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 epillumination 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 x 50 μm in size, and we have obtained spectra from cells as small as 1 x 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 red 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 (A₁) or a 3,4-dehydro-11-cis retinal (A₂) 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).

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\begin{align*}
\text{Rhodopsin} & \quad \text{Bathorhodopsin} & \quad \text{Isorhodopsin} \\
11\text{-cis} & \quad \text{trans} & \quad 9\text{-cis}
\end{align*}
\]

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 A₁ 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 A₁ (or A₂) 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-
phore determine the frequencies and intensities of these vibrational lines.

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 \times 10 \mu m. 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-Coming 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).

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. 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. 4b presents a Raman microscope spectrum obtained from a red rod photoreceptor (\(\lambda_{\text{max}} = 502\) 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 (\(\lambda_{\text{max}} = 498\) nm) in Fig. 4c 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. 4a. 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. 4a allows us to assign these bands to toad bathorhodopsin. The analogous lines in the bovine spectrum...
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 x 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 C₁₄-H, C₁₀-H, and Cₙ-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=CC 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. 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 goldfish, Carassius auratus. These photoreceptors are known to contain a 522-nm A₂ 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 A₁ 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. 5a. The addition of a coaxial 482-nm pump beam to the steady-state (Fig. 5b) causes a dramatic increase of intensity at 1,507 cm⁻¹. Since the blue pump beam...
should increase the concentration of bathorhodopsin, the 1,507 cm⁻¹ ethylenic frequency is expected for a retinal-con-formation of 2 mM, we estimate that these spectra were obtained from fewer than 10⁷ pigment molecules.

**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 Figs. 6a 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 hemeproteins in the mitochondrial electron transport chain and on chlorophyll in photosynthetic membranes.

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