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Raman Microscope Studies on the Primary Photochemistry of Vertebrate Visual Pigments with Absorption Maxima from 430 to 502 nm†

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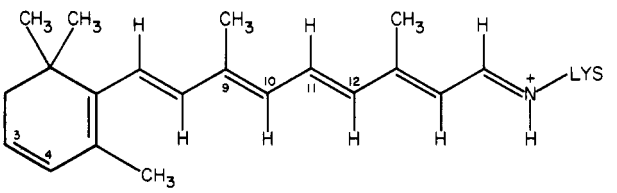
ABSTRACT: Raman microscope vibrational spectra have been recorded from single photoreceptor cells frozen at 77 K. Spectra of photostationary steady-state mixtures of visual pigments and their primary photoproducts were obtained from toad red rods (λ_{max} 502 nm), angelfish rods (λ_{max} 500 nm), gecko blue rods (λ_{max} 467 nm), and bullfrog green rods (λ_{max} 430 nm). All four photoproducts have enhanced low-wavenumber Raman lines at ~850, 875, and 915 cm^{-1} and show the anomalous decoupling of the 11- and 12-hydrogen out-of-plane (HOOP) wagging vibrations, as is observed in the bovine primary photoproduct. The low-wavenumber lines are enhanced in the resonance Raman spectrum by conformational distortion, and the uncoupling of the 11- and 12-hydrogen wags is caused by additional protein perturbations. The similarity of the HOOP modes in all four photoproducts indicates that the protein perturbations that uncouple the 11- and 12-hydrogen wags and that enhance the HOOP modes are very similar. Thus, these perturbations of the photoproduct Raman spectrum cannot be caused by the same protein–chromophore interactions that are responsible for wavelength regulation in these pigments.

Vision begins with the absorption of light by the visual pigments of the retina [for a review see Birge (1981)]. The chromophore of visual pigments is either 11-*cis*-retinal (A_1 retinal) or its 3,4-didehydro derivative (A_2 retinal) that is covalently bound as a protonated Schiff base to a lysine residue of the protein opsin (Oseroff & Callender, 1974):



Although the 11-cis A_1-protonated Schiff base absorbs at 440 nm in ethanol, A_1 visual pigments have absorption maxima that vary from about 430 to 562 nm (Dartnall & Lythgoe, 1965). This variation in pigment absorption spectrum is vital for the perception of color and for visual discrimination under a wide range of ambient light conditions (Jacobs, 1976; Levine & MacNichol, 1979). Modification of pigment absorption spectra must arise from alterations in protein sequence and differences in protein–chromophore interactions (Nathans et al., 1986).

Several types of mechanisms have been proposed to explain the molecular basis of these spectral changes, known as "opsin shifts". The protein could regulate the spectral sensitivity of the pigment by introducing ground-state twists (Blatz & Liebman, 1973), by affecting the polarizability or dielectric constant of the chromophore's environment (Blatz & Mohler, 1975; Irving et al., 1970), or by electrostatically perturbing the chromophore with charged amino acid residues (Kropf & Hubbard, 1958; Honig et al., 1976). Also, alterations in pigment spectral sensitivity could be brought about through regulation of the distance between the Schiff base nitrogen and its counterion (Blatz & Mohler, 1975; Blatz et al., 1972).

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Regeneration experiments using retinal analogues have suggested that the opsin shift in bovine rhodopsin can be explained by the presence of a negatively charged protein residue near C_{13} of the retinal chromophore (Arnaboldi et al., 1979). Calculations support the idea that electrostatic interactions can account for the substantial red shift that is observed when a protonated Schiff base is bound to bovine opsin (Honig et al., 1979). Changes in the position of this electrostatic charge in the chromophore binding pocket might then be the origin of spectral variation in pigments (Kakitani et al., 1985; Dartnall & Lythgoe, 1965).

Resonance Raman spectra have shown that the 11-*trans*-retinal chromophore in the primary photoproduct of bovine rhodopsin is also strongly perturbed by the protein (Oseroff & Callender, 1974; Eyring et al., 1980, 1982). Bathorhodopsin exhibits intense vibrations at 854, 875, and 922 cm^{-1} that have been assigned to the 12- + 14-, 10-, and 11-hydrogen out-of-plane (HOOP)[1] normal modes, respectively. These vibrations are believed to be enhanced by conformational distortion of the chromophore. Furthermore, the frequencies of these HOOP modes are anomalous. In model compounds and in bovine rhodopsin, the 11- and 12-hydrogen out-of-plane wag internal coordinates couple to form in-phase and out-of-phase normal mode combinations, while in the photoproduct these wags are uncoupled. To account for the unusual frequencies of the HOOP modes that result from this lack of coupling, it was suggested that a negatively charged protein residue might perturb the chromophore near $C_{11}=C_{12}$ (Eyring et al., 1982).

These Raman studies were performed to evaluate the idea that a charged protein residue, responsible for adjustment of spectral sensitivity in visual pigments, might also be responsible for perturbing the hydrogen out-of-plane frequencies in the primary photoproducts. Raman spectra were obtained of A_1 pigments absorbing from 430 to 502 nm as well as of their photoproducts. Pigments that absorb near 440 nm have a small opsin shift, so the specific protein–chromophore interaction that red shifts the absorption spectrum should be absent. If this same interaction is responsible for perturbing the Raman spectrum of the photoproduct, then we should observe large changes in the frequencies and intensities of the HOOP modes in photoproducts of "blue-absorbing" pigments compared to photoproducts of 500-nm pigments.

The technique of resonance Raman microscopy was developed for these studies, since it allows us to obtain spectra of pigments inside intact, *individual* photoreceptors (Barry & Mathies, 1982). The optics of a microscope are used to focus a laser on the outer segment of a single photoreceptor, and spectra of the pigment inside the outer segment can be obtained. To trap the photoproduct, these experiments must be performed on photoreceptors frozen at 77 K in a thin film of buffer. At this temperature, a steady-state mixture of the parent pigment and its primary photoproduct is formed (Yoshizawa & Wald, 1963). The composition of the steady state depends on the irradiating wavelength, so the vibrational lines in the spectrum that are due to the photoproduct can be identified by alteration of the irradiation conditions (Oseroff & Callender, 1974).

We report here spectra of A_1 pigments with absorption maxima at 502 nm (toad red rod), 500 nm (angelfish rod), 467 nm (gecko blue rod), and 430 nm (frog green rod). The hydrogen out-of-plane lines in the Raman spectra of all the primary photoproducts studied are strongly enhanced, and the frequencies of these vibrations are similar in all the pigments. We conclude that the protein perturbation responsible for the unique vibrational properties of the photoproduct is not directly involved in wavelength regulation.

[1] Abbreviations: HOOP, hydrogen out of plane; BSA, bovine serum albumin.

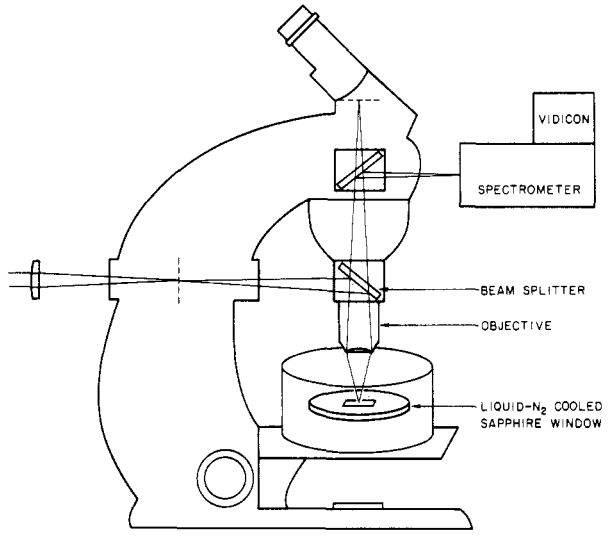

FIGURE 1: Resonance Raman microscope and liquid nitrogen cold stage as described by Barry and Mathies (1982).

Materials and Methods

Raman Microscopy. The resonance Raman microscope and liquid nitrogen cold stage is depicted in Figure 1. The laser beam is focused on the outer segment of the cell by a 40× Zeiss LD-Epiplan microscope objective. The Raman scattered light is collected by the objective and dispersed onto a multichannel detector. In these studies, the Raman probe power was typically 4–9 mW, and the coaxial pump power ranged from 7 to 14 mW. The spectral reso' 'on was 6–7 cm^{-1}, and the frequencies are accurate to ±2–3 cm^{-1}.

To isolate photoreceptors for the Raman experiments, animals were dark-adapted for 2–12 h, and the spinal column was pithed under dim red illumination. Dissection of the eye was performed in total darkness with an infrared intensifier and a dissecting microscope. Once the eye was hemisected, the retina was detached from the eyecup with a stream of Ringer's (100 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM NaH_2PO_4, pH 7.4). A small piece of the retina was placed in a drop of Ringer's on the microscope cold stage. Photoreceptors were dislodged from the retina either by brushing the tissue with a camel's hair brush or by teasing the tissue with forceps. After the piece of tissue was removed, the sample was covered with a cover slip and cooled to liquid nitrogen temperature.

In many cases the absorption maximum of a photoreceptor can be determined from cellular morphology that has been correlated with pigment content (Stell & Hārosi, 1976). However, the Raman spectrum also provides information about the absorption maximum of the pigment. The ethylenic frequency is linearly correlated with absorption maximum (Heyde et al., 1971; Aton et al., 1977). Figure 2 presents a plot of ethylenic frequency vs. absorption maximum for A_1 pigments and model compounds. Care has been taken to plot only those points for which the λ_{max} and $\nu_{C=C}$ were measured at the same temperature. Once we have identified a photoreceptor and obtained its Raman spectrum, we have used the ethylenic frequency to confirm our assignment of the photoreceptor's spectral sensitivity. Figure 2 also presents our assignment of the absorption maximum and ethylenic frequency for the new

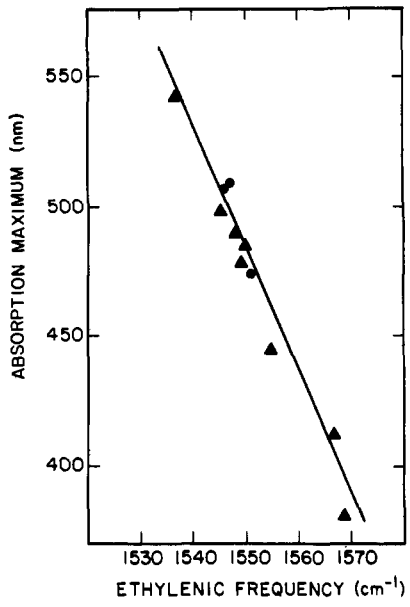

FIGURE 2: Plot of ethylenic frequency vs. absorption maximum for A_1 pigments and model compounds. The solid triangles are for bovine bathorhodopsin at 77 K, bovine rhodopsin at 20 °C, bovine isorhodopsin at 77 K, bovine isorhodopsin at 20 °C, bovine metarhodopsin I, all-trans A_1 retinal protonated Schiff base, bacteriorhodopsin's M_{412} intermediate, and bovine metarhodopsin II in order of decreasing λ_{max} (Oseroff & Callender, 1974; Yoshizawa, 1972; Eyring & Mathies, 1979; Mathies et al., 1977; Doukas et al., 1978; Braiman & Mathies, 1980). The solid circles are the Raman ethylenic frequencies of the toad red rod, angelfish rod, and gecko rod (1547, 1546, and 1551 cm^{-1}, respectively) plotted vs. our assignments of their low-temperature absorption maxima (509, 507, and 474 nm).

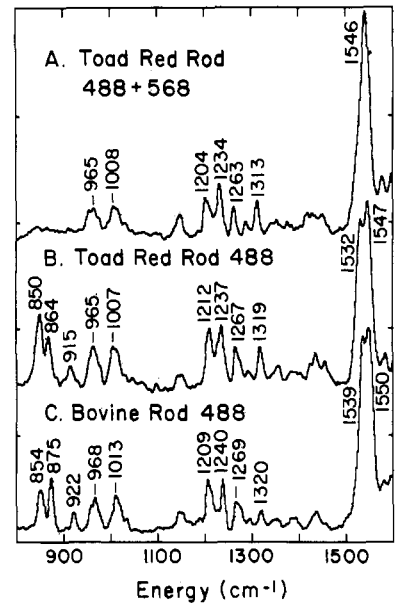

FIGURE 3: Raman microscope spectra of the 502-nm red rod from the toad. Spectrum A was recorded at 488 nm with a coaxial 568-nm pump. Spectrum B was taken with just the 488-nm probe. Spectrum C is the 488-nm spectrum of the 498-nm bovine pigment taken from Eyring et al. (1980).

pigments studied here. Because the pigment absorption maximum is known to red shift by an average of ~7 nm at 77 K (Yoshizawa, 1972), 7 nm has been added to the room temperature absorption maximum in plotting the later points.

Raman Spectroscopy on Extracted Pigments. The bullfrog *Rana catesbeiana* has a red pigment absorbing maximally at 502 nm and a green pigment absorbing at 430 nm (Makino et al., 1980). The green rod pigment can be separated from the more abundant red pigment on concanavalin A–Sepharose affinity columns. Our procedure followed the protocol described by Makino-Tasaka and Suzuki (1984) with the following modifications. The column buffers were 1% octyl glucoside, and the column was pretreated with a wash of 1 mg/mL BSA in order to prevent nonspecific binding. The pigment was eluted from the column with a linear gradient of 0–100 mM methyl D-mannoside at a flow rate of ~0.1 mL/min. Although we did not achieve complete separation of the green and red pigments under these conditions, the fractions at the beginning of the pigment elution had a lower average ratio of 502-nm/430-nm absorbance (2.0) than later fractions (2.8). The early fractions were concentrated on Amicon C-25 filters immediately before the Raman experiment. A control sample containing only red pigment was obtained by collecting all the later pigment fractions from the column and treating the pooled sample with 60 mM NH_2OH. This concentration of NH_2OH is known to selectively bleach the green pigment (Makino-Tasaka & Suzuki, 1984). The red pigment was then concentrated on Amicon filters. Raman measurements on the extracted pigments were performed by freezing the pigment solution on a stationary cold tip at 77 K as described in Eyring et al. (1980). The 413-nm probe was 50 mW in the probe-only experiments and 35 mW in the pump-plus-probe experiments. The 514.5-nm pump power was 35 mW. The scattered light was detected with a photon-counting double monochromator at a resolution of 7 cm^{-1}.

RESULTS

Because these data are obtained from a photostationary steady-state mixture of pigments at 77 K, the spectra include scattering from the parent pigment, its bathochromically shifted primary photoproduct, and a 9-cis pigment (Yoshizawa & Wald, 1963; Oseroff & Callender, 1974). Their relative concentrations are dictated by their extinction coefficients and photochemical quantum yields according to the kinetic scheme:

$$\text{11-}cis\text{-rhodopsin} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \textit{all-trans}\text{-bathorhodopsin} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{9-}cis\text{-isorhodopsin}$$

For example, with 488-nm excitation, the spectrum of bovine rhodopsin (Figure 3C) is known to contain significant contributions from all three components. The lines that are due to the red-absorbing primary photoproduct, bathorhodopsin (λ_{max} 543 nm), can be assigned by adding a red "pump beam", which is preferentially absorbed by the bathochromically shifted photoproduct. This causes a significant reduction in the concentration of bathorhodopsin. The parallel decrease in the intensity of the lines at 1539, 854, 875, and 922 cm^{-1} allows them to be assigned to bovine bathorhodopsin (Oseroff & Callender, 1974). This concept will be used to identify the vibrational lines due to the primary photoproduct in our studies as well.

The toad *Bufo marinus* is known to have two A_1 rod pigments (Hârosi, 1975). The red pigment absorbs at 502 nm and makes up most of the pigment content of the retina. The other photoreceptors contain a green pigment that absorbs at 433 nm. With 488-nm excitation, most of the rod cells in the *Bufo* preparations gave the steady-state Raman spectrum shown in Figure 3B. We conclude that this spectrum is due to the 502-nm pigment since it is very similar to that of the 500-nm bovine rhodopsin steady state shown in Figure 3C. The 1547-cm^{-1} component of the ethylenic line in Figure 3B is due mainly to the parent red rod pigment (see below), and this ethylenic frequency correlates well with an absorption maximum of 502 nm (509 nm at 77 K; Figure 2). Lines in

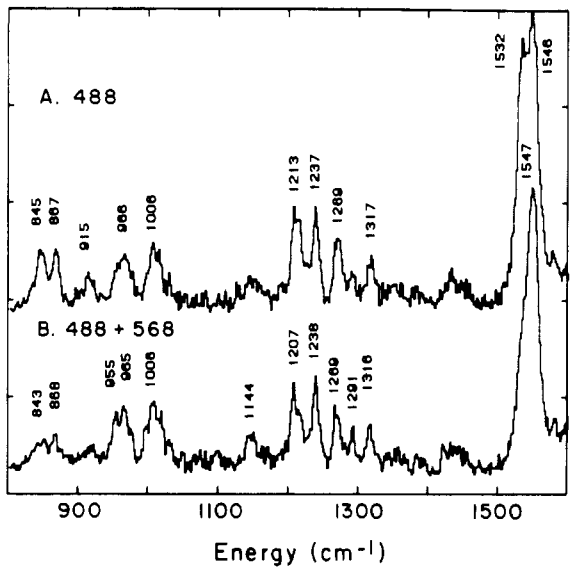

FIGURE 4: Raman microscope spectra of 500-nm angelfish rods. Spectrum A was taken with a 488-nm probe. Spectrum B was recorded at 488 nm with the addition of a coaxial 568-nm pump.

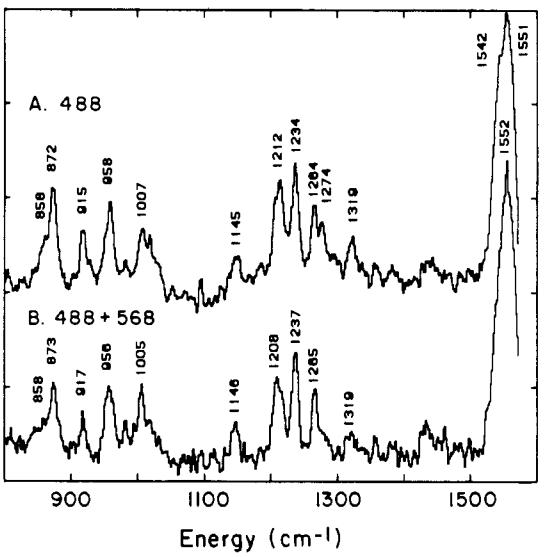

FIGURE 5: Raman microscope spectra of one outer segment from the gecko double rod. The absorption maximum of this A_1 pigment is believed to be 467 nm (see text). Spectrum A was taken with a 488-nm probe. Spectrum B was recorded at 488 nm with the addition of a coaxial 568-nm pump.

the 1100–1350-cm^{-1} fingerprint region are known to be characteristic of the geometry of the chromophore (Mathies et al., 1977). Thus, the fact that the 502-nm toad rod and the 500-nm bovine pigments have very similar fingerprint regions implies that their steady-state compositions are very similar and that the intermediates in the two steady-state mixtures have the same chromophore structure. The lines in the toad red rod spectrum at 850, 864, 915, and 1532 cm^{-1} that decrease in intensity when the 568-nm pump is added can be assigned to the red-shifted primary photoproduct. The analogous 854-, 875-, and 922-cm^{-1} bovine lines have been assigned to the 12- + 14-, 10-, and 11-hydrogen out-of-plane wags (Eyring et al., 1982). On the basis of the close frequency correspondence, we assign the toad rod lines at 850, 864, and 915 cm^{-1} to the 12 + 14, 10, and 11 HOOP modes, respectively.

From extraction measurements, angelfish (genus *Pterophyllum*) retinae have been reported to contain a mixture of an A_1 pigment absorbing at 500 nm and an A_2 pigment absorbing at 522 nm (Schwanzara, 1967). The Raman spectrum of the angelfish rods allows us to decide whether cells from the fish that we studied contain an A_1 or an A_2 pigment. We have recently shown that there are characteristic differences between A_1 and A_2 pigment quantum yields (Barry, 1984). These photochemical differences cause the A_2 steady state to exhibit Raman scattering mainly from the 9-cis pigment at all probe wavelengths. Also, with blue probe wavelengths the A_2 steady state shows very little scattering from the photoproduct. Figure 4 demonstrates that the angelfish rod pigments, on the other hand, have a photoproduct that makes a large contribution to the blue probe Raman spectrum. Lines at 1532, 915, 867, and 845 cm^{-1} decrease in intensity when a 568-nm pump beam is added to the steady state. We obtained the same Raman spectrum for all the angelfish rods we examined, and these spectra allow us to conclude that these cells do not contain an A_2 pigment. We can also rule out the possibility that the content of a single cell is a mixture of A_1 and A_2 pigments, because our spectra of the angelfish rods are very similar to the spectra of the A_1 toad and bovine rod pigments.

The fierce nocturnal Tokai gecko has an all-rod retina (Crescitelli, 1977). Most of the rods contain a 521-nm A_1 pigment. This pigment has an absorption spectrum that blue shifts in situ in response to chloride depletion and is very sensitive to chemical bleaching (Crescitelli, 1963, 1977, 1978). A smaller percentage of the gecko photoreceptors contain a 467-nm A_1 pigment that is more stable than the 521-nm pigment (Crescitelli, 1977). The rod cells containing the two types of pigments are not morphologically distinguishable. In our experiments on gecko rods, the concentration of chloride should ensure that the 521-nm pigment does not blue-shift at room temperature (Crescitelli, 1978). However, after being cooled to 77 K, most of the rods in the field gave no Raman signal. We believe that these rods contained the labile 521-nm pigment. We did find a few rods in some microscope fields that gave the Raman signal shown in Figure 5. The 1551-cm^{-1} ethylenic frequency correlates with a pigment λ_{max} of 467 nm (see Figure 2). Addition of a 568-nm pump caused a decrease in the intensity of the 858-, 872-, and 915-cm^{-1} lines as well as the disappearance of the 1542-cm^{-1} ethylenic shoulder. These lines are therefore assigned to the red-shifted primary photoproduct.

The fluorescence of the toad green rods under blue excitation prevented us from obtaining Raman microscope spectra of the primary photoproduct of the 433-nm toad pigment. As an alternative, the 430-nm pigment of the bullfrog *R. catesbeiana* was isolated by affinity chromatography (Makino-Tasaka & Suzuki, 1984). A mixture of green and red pigments that was enriched in green pigment was obtained, and a 413-nm probe was used to resonantly enhance the scattering from the green pigment. In Figure 6A we see lines at 858, 878, and 917 cm^{-1} in the low-wavenumber region, and these lines lose intensity when the 514.5-nm pump is added. We therefore assign these lines to the red-shifted photoproduct of the green pigment. Notice that the photoproduct of the 430-nm pigment has a line at 917 cm^{-1} and that the relative intensities and frequencies of all the low-wavenumber lines are similar to those seen in the other A_1 pigment spectra.

Since this experiment was performed on a mixture of pigments, we need to control for the possibility that the low-wavenumber lines observed with 413-nm excitation might be due to red rod pigment contamination. Therefore, a spectrum of the "pure" red pigment was obtained with a 413-nm probe. With 488-nm excitation, the frog red rod pigment and its photoproduct have vibrational spectra that are very similar

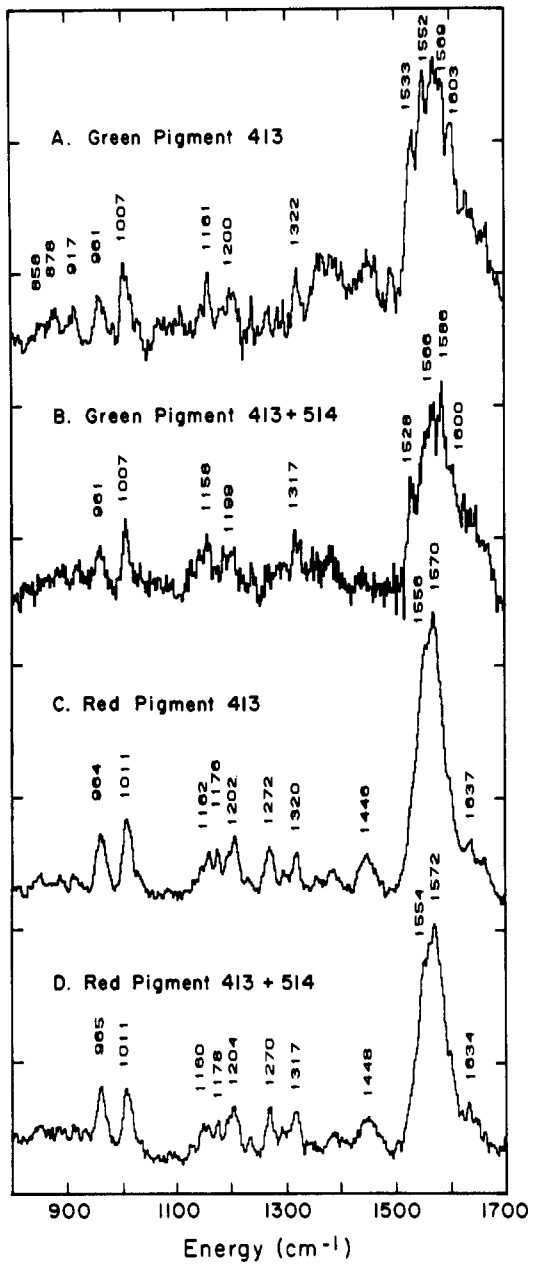

FIGURE 6: Resonance Raman spectra of solubilized bullfrog pigments enriched in the 430-nm green rod pigment (A and B). Spectra C and D present Raman data on a solubilized 502-nm red rod preparation from the bullfrog. Spectra A and C were recorded at 413 nm. Spectra B and D were recorded at 413 nm with the addition of a coaxial 514.5-nm pump.

to the toad and bovine pigments. However, with 413-nm excitation we are far from resonance with the photoproduct of this pigment, and the spectra we obtain (Figure 6C,D) show very little intensity in the low-wavenumber region. Therefore, the red pigment probably makes little contribution to the low-wavenumber region in Figure 6A.

The bullfrog has been reported to contain A_2 as well as A_1 pigments in its retina (Reuter et al., 1971; Makino et al., 1983; Makino-Tasaka & Suzuki, 1984). However, we have seen no evidence of pigment heterogeneity in our Raman studies. A blue probe spectrum of the pure red rod pigment from this organism (not shown) very closely resembled the low-temperature, steady-state spectra of the toad and bovine pigments (Barry & Mathies, 1982; Oseroff & Callender, 1974; Eyring et al., 1980; Eyring & Mathies, 1979) that are known to contain only A_1 retinal (Lythgoe, 1972). Therefore, our preparations must have little contamination from A_2 pigments.

Discussion

We report here a comparative Raman study on photoproducts of visual pigments with absorption maxima that range from 502 to 430 nm. The similarity of the probe-only spectra of pigments from toad red rods, angelfish rods, gecko rods, bullfrog green rods, and bovine rods indicates that the compositions of the steady states and the structures of the components are similar in these pigments. In particular, the photoproducts of all these pigments exhibit a familiar trio of lines from 850 to 920 cm^{-1}, and each has a strong line at ~915 cm^{-1}. By analogy with the vibrational assignments for the bovine primary photoproduct, we assign the line at ~915 cm^{-1} to an isolated C_{11} HOOP. Similarly, the ~850- and 875-cm^{-1} lines are assigned to the $C_{12}H + C_{14}H$ and to the $C_{10}H$ wags, respectively. The enhancement of the HOOP modes in these spectra indicates that the chromophores in these photoproducts are conformationally distorted (Eyring et al., 1980; Warshel & Barboy, 1982). The observation of an isolated C_{11} HOOP mode at ~915 cm^{-1} in these spectra indicates that the $C_{11}H$ and $C_{12}H$ wags are uncoupled by a similar protein perturbation in all of the photoproducts.

The mechanism of the perturbation that uncouples the C_{11} and C_{12} hydrogen wags is still obscure. It has been proposed that the C_{11} and C_{12} hydrogens are uncoupled because of a specific electrostatic interaction between the $HC_{11}{=}C_{12}H$ moiety and a negatively charged protein residue that is also responsible for λ_{max} regulation (Eyring et al., 1982). If a strong electrostatic perturbation near C_{12} does uncouple the C_{11} and C_{12} hydrogens of the photoproduct, then we have demonstrated that this charge is present in pigments having a 70-nm range of spectral sensitivity. Hence, altered positioning of this charge in the binding site cannot be responsible for λ_{max} regulation. It is also possible that the C_{11} and C_{12} HOOP modes are isolated because of large geometric distortions imposed on the carbon backbone of the chromophore by the protein. If the chromophore is severely twisted or bent around the $C_{11}{=}C_{12}$ bond following isomerization in the chromophore binding pocket, it is possible that this could isolate the $C_{11}H$ frequency. However, calculations on twisted retinals have thus far been unable to reproduce this uncoupling (Eyring et al., 1980; Warshel & Barboy, 1982). If some steric perturbation is responsible for the uncoupling of the 11 and 12 HOOP modes, then our data show that this steric effect is present in all photoproducts we have studied and is also not involved in λ_{max} regulation.

While all four photoproducts exhibit qualitatively similar spectra, the details of their distortion differ from pigment to pigment, since we observe small differences in the intensities and frequencies of the low-wavenumber lines. For example, the 858-cm^{-1} line has much less intensity in the spectrum of the gecko photoproduct (Figure 5) than in the bovine or toad photoproducts (Figure 3). This loss of intensity could be due to alterations in the excited-state or ground-state geometry of the chromophore near $C_{12}{\cdots}C_{14}$. This alteration must be caused by a change in protein structure that may play a role in determining the absorption maximum of the gecko pigment. DNA hybridization experiments have revealed that the gecko visual pigments lack strong homology to the bovine rod pigment (Martin et al., 1986).

Finally, the fact that all the primary photoproducts we have studied are strongly perturbed in a very similar fashion may be of importance for energy storage in the primary event. Bovine bathorhodopsin is known to store a large fraction (~35 kcal/mol) of the incident photon energy (Cooper, 1979). It

has been suggested that the perturbation of the HOOP modes in bovine bathorhodopsin is related to this energy storage mechanism (Eyring et al., 1982; Palings et al., 1986). If this is true, then our observations indicate that a similar large storage of energy occurs in the primary event of pigments having a wide range of spectral sensitivity.

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