Resonance Raman Microscopy of Rod and Cone Photoreceptors

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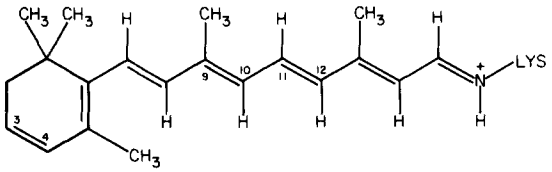
ABSTRACT We have constructed a Raman microscope that has enabled us to obtain resonance Raman vibrational spectra from single photoreceptor cells. The laser beam which excites the Raman scattering is focused on the outer segment of the photoreceptor through the epiillumination system of a light microscope. Raman scattering from the visual pigment in the photoreceptor is collected by the objective and then dispersed onto a multichannel detector. High-quality spectra are recorded easily from individual outer segments that are 5 × 50 μm in size, and we have obtained spectra from cells as small as 1 × 10 μm. We have used the Raman microscope to study photostationary steady-state mixtures in pigments from toad (*Bufo marinus*) and goldfish (*Carassius auratus*) photoreceptors; these photoreceptors were frozen in glycerol glasses at 77°K. Comparison of our toad red rod spectra with previously published spectra of bovine rod pigments demonstrates that the conformation of the chromophore in the first photointermediate, bathorhodopsin, is sensitive to variations in protein structure. We have also studied the first photointermediate in the goldfish rod photostationary steady-state. This bathoporphyropsin has a much lower ethylenic stretching frequency (1,507 cm^{-1}) than that observed in the toad and bovine bathoproducts (~1,535 cm^{-1}). Preliminary results of our work on goldfish cone pigments are also reported. These are the first vibrational studies on the vertebrate photoreceptors responsible for color vision.

The first molecular event in vision is the absorption of a photon by the visual pigment in the photoreceptors of the retina. All known visual pigments contain an 11-*cis* retinal (A_1) or a 3,4 dehydro-11-*cis*-retinal (A_2) chromophore which is covalently bound to the apoprotein, opsin, through a protonated Schiff



base linkage (1). Photon absorption produces a primary photoproduct, bathorhodopsin, that contains a distorted *trans* retinal chromophore. At liquid nitrogen temperature bathorhodopsin can be trapped in a photostationary steady-state mixture with rhodopsin and isorhodopsin (2).

$$\underset{11\text{-}cis}{\text{Rhodopsin}} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \underset{trans}{\text{Bathorhodopsin}} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \underset{9\text{-}cis}{\text{Isorhodopsin}}$$

Interactions between opsin and the chromophore have a profound effect on the absorption spectrum of the pigment. For example, an 11-*cis* retinal protonated Schiff base in solution has an absorption maximum near 440 nm, while the 11-*cis* protonated Schiff base in bovine opsin absorbs maximally at 500 nm. Further, although A_1 pigments from different vertebrate species all contain the same 11-*cis* chromophore, their absorption maxima range from 440 to 575 nm (3). This species-to-species variation in the absorption spectra of visual pigments may be explained by a variation in the placement of charged amino acid residues in the chromophore binding site (4).

Protein-chromophore interactions also appear to play an essential role in photoisomerization. The unusual Raman spectrum of bovine bathorhodopsin is a result of steric and electrostatic perturbations of the *trans* bathorhodopsin chromophore by the protein (5). Such interactions may help to explain how the protein causes an ~10-fold increase in the quantum yield for 11-*cis* → *trans* isomerization (6).

To understand how the protein modulates the absorption and photoisomerization of visual pigments, we would like to study a variety of A_1 (or A_2) pigments that have different absorption spectra. Differences in the structure of the chromophore from pigment to pigment would then reflect variations in the chromophore's protein environment. Resonance Raman vibrational studies can provide such comparative structural information. In resonance Raman experiments laser excitation within the absorption band of the pigment results in resonance enhancement of only the retinal chromophore vibrations (7). The configuration and conformation of the chromo-

For a review of visual photochemistry, see Birge, R. R. (1), and references therein.

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 AUGUST 1982 479-482

phore determine the frequencies and intensities of these vibrational lines (8).

To circumvent the difficult biochemical purification of pigments which would otherwise be required in these studies, we have developed an *in situ* vibrational technique, Raman microscopy. Laser excitation of the sample is performed with an optical microscope. By using multichannel detection we are able to perform resonance Raman experiments on single photoreceptors as small as 1 × 10 μm. The development of a 77°K cold stage allows us to trap and study parent pigments and their primary photoproducts in a steady-state mixture. Although Raman microscopes and microprobes have been used previously to obtain vibrational spectra of small samples (9–11), they have not been used to study chemical processes inside intact cells. We report here the results of our microscope studies on the 502-nm toad rod pigment and on the 522-nm goldfish rod pigment. We also report studies on the pigments in the two outer segments of the goldfish double cone.

MATERIALS AND METHODS

Bufo marinus are obtained from Lemberger Associates, Germantown, WI. Comet goldfish (3–4 inches long) are obtained locally. Animals are dark-adapted ~12 h before they are sacrificed. The eye is enucleated and hemisected, and then the retina is detached from the pigment epithelium with a stream of Ringer's solution. The intact retina is transferred to a petri dish containing a layer of Dow-Corning 184 Sylgard polymer. The retina is oriented with its vitreal side adhering to the Sylgard and is diced with a razor blade. The pieces of retina are dislodged from the Sylgard with a gentle stream of Ringer's and transferred to a microcentrifuge tube. Shaking of the centrifuge tube dislodges the photoreceptors, which are then pelleted by low-speed centrifugation. The supernatant is removed, and glycerol is added to the pellet to give an ~80% vol/vol solution. All operations are performed under dim red illumination.

A schematic of the Raman microscope is given in Fig. 1. The rationale and optics for these low-temperature, double-beam experiments on visual pigments have been thoroughly described in references 12 and 13. Also, the design and operation of the vidicon spectrometer has been given by Mathies and Yu (14). The size of the focused laser beam (5–15 μm) and its alignment with respect to the entrance slit and aperture are determined by viewing the fluorescence from a slide coated with rhodamine-6G. The diameter of this focused beam is adjusted by changing the position of the 190-mm lens. Laser excitation is provided by Spectra-Physics 165 argon-ion and 171-01 krypton-ion lasers.

All spectra are obtained from photoreceptors frozen at 77°K on the cold stage described in Fig. 2. A photomicrograph of toad rod receptors frozen on the cold stage is presented in Fig. 3. Typical spectra are averages of several 135-s integrations; each integration is performed on a separate, single photoreceptor. Unless otherwise stated, the spectral slit width is 7–9 cm^{-1}. Background signal due to the detector and to Raman scattering from glycerol is obtained by performing a separate integration with the beam spot displaced from the photoreceptor. These background scans are then subtracted from the data scans. A PDP 11/23 is used to remove fluorescence backgrounds and to digitally smooth the data.

RESULTS AND DISCUSSION

Fig. 4*b* presents a Raman microscope spectrum obtained from a red rod photoreceptor (λ_{max} = 502 nm) of the toad using 488-nm excitation. The striking similarity of this spectrum to the one obtained under identical conditions from extracted bovine rod pigments (λ_{max} = 498 nm) in Fig. 4*c* indicates that the compositions of these two steady-states are very similar. When a coaxial 568-nm pump beam is introduced in the toad rod experiment, we obtained the Raman spectrum in Fig. 4*a*. This pump beam should decrease the concentration of the red-absorbing intermediate, bathorhodopsin, in the steady-state mixture. The disappearance of the 850, 864, 915, and 1,532 cm^{-1} lines in Fig. 4*a* allows us to assign these bands to toad bathorhodopsin. The analogous lines in the bovine spectrum

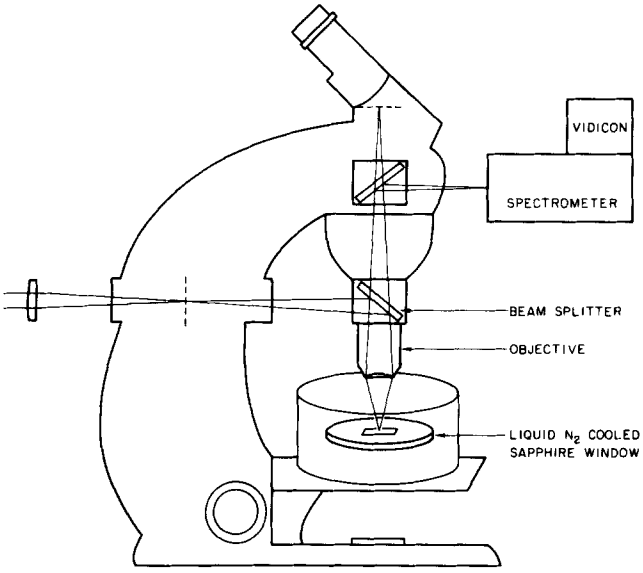


FIGURE 1 Raman microscope and liquid nitrogen cold stage. A Zeiss Universal microscope with a II-C vertical illuminator is coupled to a Spex 1400 3/4-meter double monochromator. The detector is a dry ice-cooled intensified vidicon and multichannel analyzer (PAR 1205A/1205D). This spectrograph can simultaneously observe up to 800 cm^{-1} of the Raman spectrum. A 190-mm lens expands the laser beam to fill the aperture of the objective. Long-working length Zeiss LD-epiplan objectives (× 16, NA 0.3 or × 40, NA 0.6) focus the laser beam on the photoreceptors *and* collect Raman scattering. The second surface reflection from the beamsplitter and scattering from the surrounding medium are rejected by an image plane aperture at the spectrometer entrance slit.

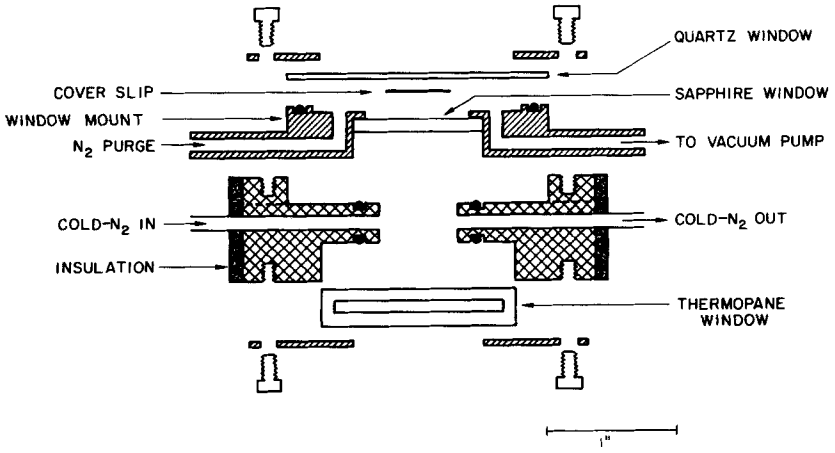


FIGURE 2 Cross-sectional view of the liquid nitrogen cold stage. The photoreceptor preparation is placed on the sapphire window and covered with a cover slip. The sapphire window is cooled by flowing cold nitrogen gas or liquid nitrogen through the central cavity in the linen-based phenolic block. An evacuated thermopane window provides thermal insulation below this central cavity. The 0.05-inch chamber above the sapphire window is sealed by a 0.05-inch thick quartz window. This chamber is purged with dry nitrogen during cooling of the dewar to reduce condensation and is evacuated after the sample is frozen.

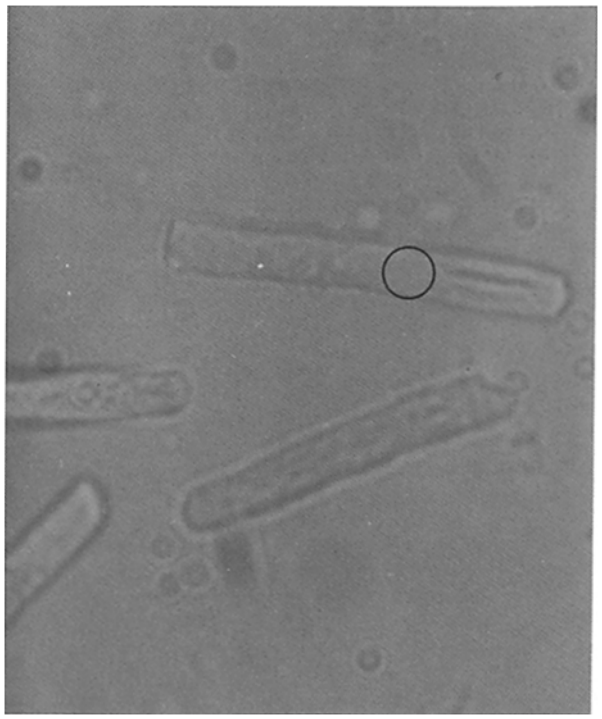


FIGURE 3 Photomicrograph of a toad rod preparation in an aqueous sucrose/glycerol glass 77°K. The circle indicates the area of the photoreceptor that is illuminated by the laser in a typical Raman experiment. These red rod cells are 5 × 50 μm in size.

were assigned to bovine bathorhodopsin using this double beam technique (12, 13).

In bovine pigments the 854, 875, and 922 cm^{-1} lines have been assigned to the hydrogen out-of-plane wagging vibrations of the C_{14}-H, C_{10}-H, and C_{11}-H hydrogens of bathorhodopsin, respectively (5). Since the frequencies of these bathorhodopsin "low wavenumber" lines are nearly identical in the toad and in the bovine spectra, they can be assigned to similar normal modes. However, the intensities of the low wavenumber lines in the two spectra are different. Fig. 4 *b* and *c* have been plotted so that the ethylenic C=C stretches (~1,540 cm^{-1}) have the same amplitude. In the toad pigment the 850 cm^{-1} line is significantly more intense than the 854 cm^{-1} line in the bovine spectrum. The intensities of the low wavenumber lines in bovine bathorhodopsin are now known to be induced by out-of-plane distortions of the *trans* retinal chromophore (15). The intensity differences between the low wavenumber lines of the two bathopigments demonstrate that the chromophores have different conformations.[1] Since the chromophores are identical, differences in their conformations must be caused by differences in protein sequence or in protein conformation.

Fig. 5 presents Raman spectra of rod photoreceptors from the comet goldfish. These photoreceptors are known to contain a 522-nm A_2 pigment (17, 18). Studies on the 522-nm rod pigment of an organism which is closely related to the goldfish, the carp (*Carassius carassius*), have demonstrated that three intermediates are present in the 77°K steady-state. These intermediates are distinguishable by their low temperature absorption spectra and were named in analogy with previously studied A_1 intermediates: porphyropsin (λ_{max} = 528 nm); isoporphyropsin (λ_{max} = 514 nm); and bathoporphyropsin (λ_{max} = 592 nm) (19). Our analysis of the goldfish 522-nm pigment is based on the assumption that carp and goldfish rod steady-states are similar.

Double beam experiments on the goldfish rod steady-state can be used to assess the contribution of bathoporphyropsin to the Raman spectrum. The ethylenic region of a 583-nm probe spectrum is shown in Fig. 5 *a*. The addition of a coaxial 482-nm pump beam to the steady-state (Fig. 5 *b*) causes a dramatic increase of intensity at 1,507 cm^{-1}. Since the blue pump beam

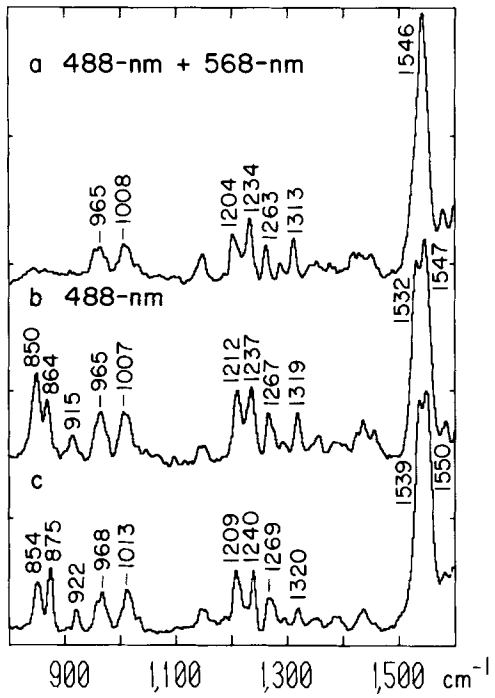


FIGURE 4 Resonance Raman microscope spectra of red rod photoreceptors from the toad, *Bufo marinus*. Spectrum *a* was obtained with a 2-mW, 488-nm probe beam and a coaxial 10-mW, 568-nm pump. Spectrum *b* was generated with a 488-nm probe beam alone (5 mW). In *c* we present a resonance Raman spectrum of bovine visual pigments that have been solubilized in Ammonyx-LO (488-nm probe) from Eyring, et al. (5).

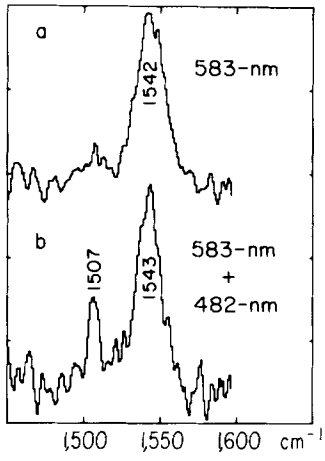


FIGURE 5 Resonance Raman microscope spectra of rod photoreceptors from the goldfish, *Carassius auratus*. Spectrum *a* was obtained with 2 mW of 583-nm excitation. Spectrum *b* was generated using a 2-mW, 583-nm probe and a coaxial 2-mW, 482-nm pump beam.

[1] The possibility that the intensity differences between toad and bovine pigments are due to the two-dimensional ordering of membranes (16) within the toad photoreceptors can be excluded. The depolarization ratios of the low wavenumber lines in the toad spectrum are identical, so any polarization sensitivity of the detection optics will not alter their relative intensities. Also, we have observed the same pattern of intensities in spectra of toad rod pigments in membrane pellets; these pellets contain randomly oriented outer segment fragments.

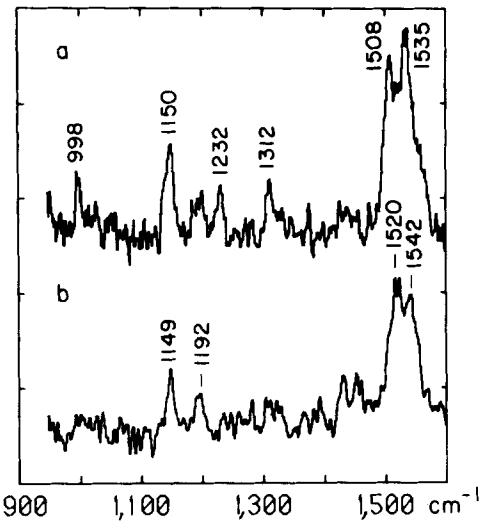


FIGURE 6 Resonance Raman microscope spectra of the two outer segments of the double cone from the goldfish. The probe wavelength is 530 nm (3 mW); spectral slit width is 6 cm^{-1}. Each spectrum was obtained using a single, 135-s integration on one of the two outer segments. Based on a cell volume of 10 μm^3 and a pigment concentration of 2 mM, we estimate that these spectra were obtained from fewer than 10^7 pigment molecules.

should increase the concentration of bathoporphyropsin, the 1,507 cm^{-1} line can be assigned to the bathointermediate. This 1,507 cm^{-1} ethylenic frequency is expected for a retinal-containing pigment absorbing near 600 nm (20, 21). In future work we will examine the low wavenumber region of this spectrum to determine how the chromophore conformation in this bathoporphyropsin differs from the conformation of the toad and bovine bathoproducts.

Prospects

The development of the Raman microscope has enabled us to obtain the first resonance Raman spectra of visual pigments and their photoproducts inside the outer segments of single photoreceptors. Because the Raman microscope makes it feasible to observe photochemical isomerization inside intact cells, it is now possible to study pigments that are exceedingly difficult to isolate from vertebrate retinas. For example, each Raman spectrum in Fig. 6 was obtained by selectively exciting just one of the two outer segments of the goldfish double cone. The spectra in Fig. 6 *a* and *b* have different ethylenic stretching frequencies, confirming the absorption microspectrophotometric finding that the two outer segments of the double cone contain different pigments (22, 23).

These teleost cone spectra demonstrate that we can now perform vibrational studies on the pigments responsible for color vision in vertebrates. Improvement of our Raman microscope should permit the extension of this work to primate cones. Comparative vibrational studies of a wide variety of pigments will allow us to assess the role of protein-chromophore interactions in visual photochemistry.

Finally, we would like to stress the wide applicability and great potential of single-cell vibrational spectroscopy. It is now feasible to study cellular processes which involve chromophore-containing proteins in many other animal and plant tissues. The Raman microscope makes it possible, for example, to perform *in situ* vibrational studies on heme proteins in the mitochondrial electron transport chain and on chlorophyll in photosynthetic membranes.

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