# A Resonance Raman Study of the C═N Configurations of Octopus Rhodopsin, Bathorhodopsin, and Isorhodopsin†

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ABSTRACT: The resonance Raman spectra of octopus rhodopsin, bathorhodopsin, and isorhodopsin at 120 K have been obtained as well as those of pigments regenerated with isotopically labeled retinals near the C14−C15 bond. Deuteration of the Schiff base nitrogen induces relatively large changes in the C−C stretch region between 1100 and 1300 $cm^{-1}$, including a large frequency shift of the C14−C15 stretch mode located at 1206−1227 $cm^{-1}$ in the three octopus species, as revealed by the Raman spectra of their 14,15-$^{13}C_2$ derivatives. Such results are different compared to those of the bovine pigments, in which no significant frequency shift of the C14−C15 stretch mode was observed upon Schiff base N deuteration. In an earlier Raman study of a Schiff base model compound which contained only one single bond adjacent to two double bonds, we have found that the stretch mode of this C−C single bond at 1232 $cm^{-1}$ shifts up by 15 $cm^{-1}$ and its intensity is also greatly reduced upon Schiff base N deuteration when the C═N configuration is anti [Deng et al., (1994) *J. Phys. Chem. 98*, 4776−4779]. The same study has also shown that when the C═N configuration is syn, the C−C stretch mode should be at about 1150 $cm^{-1}$. Since the C14−C15 stretch mode frequency is relatively high in the spectra of octopus rhodopsin and bathorhodopsin (>1200 $cm^{-1}$) and since the normal mode pattern near the Schiff base is similar to the model, we suggest that the C═N configuration in these two species is anti. The different responses of the C14−C15 stretch mode to the Schiff base nitrogen deuteration in bovine and octopus pigments are due to the fact that the coupled C14−C15 stretch and the C12−C13 stretch motions in the model compound or in bovine rhodopsin are altered in octopus rhodopsin so that the stretch motion of the C14−C15 bond is more localized, similar to the C−C stretch motion in the small Schiff base model compound. In clear contrast with the bovine rhodopsin Raman spectrum, which is very similar to that for the 11-*cis*-retinal Schiff base, the drastically different octopus rhodopsin spectrum indicates large protein perturbations on the C11═C12−C13 moiety, either by steric or by electrostatic interactions. Further studies are required to determine if such spectral differences indicate a difference of the energy conversion mechanisms in the primary photochemical event of these two pigments.

The visual pigments, called rhodopsins, consist of a chromophore, the 11-*cis* isomer of retinal, covalently bound via a protonated Schiff base to the $\epsilon$-amino group of a lysine residue in the apoprotein opsin. Upon absorption of a photon by rhodopsin, the chromophore isomerizes from the 11-*cis* to an *all-trans* configuration, and its primary photoproduct, bathorhodopsin, is formed. About two-thirds of the photon's energy is converted to chemical energy in this photoreaction in either bovine rhodopsin or octopus rhodopsin (Cooper, 1979; Cooper et al., 1986). An intriguing question is just how the protein pigment accomplishes this extremely efficient conversion.

In classical terms, energy can be stored in bathorhodopsin in a variety of different forms: conformational strain about the retinal chromophore polyene chain or about the chromophore−protein linkage, steric interactions between the chromophore and protein binding pocket, and electrostatic interactions between the retinal chromophore and protein (Birge et al., 1988; Callender et al., 1989; Eyring et al., 1982; Honig et al., 1979; Warshel & Barboy, 1982). Electrostatic interactions certainly play an important role since the isomerization of the retinal chromophore around its C11═C12 bond induces a large displacement of the Schiff base hydrogen, which carries most of the formal positive charge of the retinal protonated Schiff base (RPSB), against the electrostatic field generated by the protein (Honig et al., 1979). This is illustrated in Scheme 1, where the electrostatic field of the protein is represented simply by a putative negatively charged residue that serves as a counterion to the Schiff base positive charge. In bovine rhodopsin, this counterion has been identified as the carboxylate of Glu-113; in octopus rhodopsin, the counterion is not yet determined. It is known that the active site is more complicated than this (it certainly contains structural water molecules, for example) and the field at the Schiff base

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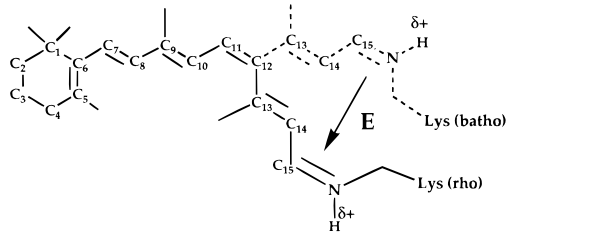
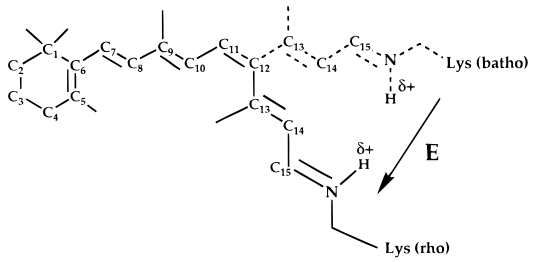
Scheme 1




a) 11-cis to 11-trans isomerization with the C=N syn configuration




b) 11-cis to 11-trans isomerization with the C=N anti configuration

position will be made up of contributions from the counterion, any structural water molecules, and other charged and polar protein residues.

In this paper, the configurations of the chromophore and the Schiff base bond are determined by obtaining resonance Raman spectra of the bound chromophore in octopus rhodopsin, its photoproduct bathorhodopsin, and the artificial pigment isorhodopsin, which is based on the 9-*cis* configuration of the retinal chromophore. As can be seen in Scheme 1, the distance that the positive charge moves in the 11-*cis* to 11-*trans* isomerization in the rhodopsin to bathorhodopsin photoconversion is substantially greater for a C═N syn configuration (Scheme 1a) than for a C═N anti configuration (Scheme 1b). In contrast, the required movement of the Lys residue is relatively small for the syn configuration. In the anti configuration, the required movement of the Lys residue in such a reaction is large, and considerable distortion of the retinal chain in bathorhodopsin and/or rhodopsin is expected. Thus, the structure of the Schiff base C═N bond is an important structural factor in understanding energy conversion in visual pigments.

It has been shown that the C═N configuration of retinal as a protonated Schiff base (RPSB) can be determined by Raman spectroscopy based on the shift of the C14−C15 stretch frequency upon Schiff base N deuteration as well as on the shifts of other marker bands (Smith et al., 1984; Deng et al., 1994; Palings et al., 1987). In bovine rhodopsin and bathorhodopsin, such studies (Palings et al., 1987) have assigned the C═N configuration to be anti since there is little shift ($<5$ cm$^{-1}$) and intensity change of the C14−C15 stretch mode upon Schiff base N deuteration. In contrast, a C═N syn configuration is expected to result in a large ($\sim$50 cm$^{-1}$) shift of the C14−C15 stretch mode, as has been observed in dark-adapted bacteriorhodopsin (Smith et al., 1984).

In the vibrational spectroscopy of octopus visual pigments, large spectral changes are observed in the octopus rhodopsin C−C stretch mode region upon Schiff base N deuteration, while the changes in octopus bathorhodopsin are relatively minor (see below; Deng et al., 1991b). It is therefore possible that, besides the isomerization around the C11═C12 bond, the photoreaction in octopus pigment might also involve an isomerization around the C═N bond, from a syn configuration in rhodopsin to an anti configuration in bathorhodopsin. In this paper, octopus pigments are regenerated with isotopically labeled retinals near the C14−C15 bond and studied by Raman spectroscopy. This permits assignments of marker bands relevant to probing the issue of the C═N configuration in octopus rhodopsin and its photoproduct bathorhodopsin. In addition, these studies determine the conformation of the C12−C13 single bond in octopus rhodopsin and the nature of the internal coordinates that make up the observed bands.

## MATERIALS AND METHODS

The synthesis and characterization of the 14,15-$^{13}C_2$- and 12,13-$^{13}C_2$-labeled retinals have been described (Lugtenburg et al., 1988). Regenerated octopus rhodopsin with labeled chromophores was prepared as previously described (Deng et al., 1991a; Koutalos et al., 1989). One resonance Raman experiment was performed on octopus rhodopsin at 10 °C with the rapidly flowing sample method as described previously (Pande et al., 1987), except a cylindrical lens was used to focus the 5 mW of 457.9 nm probe laser beam in order to minimize the composition of metarhodopsin in the rhodopsin spectrum, and a stronger 568.2 nm laser light (400 mW instead of 60 mW) was used to irradiate the cooled sample reservoir to convert the acid metarhodopsin back to rhodopsin. The modification of the method eliminates most of the acid metarhodopsin that contributes to the rhodopsin spectrum published previously (Pande et al., 1987).

Other resonance Raman experiments were performed at 120 K in a liquid nitrogen cold finger dewar, as previously described, with the dual-beam pump−probe technique (Deng et al., 1991a; Oseroff & Callender, 1974). The native or isotopically labeled membranes were centrifuged to a thick pellet that was applied to the sample tip of the coldfinger and then cooled down to 120 K. The photostationary mixtures of rhodopsin ($\lambda_{max} = 472$ nm), isorhodopsin ($\lambda_{max} = 462$ nm), and bathorhodopsin ($\lambda_{max} = 540$ nm) at 120 K were obtained and their Raman spectra taken with the "probe" beam (457.9 nm line from an $Ar^+$ laser). The composition of the mixture can be changed by simultaneously irradiating the sample with the second high-intensity "pump" laser beam. For each sample, three Raman spectra were taken: The first was a probe-only spectrum. The second was a pump−probe (575 nm pump from a dye laser) spectrum with a pump:probe intensity ratio of 50:1. The third was also a pump−probe (with 553 nm pump) with a pump:probe intensity ratio of 40:1. These three type of spectra were taken sequentially in a relatively short time period, and the measurements were repeated many times and the data pooled to reduce the systematic error due to drifts in optical alignment.

Although each of the three measured Raman spectra contains all three species (rhodopsin, bathorhodopsin, and isorhodopsin), specific marker bands can be assigned to a specific species. Based on the knowledge of the characteristic bands of each species, it is possible to determine constituent ratios in each spectrum. Previous Raman and FTIR studies have shown that the 887 and 940 cm$^{-1}$ bands are unique to bathorhodopsin, the 956 and 1325 cm$^{-1}$ bands unique to isorhodopsin, and the 973 cm$^{-1}$ band unique to rhodopsin (Bagley et al., 1989; Deng et al., 1991a). Based

on such information, we can see immediately that the first spectrum (457.9 nm probe only) is a bathorhodopsin-rich one (bands in 940 and 887 $cm^{-1}$ are intense), while the second one (weak 457.9 nm probe + strong 575 nm pump) and the third one (weak 457.9 nm probe + strong 553 nm pump) are bathorhodopsin-poor spectra (the bands in 940 and 887 $cm^{-1}$ are small). Careful examination shows that the third spectrum has more isorhodopsin and less rhodopsin than the second one (as indicated by the larger intensity ratio of the 956 $cm^{-1}$/973 $cm^{-1}$ bands). Thus, by subtracting a fraction of the first spectrum from the second one, adjusted to eliminate the 887 and 940 $cm^{-1}$ bands, a bathorhodopsin-free spectrum is obtained. Similarly, by subtracting the first spectrum from the third one, another bathorhodopsin-free spectrum is also obtained. Note that although both difference spectra are bathorhodopsin-free, the rhodopsin:isorhodopsin ratios of each one are different. The first difference spectrum, obtained by subtraction of the first "measured" spectrum from the second "measured" one, has more rhodopsin and less isorhodopsin than the other difference spectrum. Thus, by subtracting the second difference spectrum from the first one, a pure rhodopsin spectrum is obtained. This spectrum is identical to the spectrum obtained from the flow experiment at ambient temperature (see Figure 1). Next, by subtracting the first difference spectrum from the second one, a pure isorhodopsin spectrum is obtained. This is the first resonance Raman spectrum of octopus isorhodopsin, and it confirms the FTIR spectrum (Bagley et al., 1989).

The pure bathorhodopsin spectrum, obtained by subtraction of the pure rhodopsin and isorhodopsin spectra, obtained as described above, from the first (probe only) bathorhodopsin-rich spectrum, is identical to a previous one obtained by a slightly different method (Deng et al., 1991a,b).

From the parameters which are used to perform the subtractions, it is determined that the first 457.9 nm probe-only spectrum contains 57% bathorhodopsin, 22% rhodopsin, and 21% isorhodopsin; the second spectrum (457.9 nm probe pluse 575 nm pump) contains 22% bathorhodopsin, 40% rhodopsin, and 38% isorhodopsin; and the third (457.9 nm probe plus 553 nm pump) contains 22% bathorhodopsin, 28% rhodopsin, and 50% isorhodopsin. The composition of the probe-only spectrum thus determined is consistent with the chemical extraction result (Pande et al., 1987), of 58% bathorhodopsin, 22% rhodopsin, and 20% isorhodopsin. The composition of the second one confirms the estimation made by Deng et al. (1991a). We estimate that these compositions are accurate to about 5%.

## RESULTS

*Rhodopsin.* Figure 1 shows the resonance Raman spectra of octopus rhodopsin obtained by the rapid flow method at 10 °C (a) and by the pump−probe method at 120 K (b). It can be seen that the Raman spectra of the octopus rhodopsin obtained by the two different methods are very much the same, with some minor differences in the band positions and band width as might be expected from the difference in sample temperature. However, the octopus rhodopsin spectrum presented here is significantly different from the spectrum published in a previous study from our lab (Pande et al., 1987). Specifically, bands at 1004 and 1274 $cm^{-1}$ assigned to the octopus rhodopsin in the earlier study are not found in the present spectrum. Since it was known that the sample used in the previous study contained about 25% acid metarhodopsin, which has strong Raman bands at these two positions (Pande et al., 1987), these bands in the previous rhodopsin spectrum can now be assigned to acid meta-rhodopsin.

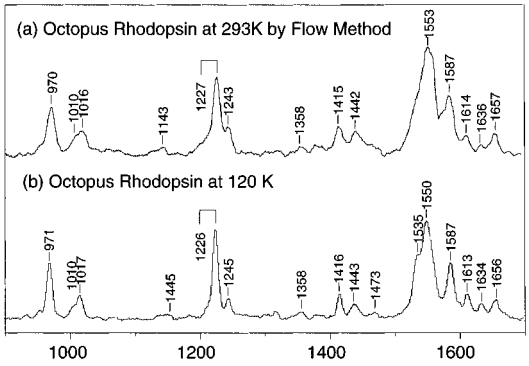



FIGURE 1: Raman spectra of octopus rhodopsin (a) obtained by the rapid flowing method at 10 °C and (b) by the pump−probe method at 120 K.

Like bovine rhodopsin, regeneration experiments of the apoprotein with retinal isomers suggest that octopus rhodopsin contains an 11-*cis*-retinal chromophore (Deng et al., 1991a; Koutalos et al., 1989). However, the Raman spectrum suggests that the chromophore in octopus rhodopsin does not have the same 11-*cis*-conformation as bovine rhodopsin. For example, a band near 1270 $cm^{-1}$, assigned to the 11H+12H rock mode (Curry et al., 1985; Eyring et al., 1982) in bovine rhodopsin and found in the Raman spectra of 11-*cis*-retinal and its Schiff base derivatives in solution as well, is missing in octopus rhodopsin. Furthermore, a band at 997 $cm^{-1}$ found in the bovine rhodopsin spectrum apparently shifts up to 1010 $cm^{-1}$ in the octopus rhodopsin spectrum (as a shoulder to the 1017 $cm^{-1}$ band, Figure 1). This band has been assigned to the C13−$CH_3$ rock mode (Callender et al., 1976; Curry et al., 1985; Eyring et al., 1982) and shows a 5 $cm^{-1}$ downshift upon $^{13}C$ labeling of C13 (Palings et al., 1987), similar to the shift of the 1010 $cm^{-1}$ band in the octopus rhodopsin 12,13-$^{13}C_2$ derivative (see below, Figure 2e). This rock mode normally appears at about 1018 $cm^{-1}$ in RPSB's, but shifts to 997 $cm^{-1}$ in bovine rhodopsin. This has been interpreted structurally in terms of an 11-*cis*, 12-*s-trans* configuration of the retinal chromophore and results from the methyl group on the C13 being in close contact with the hydrogen on C10 (i.e., 10H). The relatively higher frequency of this mode in the octopus rhodopsin spectrum suggests that the interaction between C13−$CH_3$ and 10H is weaker relative to bovine rhodopsin, but the conformation about the 12C−13C single bond is 12-*s-trans* as in bovine rhodopsin. A detailed analysis of the Raman spectrum of octopus rhodopsin will be published elsewhere. In this paper, we will concentrate our attention to isotopic effects and concomitant assignments on bands having to do with the C=N moiety in order to determine the C=N configuration of the RPSB chromophore in octopus rhodopsin and bathorhodopsin.

The Raman spectra of octopus (a) rhodopsin and its (b) ND, (c) 14,15-$^{13}C_2$, (d) 14,15-$^{13}C_2$ ND, and (e) 12, 13-$^{13}C_2$ derivatives are shown in Figure 2. Only two bands are observed in the C−C stretch region in the octopus rhodopsin spectrum between 1100 and 1300 $cm^{-1}$: a very strong band

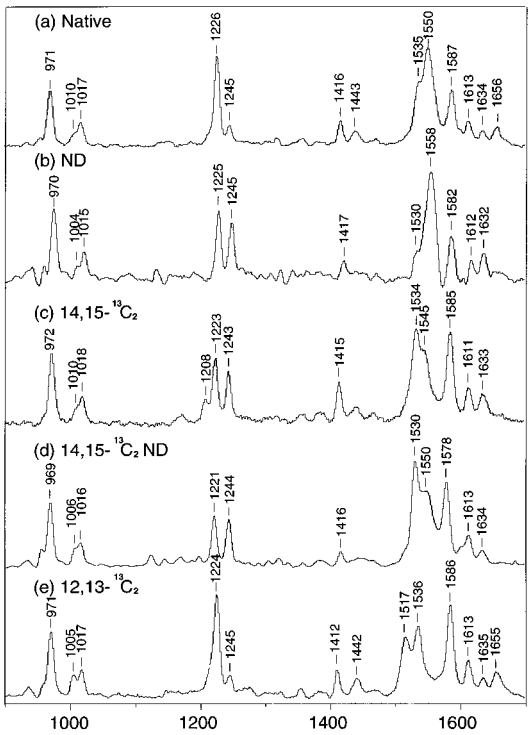



FIGURE 2: Raman spectra of octopus (a) rhodopsin and its (b) ND, (c) 14,15-$^{13}C_2$, (d) 14,15-$^{13}C_2$ ND, and (e) 12,13-$^{13}C_2$ derivatives.

at 1226 cm$^{-1}$ and a weaker band at 1245 cm$^{-1}$ (Figure 2a). This spectral pattern is unusual compared to the Raman spectrum of bovine rhodopsin, where four Raman bands are observed at 1190, 1217, 1240, and 1269 cm$^{-1}$ (Callender et al., 1976; Mathies et al., 1976), or with that of the 11-*cis*-retinal protonated Schiff base (RPSB), which also shows four bands in this region that are very similar in positions and intensities to the bovine rhodopsin spectrum (Palings et al., 1987). The stretch motions of the single bonds in highly conjugated systems, such as the various isomers of RPSB, are normally coupled, and the stretch motion of one single bond contributes to several vibrational modes. Such coupling has been demonstrated by the Raman studies of bovine rhodopsin by the use of $^{13}$C-labeled retinal derivatives. For example, two bands at 1190 and 1240 cm$^{-1}$ in the Raman spectrum of bovine rhodopsin shift down by 22 and 12 cm$^{-1}$, respectively, in the 14,15-$^{13}C_2$ derivative. The 1240 cm$^{-1}$ Raman band is also sensitive to the $^{13}$C labeling at the C13 position, downshifting by 5 cm$^{-1}$ in the 13-$^{13}$C derivative (Palings et al., 1987). Similar results have been observed in the Raman studies of the 11-*cis* RPSB (Palings et al., 1987), suggesting that for a 11-*cis* RPSB in solution, as for bovine rhodopsin, the C14−C15 stretch is coupled with the C12−C13 stretch.

In clear contrast, coupling between the C14−C15 and the C12−C13 stretches is absent in octopus rhodopsin. As shown in Figure 2a−e, large spectral changes are observed in the C−C fingerprint region of octopus rhodopsin Raman spectra when the C14−C15 bond is $^{13}$C labeled (Figure 2c) or the Schiff base nitrogen is deuterated (Figure 2b,d). However, no significant shift is observed for the 1226 or 1245 cm$^{-1}$ band in the Raman spectrum of the 12,13-$^{13}C_2$ derivative (Figure 2e).

The intensity at 1226 cm$^{-1}$ arises from more than one mode, one of which can be assigned to have significant C14−C15 stretch character based on the data in Figure 2. In the 14,15-$^{13}C_2$ derivative, the 1226 cm$^{-1}$ band intensity decreases with concomitant intensity increase of the 1245 cm$^{-1}$ band. A new band at 1208 cm$^{-1}$ also appears and so can be assigned to the $^{13}$C14−$^{13}$C15 stretch mode (Figure 2c). When the Schiff base nitrogen is deuterated, the intensity of the 1226 cm$^{-1}$ band also decreases with a simultaneous intensity increase of the 1245 cm$^{-1}$ band (Figure 2b). Deuterium labeling at the C14 position induces a spectral change similar to that of the deuterated Schiff base, and deuterium labeling at C15 shifts the entire 1226 cm$^{-1}$ band to 1237 cm$^{-1}$ (data not shown). Since isotopic labeling at any other position along the retinal chain induces only minor changes of the 1226 cm$^{-1}$ band (<3 cm$^{-1}$; data not shown), the molecular motions associated with this band are localized to the C14−C15═N−C moiety, with stretch motions of C14−C15 and perhaps also from the lysine N−C.

The sensitivity of the C14−C15 stretch mode to Schiff base nitrogen deuteration has been utilized in the determination of the Schiff base C═N configuration in a number of retinal Schiff base containing pigments (Palings et al., 1987; Smith et al., 1984). In the Raman studies of bacteriorhodopsin and its photointermediates, a large shift (∼50 cm$^{-1}$) of the relatively low-frequency C14−C15 stretch mode (∼1170 cm$^{-1}$) is observed in the C═N syn configuration upon Schiff base N deuteration, whereas a very small shift (<5 cm$^{-1}$) or not shift of the C14−C15 stretch mode at relatively high frequency (∼1200 cm$^{-1}$) is observed for the C═N anti configuration (Smith et al., 1984). In the Raman studies of bovine rhodopsin, bathorhodopsin, and isorhodopsin, no significant shifts of the respective C14−C15 stretch modes were observed upon Schiff base N deuteration. It was, therefore, concluded that their chromophores contain the C═N anti configuration (Palings et al., 1987).

The present Raman results show that the C14−C15 stretch at 1226 cm$^{-1}$ in octopus rhodopsin is significantly changed upon Schiff base N deuteration: either its intensity is reduced from the native spectrum (Figure 2b) or it shifts to underneath the 1245 cm$^{-1}$ band. Such changes are analogous to the large deuterium effect observed in the Raman studies of the dark-adapted bacteriorhodopsin, which contains a retinal chromophore with the C═N syn configuration (Smith et al., 1984). However, the C14−C15 stretch frequency of the octopus rhodopsin (1226 cm$^{-1}$) is much higher than that expected for a C═N syn RPSB (<1170 cm$^{-1}$). Moreover, the C14−C15 stretch mode in octopus rhodopsin is not coupled with C12−C13 stretch, unlike the analogous chromophores previously studied (which may be responsible for its comparatively high frequency). Both these observations suggest that the syn−anti rule based on the deuterium shift of the C14−C15 stretch mode developed for "normal" RPSB's may not be applicable to octopus rhodopsin.

We have recently carried out extensive normal mode calculations, based on vibrational measurements of a series of isotopically substituted analogs, of a truncated RPSB model (Deng et al., 1994). This compound contains all the elements of a RPSB from the C13 position toward and including the protonated Schiff base. Since the C14−C15 stretch mode in octopus rhodopsin is not coupled with C12−C13 stretch, it is more appropriate to compare its deuterium shift with that observed in the truncated RPBS model compound. Indeed, the C14−C15 stretch mode in the model compound, whether labeled by $^{13}$C or not, shows a significant upward shift (15−18 cm$^{-1}$) upon Schiff base N deuteration,

and its intensity is also greatly reduced (Deng et al., 1994), in excellent general agreement with the octopus rhodopsin data. In addition, *ab initio* calculations of a model Schiff base compound with the C=N anti configuration were found to agree very well with the Raman spectra of that compound (Deng et al., 1994). Finally, the calculations also predict that for the Schiff base model in the C=N syn configuration, the C14−C15 stretch mode has a much lower frequency (∼1150 $cm^{-1}$) and a much larger upward shift (>50 $cm^{-1}$) upon N deuteration (Deng et al., 1994). Thus, for a C14−C15 stretch mode uncoupled from the C12−C13 stretch (and other stretches of the polyene toward the ionone ring), like the one in octopus rhodopsin, its disappearance upon N deuteration is not an indication of a C=N syn configuration. Furthermore, we suggest that our data are more consistent with those of a C=N anti configuration since the C14−C15 stretch mode frequency is relatively high (>1200 $cm^{-1}$). Unfortunately, the interference of other Raman bands in the C−C stretch region excludes the possibility to assign the C14−C15 (or $^{13}$C14−$^{13}$C15) stretch mode of the deuterated octopus rhodopsin unambiguously (Figure 2b,d); thus, we cannot use the deuterium shift of the C14−C15 stretch mode to confirm this suggestion.

Model compound studies (Baasov et al., 1987) and semiempirical theoretical calculations (Deng & Callender, 1987; Gilson et al., 1988) suggest that the frequency of the Schiff base C=NH stretch mode and its shift upon N deuteration are sensitive to the strength of hydrogen bond between the protonated Schiff base and its counterion. The C=NH stretch frequency decreases and its shift upon nitrogen deuteration also decreases as the counterion's negative charge is moved away from the Schiff base nitrogen. The 24 $cm^{-1}$ deuterium shift of the Schiff base C=NH stretch mode in octopus rhodopsin (Figure 2a,b) is similar to that of the RPSB in methanol (Bagley et al., 1985) but smaller than that observed in bovine rhodopsin (∼32 $cm^{-1}$; Deng & Callender, 1987; Palings et al., 1987). Since the C=N configuration of the RPSB in both bovine and octopus rhodopsin is anti, it is reasonable to suggest that the hydrogen bonding strength on the protonated Schiff base in octopus rhodopsin is weaker than that in bovine rhodopsin.

*Bathorhodopsin.* Figure 3 shows the resonance Raman spectra of octopus (a) bathorhodopsin and its (b) ND, (c) 14,15-$^{13}C_2$, (d) 14,15-$^{13}C_2$ ND, and (e) 12,13-$^{13}C_2$ derivatives. The Raman spectra of octopus and bovine bathorhodopsins in the C−C stretch mode region look quite similar at first sight. In fact, we have previously suggested, based on this similarity, that the normal mode pattern of octopus bathorhodopsin is similar to that of bovine bathorhodopsin (Deng et al., 1991b). However, the present Raman studies on the octopus bathorhodopsin with $^{13}$C-labeled chromophore derivatives reveal differences between these two bathorhodopsins. The band at 1240 $cm^{-1}$ in bovine bathorhodopsin has relatively high intensity and is sensitive to the $^{13}$C labeling on both C14−C15 and C12−C13 bonds, suggesting that the C14−C15 and C12−C13 motions are coupled (Palings et al., 1987). The analogous 1243 $cm^{-1}$ band found in octopus rhodopsin has a much lower intensity and is not sensitive to the $^{13}$C labeling of the C14−C15 bond (Figure 3c) but shifts down by about 5 $cm^{-1}$ in the 12,13-$^{13}C_2$ derivative (Figure 3e). In addition, the bands at 1206 and 1222 $cm^{-1}$ show significant shifts in the 14,15-$^{13}C_2$ derivative (Figure 3c), but no shift is observed in the 12,13-$^{13}C_2$ derivative (Figure 3e).

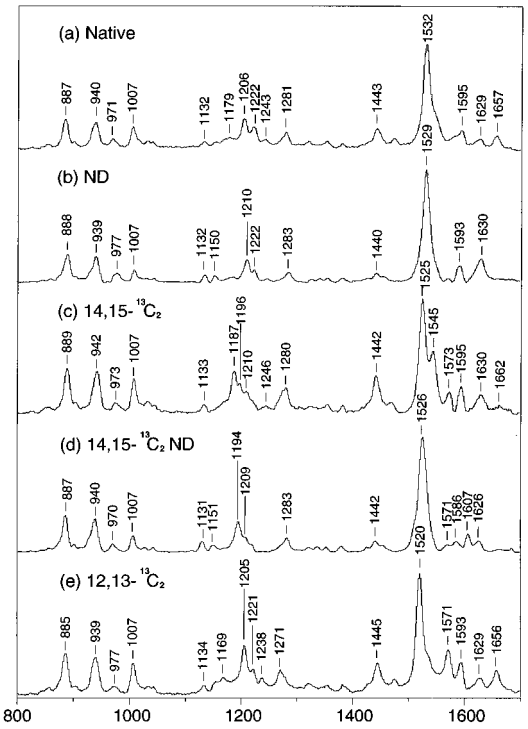



FIGURE 3: Raman spectra of octopus (a) bathorhodopsin and its (b) ND, (c) 14,15-$^{13}C_2$, (d) 14,15-$^{13}C_2$ ND, and (e) 12,13-$^{13}C_2$ derivatives.

This suggests that the coupling between C14−C15 and C12−C13 stretches observed in bovine bathorhodopsin is altered in octopus bathorhodopsin. Interestingly, the band at 1281 $cm^{-1}$ in octopus bathorhodopsin shifts down to 1271 $cm^{-1}$ in the 12,13-$^{13}C_2$ derivative (Figure 3e), suggesting that the C12−C13 stretch character in octopus bathorhodopsin shifts up in frequency compared with that of bovine bathorhodopsin (found near 1240 $cm^{-1}$; Palings et al., 1987), and becomes decoupled from C14−C15 stretch motion.

However, like that in bovine bathorhodopsin, the C14−C15 stretch motion is delocalized and contributes to several bands in this region, as evidenced by the spectral changes induced by $^{13}$C labeling at the C14 and C15 positions (Figure 3c). The band at 1206 $cm^{-1}$ seams to have two components. One shifts down to 1187 $cm^{-1}$ in the 14,15-$^{13}C_2$ derivative and disappears upon further deuteration of the Schiff base N, and the other shifts down to 1196 $cm^{-1}$ and is not affected by N deuteration (Figure 3c,d). In the bovine bathorhodopsin spectrum, the band near 1210 $cm^{-1}$ also consists of two components, but only one is sensitive to $^{13}$C labeling at the C14 and C15 positions and neither is sensitive to N deuteration (Palings et al., 1987). The band at 1222 $cm^{-1}$ in octopus bathorhodopsin also has contributions from the C14−C15 stretch and shifts down to 1210 $cm^{-1}$ in the 14,-15-$^{13}C_2$ derivative (Figure 3c) and is not sensitive to further N deuteration (Figure 3d). A similar unassigned band observed in the bovine bathorhodopsin spectrum at 1226 $cm^{-1}$ is not sensitive to $^{13}$C labeling at C14 and C15 but is sensitive to N deuteration (Palings et al., 1987). Therefore, the normal modes in the C−C fingerprint region of octopus and bovine bathorhodopsins are different despite the apparent similarities in the native spectra.

Detailed assignments of the bands in this region will be discussed elsewhere. Here we just assign the "C14−C15" stretch mode to the 1206 $cm^{-1}$ band in the native spectrum

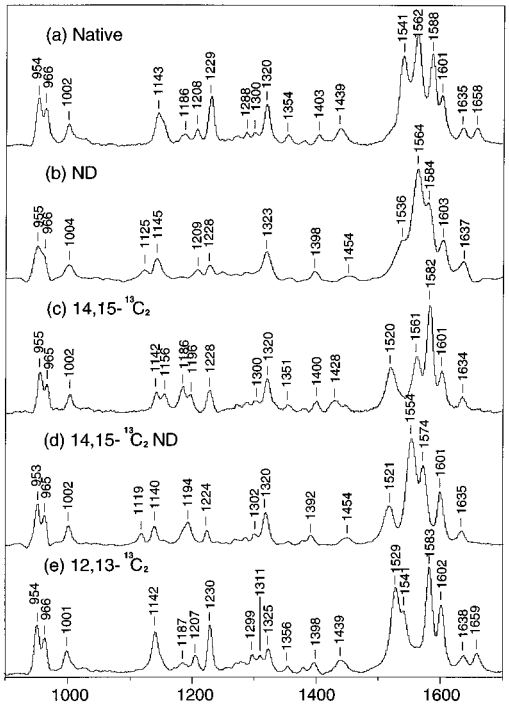



FIGURE 4: Raman spectra of octopus (a) isorhodopsin and its (b) ND, (c) 14,15-$^{13}C_2$, (d) 14,15-$^{13}C_2$ ND, and (e) 12,13-$^{13}C_2$ derivatives.

(Figure 3a), which disappears upon Schiff base N deuteration (Figure 3b). The "C14−C15" stretch mode is located at 1187 cm$^{-1}$ in the 14,15-$^{13}C_2$ derivative and also disappears upon Schiff base N deuteration (Figure 3d). Since the coupling between the C14−C15 and C12−C13 stretches is disrupted, as can be seen by a lack of shift of the 1206 cm$^{-1}$ band in the 12,13-$^{13}C_2$ spectrum (Figure 3e), and the "C14−C15" stretch character in octopus bathorhodopsin (1206 and 1222 cm$^{-1}$) is higher than that expected for a Schiff base with a C═N syn configuration, we suggest that the octopus bathorhodopsin chromophore is in a C═N anti configuration. Additional support for this suggestion can be found in the Raman spectrum of its 15D derivative, where the 15D rock mode of the RPSB with the C═N anti configuration is found at 989 cm$^{-1}$ (Deng et al., 1994), whereas in the C═N syn configuration, this mode should be observed at about 1040 cm$^{-1}$ (Deng et al., 1994; Smith et al., 1987). In the 15D spectrum of octopus bathorhodopsin, the 15D rock is found at 989 cm$^{-1}$ (Deng et al., 1991b).

The C═NH stretch frequency (1656 cm$^{-1}$, Figure 3a) and the deuterium shift (26 cm$^{-1}$, Figure 3b) of octopus bathorhodopsin are very similar to those of octopus rhodopsin (Figure 2a,b). Since the C═N configuration of the chromophore is anti in both species, it is unlikely that the Schiff base environment is changed significantly during the primary photochemical event in the octopus pigment.

*Isorhodopsin.* Figure 4 shows the resonance Raman spectra of octopus (a) isorhodopsin and its (b) ND, (c) 14,15-$^{13}C_2$, (d) 14,15-$^{13}C_2$ ND, and (e) 12,13-$^{13}C_2$ derivatives. The Raman spectrum of octopus isorhodopsin is relatively normal in that the signature band for a 9-*cis*-retinal near 1150 cm$^{-1}$ in the C−C stretch region of 9-*cis*-RPSB and bovine isorhodopsin (Curry et al., 1985; Eyring et al., 1982; Oseroff & Callender, 1974) is still present, consistent with the suggesting that octopus isorhodopsin contains a 9-*cis*-RPSB as has been found in regeneration studies (Koutalos et al., 1989). However, a comparison with bovine isorhodopsin also reaveals anomalies of the normal mode pattern in the C−C stretch region of the octopus isorhodopsin spectrum. The coupling between C14−C15 and C12−C13 stretches in the bovine isorhodopsin is evident from the isotopic shift of a strong band at 1242 cm$^{-1}$ upon $^{13}$C labeling of either C13 (−4 cm$^{-1}$) or C14,C15 (−4 cm$^{-1}$; Palings et al., 1987). The 1242 cm$^{-1}$ band was assigned to the C12−C13 stretch, and a band at 1206 cm$^{-1}$ was assigned to the C14−C15 stretch based on its ∼20 cm$^{-1}$ shift in the 14,15-$^{13}C_2$ derivative (Palings et al., 1987). In octopus isorhodopsin, the band near 1242 cm$^{-1}$ is missing, and a band at 1229 cm$^{-1}$ with similar intensity is observed (Figure 4a). This band intensity decreases by half in the 14,15-$^{13}C_2$ derivative, along with the disappearance of the 1208 cm$^{-1}$ band (Figure 4c). Concomitant appearance of a new band at 1196 cm$^{-1}$ and the intensity increase of the 1186 cm$^{-1}$ band suggest that the C14−C15 stretch shifts down from 1208−1229 cm$^{-1}$ in the native spectrum to the 1186−1196 cm$^{-1}$ region in the 14,15-$^{13}C_2$ derivative spectrum. On the other hand, the bands at 1208 and 1229 cm$^{-1}$ do not show any shift in the 12,13-$^{13}C_2$ derivative (Figure 4e), suggesting that there is no significant coupling between the C14−C15 and C12−C13 stretches. Moreover, the band intensity at 1320 cm$^{-1}$ in the octopus isorhodopsin spectrum (Figure 4a) is greatly reduced, and new bands near 1300 cm$^{-1}$ appear in the 12,13-$^{13}C_2$ derivative, showing that the C12−C13 stretch may be shifted from ∼1240 cm$^{-1}$ in a 9-*cis* RPSB or bovine isorhodopsin to 1320 cm$^{-1}$ in octopus isorhodopsin. Apparently, this substantial increase of the C12−C13 stretch character results in the decoupling of the C14−C15 stretch from the C12−C13 stretch. The high frequency of the C14−C15 stretch (1208−1229 cm$^{-1}$) indicates a C═N anti configuration in the octopus isorhodopsin. However, the spectral pattern in the fingerprint region shows very complex changes upon Schiff base N deuteration: besides the disappearance or intensity decrease of a band at 1186 cm$^{-1}$ in native or in the 14,15-$^{13}C_2$ derivative, respectively, the Schiff base N deuteration also causes the disappearance of a band at 1140−1156 cm$^{-1}$ and the appearance of a new band near 1120 cm$^{-1}$ (Figure 4b,d). Quite interestingly, this new band shows significant contribution from C14−C15 stretch motion, as suggested by its shift from 1125 cm$^{-1}$ in the native N deuterated spectrum (Figure 4b) to 1119 cm$^{-1}$ in the 14,15-$^{13}C_2$ ND derivative (Figure 4d). Such spectral changes induced by the Schiff base N deuteration do not match either those of a "normal" protonated retinal Schiff base or those of the truncated Schiff base model compound we have studied before. Thus, further experiments are necessary to determine the C═N configuration in octopus isorhodopsin.

## DISCUSSION

So far as is known, the chromophore of all visual pigments is the 11-*cis* isomer of retinal (or its 3,4-dehydro and 3-hydroxy analogues) bound to apoprotein via a protonated Schiff linkage. The essential feature of the primary photophysics is generally believed to be an 11-*cis* (rhodopsin) to 11-*trans* (bathorhodopsin) photoisomerization. In this process, which occurs on the picosecond and subpicosecond time scales, a large fraction of the photon's energy is converted to chemical energy. Within this context, it is important to verify the broad outline of the process in more than one pigment form or one species, and it is of interest to

understand how pigments might differ from one another. That there are differences is clear since, for just one example, the $\lambda_{max}$'s of pigments differ substantially, from the near-UV to the red. Resonance Raman studies, complemented by FT-IR difference spectroscopy, have provided detailed structural information of the chromophores of bovine rhodopsin and bathorhodopsin. Our studies here, and elsewhere, of octopus rhodopsin were prompted by the observation that the fraction of photon energy converted in the rhodopsin to bathorhodopsin photoconversion is large and essentially the same as that found in bovine rhodopsin based on calorimetry data. On the other hand, the $\lambda_{max}$'s of the two rhodopsins differ as do later thermal processes.

This study has concentrated on determining the structure of the chromophore in octopus rhodopsin and bathorhodopsin from the 11C═12C double bond to the protonated Schiff base linkage. This fragment undergoes large changes in geometry when rhodopsin converts to bathorhodopsin, and energy storage mechanisms must take into account the fragment's changes in conformation and geometry. As has been shown previously, the present results are in agreement with an 11-*cis* to 11-*trans* photoconversion. Our studies here suggest that the conformation of octopus rhodopsin is 11-*cis*, 12-*s*-*trans* and the Schiff base C═N bond is anti. The geometry of octopus bathorhodopsin, besides being 11-*trans*, is also anti about the Schiff base C═N bond. These structural attributes are just like those found in bovine rhodopsin and bathorhodopsin, respectively, and suggest a relatively simple reaction coordinate (i.e., simple rotation about the C11═C12 double bond). Furthermore, since the frequency of the Schiff base C═NH stretch mode and its shift upon N deuteration are similar in the octopus rhodopsin and bathorhodopsin resonance Raman spectra, it is unlikely that the Schiff base environment is changed significantly in the octopus during the photoreaction. This too is found in the corresponding bovine pigments, although the relatively larger shift in the C═NH stretch frequency in the bovine pigments ($\sim$32 cm$^{-1}$) compared to the octopus pigments ($\sim$25 cm$^{-1}$) indicates that the hydrogen bond between the protonated Schiff base and the apoprotein counterion is stronger in bovine than octopus.

On the other hand, the normal mode pattern is not the same in the bovine and octopus pigments, and this has structural implications. Vibrational studies of bovine rhodopsin reveal that its spectrum is quite similar compared with that of the 11-*cis* RPSB in solution, suggesting that there is no substantial conformational distortion of its chromophore upon binding. On the other hand, the Raman studies of octopus rhodopsin show that substantial perturbations around the retinal chain, especially near the C11═C12−C13 moiety, are present. Such perturbations apparently involve some twist around the C12−C13 bond so that the interaction between the C13−$CH_3$ group and 10H becomes weaker than that found in bovine rhodopsin. The perturbations also appear to result in the decoupling of the C12−C13 stretch and the C14−C15 stretch, and eliminate the 11H+12H rock mode observed in the solution spectrum of 11-*cis*-retinal and its Schiff base derivatives. The decoupling of the C12−C13 stretch motion from that of the C14−C15 stretch is also found in octopus bathorhodopsin. It is unclear if such anomalies in the normal modes of the octopus rhodopsin are mainly caused by the twist of the chromophore or by external effects from surrounding protein. Previous Raman studies of octopus bathorhodopsin suggest that the distortion of the chromophore is less than that in bovine bathorhodopsin (Deng et al., 1991a), and no external charge is in close contact. Further studies are required to determine the nature of the perturbations on the chromophore of the octopus rhodopsin and bathorhodopsin, and these are underway. By themselves, they do not seem sufficient to modify much the mechanism of how energy is converted in essential ways. However, these differences may help define how the retinal chromophore is interacting with the protein pocket.

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