band at 887 cm^{-1} to $C_{10}H$ and $C_{14}H$ HOOP modes, and the band at 940 cm^{-1} to $C_{11}H{=}C_{12}H$ A_u-like HOOP mode. Except for a 26 cm^{-1} downward shift, the $C_{11}H{=}C_{12}H$ A_u-like wag appears to be little disturbed in octopus bathorhodopsin from the chromophore in solution since its changes upon deuterium labeling are close to those found in solution model-compound studies. We found also that the $C_{10}H$ and $C_{14}H$ HOOP wags are also similar to those in the model-compound studies. However, we have found that the interaction between the C_7H and C_8H HOOP internal coordinates of the chromophore in octopus bathorhodopsin is different from that of the chromophore in solution. The intensity of the $C_{11}H{=}C_{12}H$ and the other HOOP modes suggests that the chromophore of octopus bathorhodopsin is somewhat torsionally distorted from a planar trans geometry. Importantly, a twist about $C_{11}{=}C_{12}$ double bond is inferred. Such a twist breaks the local symmetry, resulting in the observation of the normally Raman-forbidden $C_{11}H{=}C_{12}H$ A_u-like HOOP mode. The twisted nature of the chromophore, semi-quantitatively discussed here, likely affects the λ_{max} of the chromophore and its enthalpy. The nature of the HOOP modes of octopus bathorhodopsin differs substantially from those found in bovine bathorhodopsin. Thus, while the λ_{max} values for the two batho products are essentially the same as is their enthalpies (relative to their respective rhodopsins), it seems likely that the various molecular factors that determine these quantities are present to different degrees. For example, the twist about $C_{11}{=}C_{12}$ double bond is likely larger in bovine bathorhodopsin than in octopus bathorhodopsin to account for the decoupling of $C_{12}H$ wag from $C_{11}H$ wag in bovine bathorhodopsin. This difference can be caused by the perturbation of a negatively charged protein residue in bovine bathorhodopsin (Eyring et al., 1982), which is much weaker in octopus bathorhodopsin. These issues are discussed in some detail.

The visual pigment rhodopsin consists of an 11-*cis*-retinal chromophore (see Figure 1) covalently bound via a protonated Schiff base to the apoprotein opsin. Upon absorption of a photon by the pigment, the chromophore isomerizes from the 11-*cis* to an all-*trans* configuration, and a transient species, bathorhodopsin, is formed. Bathorhodopsin's absorption maximum is invariantly red-shifted from that of the primary pigment. Some two-thirds of the photon's energy is converted to chemical energy in this photoreaction in either bovine (Cooper, 1979; Schick et al., 1987) or octopus rhodopsin (Cooper et al., 1986). This energy is used to drive subsequent thermal reactions, leading eventually to visual excitation.

Resonance Raman spectroscopy has been widely used to obtain direct information on the chromophore structures of visual pigments. The use of low-temperature studies, coupled with the pump-and-probe technique (Oseroff & Callender, 1974), allows the study of the bathorhodopsin intermediate. This has been done for the bovine pigment in some detail (Aton et al., 1980; Eyring & Mathies, 1979). Three intense Resonance Raman bands, at 853, 875, and 920 cm^{-1}, provide a unique signature of the chromophore structure for bovine bathorhodopsin (Oseroff & Callender, 1974). These bands have been assigned to hydrogen out-of-plane (HOOP)[1] vibrations of the chromophore on the basis of their isotopic shift upon deuterium labeling of the chromophore (Eyring et al., 1980, 1982; Palings et al., 1989). It has been suggested that the unusually large intensities of these Raman bands arise from a relatively large torsional distortion of the chromophore's polyene chain between the ground and electronic states (Eyring et al., 1982).

We previously reported resonance Raman data of bathorhodopsin from the octopus visual pigment (*Paroctopus defleini*, Pande et al., 1987). These studies were undertaken

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[1] Abbreviations: HOOP, hydrogen out of plane.

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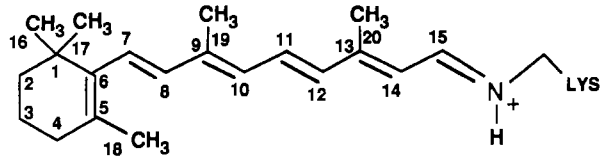

FIGURE 1: Labeling convention for the all-*trans*-retinal protonated Schiff base.

because of a number of interesting similarities and differences between the bovine and octopus pigments. Both pigments systems absorb the energy of a single photon, at ca. 55 kcal/mol, and convert the same fraction of this light energy to chemical energy, some 32 kcal/mol. On the other hand, there is a larger difference between the λ_{max}'s of octopus rhodopsin (λ_{max} = 472 nm) and bathorhodopsin (λ_{max} = 540 nm) compared to the bovine counterparts (500 and 543 nm, respectively). Also, the photochemical cycles of these two species differ. The chromophore eventually detaches from the apoprotein in the vertebrate pigment but does not in the invertebrate one. We found in our earlier study that the HOOP modes in the octopus bathorhodopsin spectrum differ in both positions and relative intensities from their counterparts in the bovine pigment spectrum.

We report here an extensive study of the HOOP modes in octopus bathorhodopsin. We have optimized the experimental set-up so as to achieve a spectrum containing essentially pure bathorhodopsin. We find that octopus bathorhodopsin contains two dominant HOOP modes, at 940 and 887 cm^{-1}, and several much weaker bands. Two of the bands that appeared to be associated with bathorhodopsin in our previous study of more complicated mixtures of species (Pande et al., 1987) are found not to be bathorhodopsin bands. In addition, we report on the isotopic effect on bathorhodopsin's HOOP modes as a result of selective labeling of the retinal polyene chain (C_7D, C_8D, C_7, C_8D_2, $C_{10}D$, $C_{11}D$, C_{11}, $C_{12}D_2$, $C_{14}D$, $C_{15}D$, C_{14}, $C_{15}D_2$, and ND). This permits us to assign the Raman bands observed in the 700-1000-cm^{-1} HOOP-mode region to particular internal coordinates. The 887- and 940-cm^{-1} bands can be assigned to $C_{10}H$, $C_{14}H$, and $C_{11}H{=}C_{12}H$ A_u-like HOOP vibrational modes, respectively, on the basis of their shifts upon deuterium labeling. A comparison between the assignments in octopus bathorhodopsin and bovine bathorhodopsin HOOP modes reveals several interesting differences. First, part of the observed band at 853 cm^{-1} found in bovine bathorhodopsin has been assigned to the $C_{12}H$ HOOP vibration mode. The interesting feature of this mode is that it is decoupled from that of $C_{11}H$ and shifted down by more than 100 cm^{-1} compared with the coupled $C_{11}H{=}C_{12}H$ A_u-like HOOP vibrational mode in all-*trans*-retinal in solution (Curry et al., 1982). The irregular behavior of this $C_{12}H$ HOOP mode has been used in arguments that suggest a protein charge perturbation near C_{12} of bovine bathorhodopsin (Eyring et al., 1982). In octopus bathorhodopsin, in contrast, the $C_{12}H$ HOOP vibration is still well coupled with $C_{11}H$ to form an A_u-like HOOP mode at 940 cm^{-1}. The mode here is some ~26 cm^{-1} down shifted from the corresponding mode in all-*trans*-retinal, indicating a less disturbed $C_{11}{=}C_{12}$ bond of the retinal chromophore in octopus bathorhodopsin compared with that in bovine bathorhodopsin. The other intense HOOP band at 887 cm^{-1}, which is assigned to $C_{10}H$ and $C_{14}H$ HOOP vibrations, is also much less disturbed compared to the analogous modes in free retinal. In bovine bathorhodopsin, the frequencies of these wags are quite low. Finally, we have found that C_7H and C_8H HOOP vibrations have a different coupling than that found in all-*trans*-retinal or in the bovine bathorhodopsin chromophore.

We discuss how these results bear on various theories concerning the molecular mechanisms of converting light energy to chemical energy in the rhodopsin-to-bathorhodopsin photoconversion and on the question regarding which factors play important roles in determining the red-shifted absorption maxima in octopus and bovine bathorhodopsin.

Materials and Methods

Octopus microvillar membranes were prepared and bleached as previously described (Koutalos et al., 1989). The synthesis and characterization of the C_7D, C_8D, C_7, C_8D_2, $C_{10}D$, $C_{11}D$, C_{11}, $C_{12}D_2$, $C_{14}D$, $C_{15}D$, C_{14}, $C_{15}D_2$, and ND retinals for both 11-*cis* and 9-*cis* configurations were described by Lugtenburg et al. (1988).

Each labeled chromophore in ethanol was added to a 10-mL opsin suspension, pH 7.0, with a retinal/opsin molar ratio of 2:1. After incubation for 60 min at room temperature, 100 μL of 2.0 M hydroxylamine, pH 6.5, was added. The regenerated membranes were washed once with 5 mM Tris buffer, pH 7.2, then twice with 4% bovine serum albumin (fatty acid free, Sigma Chemical Co.) in 5 mM Tris, pH 7.2, to remove excess chromophore. After a final wash with 5 mM Tris, pH 7.2, the membranes were stored at −60 °C until further use. About 50% of the opsin was regenerated by this procedure. No difference was observed in the Raman spectra of the native pigment and that regenerated with either 11-*cis* or 9-*cis* unlabeled retinals. All operations were carried out in dim red light.

Resonance Raman experiments were performed at 80 K in a liquid nitrogen coldfinger Dewar, as previously described (Aton et al., 1980), with the dual-beam pump-probe technique (Oseroff & Callender, 1974). The regenerated membranes were centrifuged to a thick pellet that was applied to the sample tip of the coldfinger and then cooled down to 80 K. The photostationary mixtures of rhodopsin (λ_{max} = 472 nm), isorhodopsin (λ_{max} = 462 nm), and bathorhodopsin (λ_{max} = 540 nm) (Tsuda et al., 1980, 1982) at 80 K are obtained and their Raman spectra taken with the "probe" beam (457.9-nm line from an Ar^+ laser). The composition of this mixture can be changed by simultaneously irradiating the sample with a second high-intensity "pump" laser beam (e.g., in the experiments described here, the 568.2-nm line from a Kr^+ ion laser). In our earlier studies, we have found that when the pump beam is such that its wavelength overlaps only the bathorhodopsin absorption band, the relative amounts of isorhodopsin and rhodopsin are virtually the same as in the "probe-only" spectrum (Deng & Callender, 1987; Narva & Callender, 1980). Therefore, it is possible to obtain an almost pure bathorhodopsin Raman spectrum by subtracting the appropriate amount of a pump-probe spectrum from a probe-only spectrum. For each sample, two Raman spectra were taken. The first was a probe-only spectrum with 58% bathorhodopsin, 22% rhodopsin, and 20% isorhodopsin as determined by chromophore extraction (Pande et al. 1987). The second is a pump-probe with a pump/probe intensity ratio of 12:1. We estimate that the bathorhodopsin component in this spectrum is about 15–20% and the rhodopsin/isorhodopsin ratio is still close 1.1:1 on the basis of the relative intensities of the marker HOOP modes found in rhodopsin and in isorhodopsin (970 and 960 cm^{-1}, respectively, see the text for details). While Palings et al. (1987) have found that using a spinning cell can improve the resolution of the bathorhodopsin Raman spectrum, we have noticed that there is no significant difference between their bovine bathorhodopsin Raman spectrum and the spectrum taken by our conventional method, except that some relative Raman band intensity differences result from a dif-

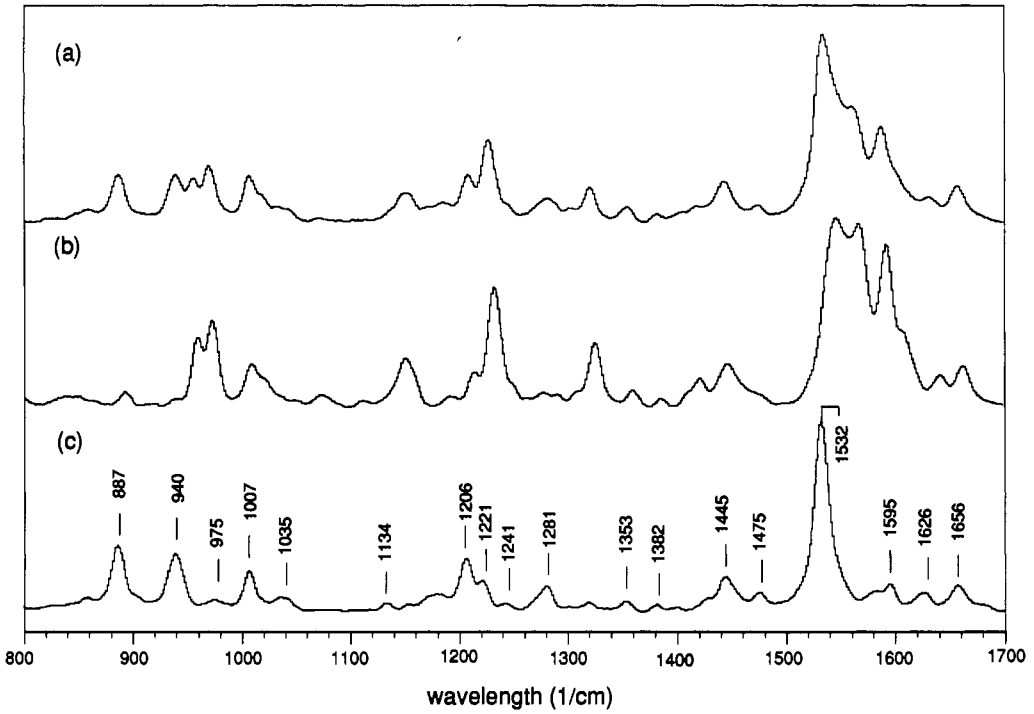

FIGURE 2: Resonance Raman spectra of octopus rhodopsin and its photoproducts at 80 K. (a) Excitation by the probe beam alone, at 457.9 nm, representing a mixture of rhodopsin, isorhodopsin, and bathorhodopsin spectra. (b) Probe beam and a coaxial 568.2-nm pump beam. (c) Spectrum a minus 0.5 times spectrum b, representing predominantly the bathorhodopsin spectrum.

ferent laser excitation wavelength (Deng & Callender, 1987).

The Raman spectra were taken with an optical multichannel analyzer (OMA) system, which consists of a triplemate spectrometer (Spex Industries, Metuchen, NJ) and a model DIDA-100 reticon detector connected to a ST-100 detector controller (Princeton Instruments, Trenton, NJ), which is interfaced to a MacIntosh II microcomputer (Apple Computer, Cupertino, CA). With the laser excitation beam at 457.9 nm, a spectral window of about 1400 cm^{-1} with resolution of 8 cm^{-1} can be detected simultaneously. The Raman band positions are calibrated against the known Raman peaks of toluene and are accurate to ± 1.5 cm^{-1}.

Results

Octopus Bathorhodopsin Spectrum. Figure 2 shows the low-temperature resonance Raman spectra of octopus rhodopsin obtained at 80 K by irradiating the sample with a 457.9-nm probe beam without (a) or with (b) a coaxial 568.2-nm pump beam. In panel c, the difference spectrum of spectrum a − (0.5 × spectrum b) is shown. Two main differences in the HOOP region (700–1000 cm^{-1}) are apparent between Figure 2a and figure 2b. In the first place, a decrease in the intensity of the Raman bands at 887 and 940 cm^{-1} is seen. Since the 568.2-nm light can only be effectively absorbed by octopus bathorhodopsin (λ_{max} = 540 nm), far away from absorption maxima of rhodopsin and isorhodopsin (λ_{max} = 472 and 462 nm, respectively), we assign these two bands to the hydrogen out-of-plane (HOOP) vibrational modes of octopus bathorhodopsin. Secondly, an increase in the intensities of the 960 and 970 cm^{-1} bands upon 568.2-nm irradiation is observed. On the basis of our previous Raman (Pande et al., 1987) and FTIR (Bagley et al., 1989) studies on octopus rhodopsin, we can assign these two bands to the octopus isorhodopsin and rhodopsin HOOP modes, respectively. The intensity ratios of the bands at 960 and 970 cm^{-1} do not change upon irradiation with the pump beam at 568.2 nm (compare Figures 2a and 2b), unlike what is observed in the bovine pigment (Oseroff & Callender, 1974). Since the absorption maxima of octopus rhodopsin (472 nm) is lower than that in bovine rhodopsin (500 nm), the 568.2-nm laser light cannot drive octopus rhodopsin to isorhodopsin as effectively; therefore, the relative amounts of octopus rhodopsin and isorhodopsin are determined only by the 457.9-nm probe laser beam. We estimate that the contribution of octopus rhodopsin in Figure 2c is about 5% or less, the rest of the spectrum being bathorhodopsin.

Deuteration Results and Assignment of the Bathorhodopsin HOOP Modes. The hydrogen out-of-plane vibrational modes of retinal have been extensively studied and characterized both in the free chromophore and when bound to bovine bathorhodopsin (Curry, et al., 1985; Palings et al., 1989). In general, C–H HOOP modes are found between 850 and 970 cm^{-1}, and deuterated C–D HOOP modes are in the 690–760-cm^{-1} region. We therefore limit our discussion on the labeled octopus pigments to this part of the bathorhodopsin Raman spectrum (600–1000 cm^{-1}). The main features of the octopus bathorhodopsin HOOP modes are two major Raman bands at 887 and 940 cm^{-1} and several small bands at 975, 861, 795, and 742 cm^{-1} (Figures 2c and 3a). All these bands, including the very weak ones, repeatedly appear in one or the other bathorhodopsin spectra with deuterium-labeled chromophores.

We applied the same subtraction procedure described above to a series of octopus pigments that were regenerated with deuterated chromophores. Shown in Figure 3 are the expanded HOOP regions of the resultant bathorhodopsin spectra from pigments labeled at the indicated position(s). The empirical assignments for the octopus bathorhodopsin HOOP bands may be given on the basis of the data in this figure.

The only effect on deuterating the chromophore Schiff base nitrogen in the HOOP mode region (Figure 3b) is an increase in intensity of the 981-cm^{-1} band. We assign this to the presence of a N–D in-plane bending mode. Since most of the N–D and C–D in-plane bending modes are the 960–990-cm^{-1} region, we will not discuss any bands in this region in the following spectra, unless we believe they originate from a HOOP mode. We also found that bathorhodopsin's protonated

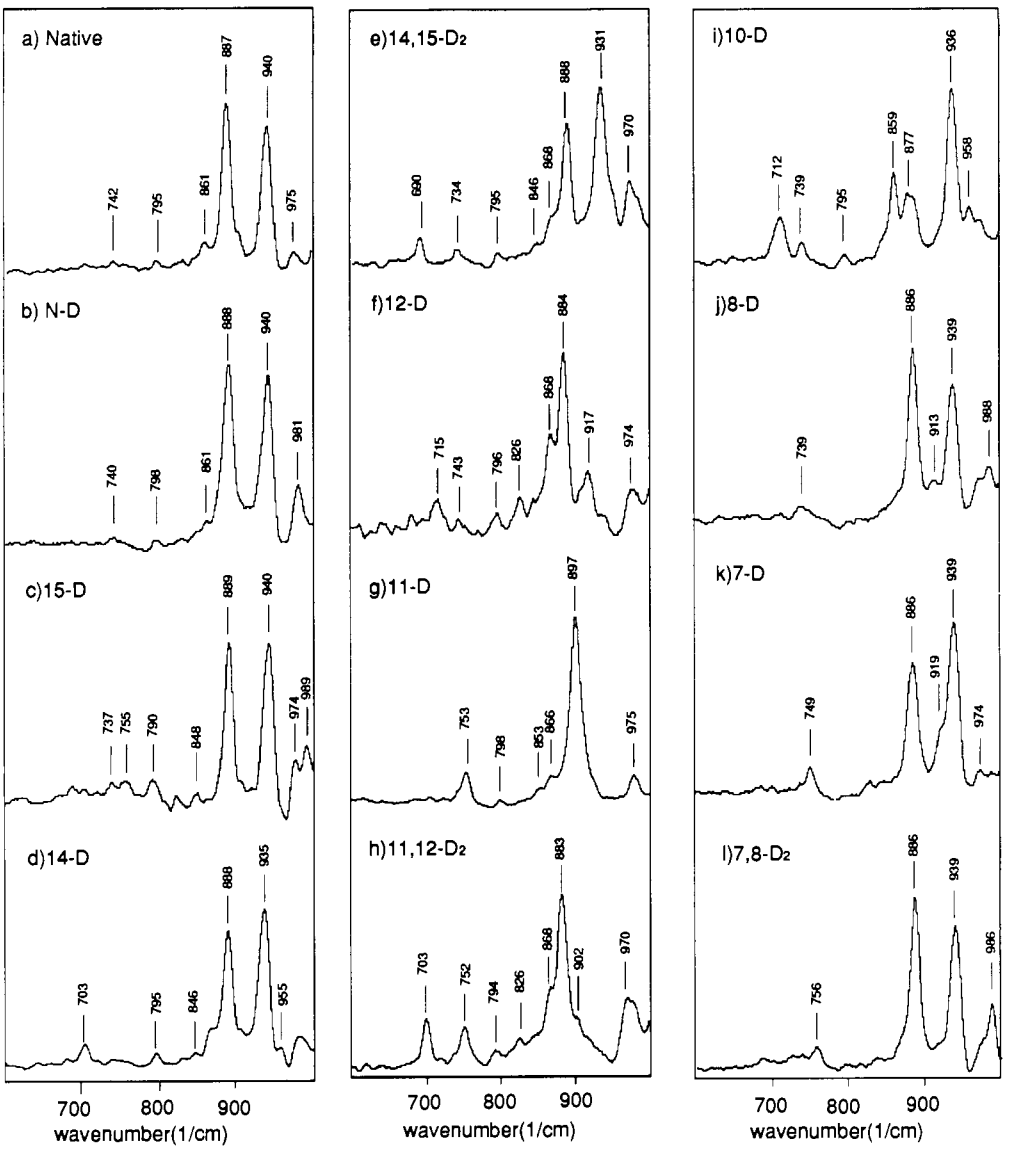

FIGURE 3: Resonance Raman spectra of HOOP modes from octopus bathorhodopsin and its deuterium-labeled derivatives obtained as in Figure 2: (a) native pigment; (b) ND; (c) $C_{15}D$; (d) $C_{14}D$; (e) C_{14} and $C_{15}D_2$; (f) $C_{12}D$; (g) $C_{11}D$; (h) C_{11} and $C_{12}D_2$; (i) $C_{10}D$; (j) C_8D; (k) C_7D; and (l) C_7 and C_8D_2.

Schiff base mode at 1656 cm^{-1} shifted to 1631 cm^{-1} upon deuteration of the Schiff base nitrogen (data not shown). The shift in frequency of this mode is of importance (see below).

The main effect of deuterating the C_{15} position (Figure 3c) is the appearance of the small band at 755 cm^{-1}, which we assign to the $C_{15}D$ HOOP mode.

Deuteration at the C_{14} position (Figure 3d) results in an intensity decrease of the band at 887 cm^{-1} and a concomitant appearance of a new band at 703 cm^{-1}. We therefore assign the 703-cm^{-1} band to the $C_{14}D$ HOOP mode and the 887-cm^{-1} band partially to the $C_{14}H$ HOOP mode. This assignment is confirmed by the spectrum from a chromophore in which both the C_{14} and C_{15} positions are deuterated (Figure 3e); the band intensity at 887 cm^{-1} again decreases, and a new band at 690 cm^{-1} with fairly strong intensity appears. We attribute the small down shift of the new band (690 cm^{-1}) relative to $C_{14}D$ singly deuterated chromophore (703 cm^{-1}) to the coupling between the $C_{14}D$ and $C_{15}D$ HOOP modes. For comparison, the $C_{14}H$ HOOP vibration was found to be fairly isolated from other HOOP motions in all-*trans*-retinal with its frequency at 876 cm^{-1} (Curry et al., 1985). It is interesting to point out that in bovine bathorhodopsin the $C_{14}H$ and $C_{14}D$ HOOP modes are also isolated and show fairly strong Raman bands at 850 and 693 cm^{-1}, respectively (Palings et al., 1989).

Table I: Spectral Location of the HOOP Modes Associated with $C_{12}H$ and $C_{11}H$ Hydrogen Out-of-Plane Vibrational Motions As Seen in Free Retinal [from the IR Data of Curry et al. (1982)] and in Octopus Bathorhodopsin (Data from this Report)[a]

deuterium position	$C_{11}D$	$C_{12}D$	$C_{11},C_{12}D_2$
free retinal	917 s, 740 s	906 s, 862 s, 815 m, 722 m	887 m, 864 s, 814 m, 742 s, 708 s
octopus bathorhodopsin	897 s, 753 s	917 s, 868 s, 826 m, 715 m	902 m, 868 s, 826 m, 752 s, 703 s

[a] s, strong intensity; m, moderate intensity.

Deuteration at the C_{12} position (Figure 3f) eliminates the strong Raman band at 940 cm^{-1} completely, with the concomitant appearance of two strong bands at 917 and 868 cm^{-1} and two moderate bands at 826 and 715 cm^{-1}. Deuteration at the C_{11} position (Figure 3g) also eliminates the band at 940 cm^{-1}, with the appearance of two strong bands at 897 and 753 cm^{-1}. With the use of vibrational spectroscopy and normal mode calculations, it has been argued (Curry et al., 1982) that the hydrogens trans to a double bond couple strongly to form an A_u-like HOOP mode that is IR active and an additional B_g-like HOOP mode. In the present study, we find surprising

similarities between the IR spectra of all-*trans*-retinal and the Raman spectra of octopus bathorhodopsin with regard to effects of deuteration at the C_{11} and/or C_{12} positions as illustrated in the Table I.

This close correspondence between all-*trans*-retinal and octopus bathorhodopsin enables us to make the following assignments in the octopus bathorhodopsin spectra by using the previous assignments for all-*trans*-retinal (Curry et al., 1982): the 940 cm^{-1} band to the $C_{11}H{=}C_{12}H$ A_u-like HOOP mode; the bands at 917 and 715 cm^{-1} to the decoupled $C_{11}H$ HOOP and $C_{12}D$ HOOP modes, respectively, in the $C_{12}D$-labeled chromophore; the bands at 897 and 753 cm^{-1} to the $C_{12}H$ HOOP and $C_{11}D$ HOOP modes, respectively, in $C_{11}D$-labeled chromophore; and the bands at 752 and 703 cm^{-1} to the $C_{11}D$ and $C_{12}D$ HOOP modes, respectively.

Deuteration at the C_{10} position (Figure 3i) lowers most of the band intensity at 887 cm^{-1} with concomitant increase at 712 cm^{-1}. This shift, which is identical with that observed in all-*trans*-retinal (Curry et al., 1982), suggests that the force constant of this coordinate changes little upon binding to the opsin. However, a new peak appeared at 859 cm^{-1}, which is not observed in all-*trans*-retinal, indicating that this HOOP motion may be coupled with other modes more strongly than the free chromophore.

Upon deuteration of the C_8 position (Figure 3j), the intensity of the 940-cm^{-1} band decreases and two weak bands at 913 and 739 cm^{-1} appear. Deuteration at the C_7 position (Figure 3k) results in intensity decrease of the band at 887 cm^{-1} with a concomitant appearance of a shoulder at 919 cm^{-1} and a new band, with moderate intensity, at 749 cm^{-1}. We assign the bands at 913 and 739 cm^{-1} to the C_7H and C_8D HOOP modes, respectively, in the C_8D-labeled chromophore. The bands at 919 and 749 cm^{-1} are assigned to the C_8H and C_7D HOOP modes, respectively, in the C_7D-labeled chromophore. These assignments are tentative, and C_7H and C_8H HOOP modes are apparently coupled to each other and with other HOOP modes. When the chromophore is deuterated at both the C_7 and C_8 positions, a $C_7D{=}C_8D$ HOOP mode is observed at 756 cm^{-1}. This frequency is substantially higher than that found in all-*trans*-retinal in solution and bovine bathorhodopsin (720 and 728 cm^{-1}, respectively; Curry et al., 1985; Palings et al., 1989).

Discussion

Apart from a band found at 1240 cm^{-1} in bovine bathorhodopsin but absent in octopus bathorhodopsin, the Raman spectrum of bovine bathorhodopsin and octopus bathorhodopsin given in Figure 2C are very close in the fingerprint region. This has been pointed out previously and discussed in resonance Raman (Pande et al., 1987) and FTIR (Bagley et al., 1989) studies. The close vibrational correspondence has lead to a number of structural conclusions. One is that the primary photophysics involves a photoisomerization of the chromophore from an 11-*cis* to an 11-*trans* structure. The fingerprint region from 1100 to 1400 cm^{-1} is sensitive to the isomeric form of the chromophore, and the pattern for bovine bathorhodopsin pigments has been analyzed in terms of an all-*trans* configuration (Palings et al., 1987); the close correspondence between the two batho pigments in the fingerprint region suggests that the configuration of octopus bathorhodopsin is very much like that of the bovine pigment. Another key attribute to visual pigments and some of their photoproducts is that the retinal chromophore is covalently linked to the apoprotein by a protonated Schiff base, a positively charge moiety. The positive charged nature of the Schiff base is of importance to pigment color regulation. Moreover, the separation of the protonated Schiff base from its putative apoprotein counterion during chromophore isomerization can result in substantial electrostatic potential energy. It has been proposed that much (Birge et al., 1988) if not most (Honig et al., 1979b) of the energy difference between rhodopsin and bathorhodopsin (see below) is electrostatic in nature. Both bovine and octopus batho products show a band at 1656 cm^{-1}, characteristic of a protonated Schiff base linkage (Pande et al., 1987).

The "low-frequency" modes in the 700–1000-cm^{-1} region found in bathorhodopsin (Oseroff & Callender, 1974) and identified as HOOP modes by isotopic substitution studies (Eyring et al., 1980) have long been of interest. They generally are found only weakly in the spectra of retinals and retinal chromophores, and the pattern found in bovine bathorhodopsin, at 853, 875, and 921 cm^{-1}, seems particularly characteristic of the primary photoproduct of visual pigments.[2] For example, a very similar pattern has been found in the Raman spectra of the bathorhodopsins from several vertebrate bathorhodopsin pigments (Barry & Mathies, 1987) having varying absorption maxima. With this in mind, we undertook this study to investigate, in a detailed isotopic investigation, the HOOP modes of octopus bathorhodopsin. Our earlier studies (Pande et al., 1987) had suggestd that the HOOP modes in octopus rhodopsin differed from that found in these other species. Our data now allow us to make qualitative normal mode assignments.

The internal coordinate characters of the HOOP modes in octopus bathorhodopsin are similar to those found for free (solution) all-*trans*-retinal. In particular, the band at 940 cm^{-1} in octopus bathorhodopsin responds very much to isotopic substitution as a band found at 966 cm^{-1} in all-*trans*-retinal (see Results; Table I). In the studies of Curry et al. (1982, 1985) on the normal modes of isomers of retinals, it was found that HOOP modes are quite localized compared to other vibrational modes in retinal. Therefore, it is a good approximation to use local symmetry to understand the HOOP modes. This 966-cm^{-1} mode was calculated to be a coupled $C_{11}H{=}C_{12}H$ wagging motion where one of the hydrogens rotates clockwise while the other rotates counterclockwise (looking down the polyene axis) across the trans $C_{11}{=}C_{12}$ bond. For a planar molecule, the local point symmetry of the trans $HC_{10}{-}C_{11}{=}C_{12}{-}C_{13}H$ moiety is C_{2h}; the 966-cm^{-1} mode belongs to the A_u representation, which is IR allowed but Raman forbidden.

While the octopus mode at 940 cm^{-1} is like the 966-cm^{-1} mode in all-*trans*-retinal, there are important differences. For one thing the frequencies differ by 26 cm^{-1}, which suggests that the basic force constant or coupling constant of the 940-cm^{-1} mode is smaller than that for the 966-cm^{-1} mode. More importantly, the 966-cm^{-1} band is strong in the IR but very weak in the Raman spectrum of all-*trans*-retinal, consistent with the A_u-like symmetry assignment. The octopus bathorhodopsin 940-cm^{-1} mode, on the other hand, is strong in both the IR (Bagley et al., 1989) and in the Raman (Figure 2c) spectra. The local planar symmetry in the ground state appears broken. If we assume that the $C_{11}H{=}C_{12}H$ wagging motion is localized to the $HC_{10}{-}C_{11}{=}C_{12}{-}C_{13}H$ moiety, then the local symmetry can be broken by an out-of-plane twist about the $C_{11}{=}C_{12}$ double bond. It has been shown previously that a twist of the hydrogens out of the polyene plane of even a modest 5° can produce a very significant HOOP-mode intensity (Eyring et al., 1980, 1982; Warshel & Barboy 1982).[3]

[2] The 853-cm^{-1} band has recently been resolved into three bands at 838, 850, and 858 cm^{-1} (Palings et al., 1989).

Using the formulas derived previously relating Raman intensity to twist angle [see e.g., Eyring et al. (1982)], we obtain an estimated twist of 4.7° about the C_{11}=C_{12} bound (assuming the twist in the excited state is small). The energy needed to produce this modest twist about a double bond need not be large. For example, a twist of 90° about a double bond might normally take ~25 kcal/mol compared to ~5 kcal/mol for a single bond. However, the π-electron structure is quite delocalized in these red-shifted retinal pigments, suggesting that double bonds take on considerable single-bond character and vice versa. Assuming a reasonable value, we think, of ~15 kcal/mol for a 90° twist about the C_{11}=C_{12} double bond in octopus bathorhodopsin, we can estimate that a 4.7° twist would require about 1.2 kcal/mol (= 1.5 sin 4.7°) in energy, not a large number compared to the 32 kcal/mol of energy stored in octopus bathorhodopsin.[4]

The strong band at 887 cm^{-1} consists of two degenerate modes, one of moderate intensity, the $C_{14}H$ wag, and the intense $C_{10}H$ wag. These modes are close to their positions found in all-*trans*-retinal, at 876 and 887 cm^{-1}, respectively (Curry et al., 1982, 1985). Both the C_{14} and C_{10} carbons are connected across single bonds to adjacent carbons with CH groups or across a double bond with a bonded methyl groups (see Figure 1). The coupling across single bonds to an adjacent CH wag is weak because coupling across π-electron orbitals is much stronger than across σ orbitals (Curry et al., 1982; Palings et al., 1989). For this reason, there is no clear local symmetry for the $C_{14}H$ and $C_{10}H$ wags. In general, it is expected that both bands will be weak in their Raman spectra, as is found for all-*trans*-retinal. From the rather high intensity found in the $C_{14}H$ and $C_{10}H$ wags of octopus bathorhodopsin, we conclude that there must be some torsional distortion along either the ground state or the excited electronic state appropriate torsional coordinates.[3]

The HOOP normal-mode pattern found in bovine bathorhodopsin is quite anomalous with regard to the $C_{11}H$ and $C_{12}H$ wags (Eyring et al., 1982; Palings et al., 1989). The normal-mode character of the octopus bathorhodopsin coupled $C_{11}H$=$C_{12}H$ HOOP A_u-like mode at 940 cm^{-1}, and the similar mode in all-*trans*-retinal, is found to be decoupled in bovine bathorhodopsin to form a $C_{11}H$ HOOP band at 922 cm^{-1} and a $C_{12}H$ HOOP near 858 cm^{-1}. In the detailed normal-mode calculations on bovine bathorhodopsin performed recently (Palings et al., 1989), the $C_{12}H$ wag force constant was found to be much lower than that in all-*trans*-retinal as is the C_{11}-H-$C_{12}H$ coupling constant. There are a number of reasons why the two wags might decouple, and two have been suggested (Palings et al., 1989). One is that the $C_{11}H$ and $C_{12}H$ wag frequencies are far enough away from each other so that a partial decoupling occurs. This is consistent with the anomalously low frequency of the $C_{12}H$ wag. However, the separation of the two wags is large in octopus bathorhodopsin as well, in this case because the CH_{11} wag is higher in frequency, yet the $C_{11}H$ wag is still coupled with the $C_{12}H$ wag. For example, assuming that CD wags are reasonably localized (because their frequency is much lower than the other CH wags), the wag frequency difference can be estimated by calculating the difference in frequency between the CD_{11} wag and the CD_{12} wag. This difference is 18 cm^{-1} for all-*trans*-retinal (740 and 722 cm^{-1}, respectively), 43 cm^{-1} for bovine bathorhodopsin (742 and 699 cm^{-1}), and 38 cm^{-1} for octopus bathorhodopsin (753 and 715 cm^{-1}). Also mentioned as a possible source decoupling the two wags in bovine bathorhodopsin is a significantly reduced C_{11}=C_{12} bond order, since the coupling depends strongly on the amount of π-electron character in the double bond. The double-bond bond orders in these batho pigments are reduced. For example, the ethylenic modes of these pigments are generally believed to arise from rather complicated motions involving C=C and C=N stretching. The most intense mode often contains a large amount of C_{11}=C_{12} motion so that the fact that this ethylenic mode is at a substantially lower frequency in both bovine and octopus bathorhodopsins compared to the respective primary pigments clearly suggests that substantial π-electron delocalization with a concomitant loss of bond order in the C_{11}=C_{12} and other double bonds has occurred. Both dominant ethylenic modes lie at nearly the same frequency (at 1536 and 1532 cm^{-1} in bovine and octopus bathorhodopsins, respectively), suggesting, at first sight, that the C_{11}=C_{12} bond order is nearly the same for the two pigments. However, this must be checked out with a thorough normal-mode analysis of the ethylenic modes of the two pigments. It is very possible, even likely we believe, that the two normal modes differ in the contribution that C_{11}=C_{12} stretching makes to the coordinate.

On this basis, it is quite feasible that a larger retinal C_{11}=C_{12} bond twist in bovine bathorhodopsin is responsible for the decoupling of the $C_{12}H$ wag from $C_{11}H$ wag. This larger twist could be triggered by a negatively charged protein residue near C_{12} in bovine bathorhodopsin (Eyring et al., 1982). The placement near C_{12} was chosen since the $C_{12}H$ wag frequency (and hence its force constant) has been reduced substantially. Indeed, a negatively charged protein residue near the center portion of bovine rhodopsin's chromophore, the primary pigment, has been proposed by various experiments and theoretical modeling (Honig et al., 1979a; Koutalos et al., 1989). Recently, the studies of bovine rhodopsin by site-directed mutagenasis have revealed that glutamic acid 113 interacts with the chromophore very strongly, modulating this pigment's λ_{max} from 500 to 380 nm after modification by glutamine (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). This residue has been postulated as the negatively charged counterion to the chromophore's positively charged protonated Schiff base. Glutamic acid 122 also interacts with the chromophore, but to a much lesser extent, suggesting the possibility that this residue may be uncharged. It certainly is possible that either one of these residues is close enough to the C_{12} carbon of bovine bathorhodopsin to cause the observed unusual behavior in its $C_{11}H$ and $C_{12}H$ wags. Neither of these residues is conserved in octopus rhodopsin. The analogous residue to 113 position is a tyrosine (tyrosinate?) in octopus rhodopsin and that of 122 a phenylalanine.

The frequencies of the $C_{14}H$ and $C_{10}H$ wags in bovine bathorhodopsin, at 850 and 875 cm^{-1}, respectively, are also

[3] The intensity of a Resonance Raman band depends in general upon both ground and excited electronic state properties, while a band's position depends only on ground state properties. A band's intensity can be evaluated if the Frank–Condon vibrational overlap integrals between the ground vibrational state and the excited vibronic levels are known. The intensity of a HOOP mode depends on the scalar product of Δ, the origin shift from the ground- to the excited-state equilibrium position of the coordinate, and the HOOP normal-mode vector **Q**. For the A_u-like HOOP mode, **Q** and Δ are parallel, representing the rotation about the C=C double bond. So the intensity is proportional to Δ^2 for small displacements and harmonic potential wells (Eyring et al., 1980; Warshel, 1977; Warshel & Dauber, 1977). Assuming that the local symmetry is rigorously C_{2h} for the $C_{11}H$=$C_{12}H$ wag, the A_u mode is Raman forbidden and some ground-state symmetry-breaking factor, like torsional rotation, is necessary.

[4] The torsional angles discussed in the text, even about double bonds, are not large for delocalized π-electron systems. For example, the X-ray structure of aspartate aminotransferase reveals that the Schiff base bond of the pyridoxal phosphate cofactor has about a 10° twist (Kirsch et al., 1984).

somewhat anomalous, both being substantially lower than their counterparts in all-trans-retinal, at 876 and 887 cm^{-1}. In octopus bathorhodopsin, the $C_{10}H$ wag was at 887 cm^{-1}, which is nearly at the same position as that for all-*trans*-retinal. We do not directly observed the $C_{14}H$ wag. However, the octopus $C_{14}D$ wag is 10 cm^{-1} lower than $C_{10}D$ wag, a pattern repeated in all-*trans*-retinal.

The results reported here bear on the particular factors that are responsible for color regulation in visual pigments. A commonly used measure of the interactions between the apoprotein and the bound chromophore responsible for pigment λ_{max} is the so-called "opsin shift", the difference in energy between the visual pigment chromophore's absorption maximum and that of a protonated Schiff base in solution. A number of specific chromophore–apoprotein interaction factors have been considered [see Koutalos et al. (1989) for a recent study of bovine and octopus rhodopsins and a thorough discussion]. These include the strength of the hydrogen bond that the protonated Schiff base moiety makes with its environment [generally, but not necessarily, the weaker the hydrogen bond the more red-shifted the absorption maximum (Honig et al., 1976; Palings et al., 1987)], apoprotein charged residues surrounding the chromophore [which may shift the absorption maximum in either direction depending on charge and placement (Honig et al., 1979a)], and twists about single and double bonds [twists about double bonds yield red shifts, while twists about single bonds may yield either red or blue shifts depending on the degree of π-electron delocalization (Kakitani et al., 1985)]. Other factors may also, and probably do, play important roles.

While the bathorhodopsins of both octopus and bovine visual pigments essentially absorb at the same wavelength (540 and 543 nm, respectively), the present data and previous studies suggest that the various molecular factors responsible for the red shift are present to substantially different degrees. The data on octopus bathorhodopsin argue that there are significant twists about the $C_{11}=C_{12}$ double bond and perhaps other C–C bonds along the chromophore's polyene chain, as discussed above. Torsional distortions are almost certaintly present along bovine bathorhodopsin's polyene chain as well. However, the HOOP modes for the two pigments differ in their internal mode makeup and in their frequencies as discussed above. It thus seems reasonable to suppose that the exact nature of the polyene torsional distortion is different for the two bathorhodopsins, and this may result in a different shift in λ_{max} between the two pigments.

Moreover, while both batho products form protonated Schiff bases between chromophore and protein, the hydrogen-bonding strength appears stronger in bovine bathorhodopsin than in octopus bathorhodopsin. This conclusion is based on observation that the shift of the protonated Schiff base band, a $-C=NH^+-$ stretch, upon deuteration of the Schiff base, is 33 cm^{-1} (varying from 28 to 33 cm^{-1} depending on the study) for bovine bathorhodopsin (Eyring & Mathies, 1979; Deng & Callender, 1987; Palings et al., 1987) and 25 cm^{-1} for octopus bathorhodopsin (see Results). The position of this band and its shift upon deuteration have undergone considerable discussion (Kakitani et al., 1983; Baasov et al., 1987; Deng & Callender, 1987; Gilson et al., 1988; Palings et al., 1987). It is believed that these are markers for the hydrogen-bonding interaction of the protonated Schiff base moiety with its environment, particularly the shift in the frequency of the Schiff base band upon deuteration. The isotope shift of 33 cm^{-1} for bovine bathorhodopsin is unusually large, which suggests a particularly strong hydrogen-bonding interaction. For comparison, a shift of 24 cm^{-1} is found for models of retinal protonated Schiff bases in methanol where the Schiff base is completely solvated. Thus, the shift of 25 cm^{-1} of the protonated Schiff base mode to 1631 cm^{-1} upon deuteration suggests a weaker hydrogen bonding between the protonated Schiff base moiety and apoprotein in octopus bathorhodopsin compared to bovine bathorhodopsin. There is no clear cut rule between hydrogen bonding and wavelength regulation. It is necessary to know the specific molecular structure of the hydrogen bond. Assuming that the hydrogen bond involves a charged residue that forms a salt bridge with the protonated Schiff base, it is found that a weaker hydrogen-bond interaction will lead to λ_{max} red shifts (Baasov et al., 1987; Kakitani et al., 1985).

A third factor that must be considered in understanding the absorption maximum in bovine bathorhodopsin is the putative protein charge located near retinal's C_{12} and presumed responsible for the decoupling of the $C_{11}H$ and $C_{12}H$ wags. If present, this electrostatic interaction almost certainly would have a large effect on bovine bathorhodopsin's λ_{max} on the basis of what we know of the effects of charges on absorption maximum [see, e.g., Kakitani et al. (1985)]. This interaction is much weaker, if not absent, in octopus bathorhodopsin according to our results, as discussed above, and thus would not greatly contribute to the λ_{max} in octopus bathorhodopsin.

An outstanding feature of the absorption of light by visual pigments is that much of the light energy is converted to a very high energy photoproduct, the bathorhodopsins. One of the factors determining the high energy of bovine bathorhodopsin that has been mentioned involves a torsionally strained polyene retinal backbone extending into the alkane bonds of the attached lysine residue (Birge et al., 1988). As we discussed above, the intensity in the $C_{11}H=C_{12}H$ coupled hydrogen wagging motion and in the other HOOP modes suggest that octopus bathorhodopsin is torsionally strained. Our rough estimate of 1.2 kcal/mol for the $C_{11}H=C_{12}H$ coordinate is small compared to bathorhodopsin's 32 kcal/mol enthalpy (relative to rhodopsin). However, it seems unlikely that only one bond could be held in a nonplanar geometry without other bonds also rotating. If we take the ten bonds from retinal's C9 to the nitrogen of the Schiff base and through three alkane bonds of the lysine and assign a value of 1.2 kcal/mol of torsional strain energy, the total energy from this factor could total 12 kcal/mol. A value of 10 kcal/mol was computed for bovine bathorhodopsin by Birge et al. (1988) for this component, in rough agreement with our estimate. Presumably other factors are very important in determining energy storage. One very likely factor has to do with the changes in the electrostatic interaction between the positively charged protonated Schiff base chromophore and the surrounding negatively charged apoprotein matrix that would result from a cis to trans phototransition in the rhodopsin to bathorhodopsin conversion (Birge et al., 1988; Honig et al., 1979b). Another that has been postulated has to do with a conformational strain energy involving the chromophore and bonded atoms with nearby (yet unknown) protein residues (Birge et al., 1988; Warshel & Barboy 1982). This latter term has not yet been modeled at the level of particular atoms or molecular groups, but in an ad hoc way. In qualitative terms then, it seems likely that the chromophore plus lysine torsional distortion is present in both octopus and bovine bathorhodopsins and should be considered as a factor in their high energy. It seems unlikely to us that this term, although significant, dominates in bathorhodopsin's high energy. Also, given the differences in the nature of the HOOP modes in the two pigment systems, the

exact angular nature of the distortion is unlikely to be the same for the two batho species.

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