Modeling the resonance Raman spectrum of a metarhodopsin: Implications for the color of visual pigments

(squid rhodopsin/visual excitation/model compounds/rhodopsin thermal intermediates)

MARK SULKES, AARON LEWIS, ANN T. LEMLEY, AND ROBERT COOKINGHAM

Department of Physics, School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853

Communicated by Gordon G. Hammes, September 10, 1976

ABSTRACT

Resonance Raman spectra of an invertebrate rhodopsin are reported. The spectrum of squid acid metarhodopsin is compared with the spectra of model compounds of the retinylidene chromophore in the all-trans conformation. Correlations made between acid metarhodopsin and these crystalline model compounds with known x-ray structures indicate that the chromophore in this intermediate is an all-trans protonated Schiff base. The data suggest a mechanism for the red shift in rhodopsin.

Three years ago this laboratory reported the resonance Raman spectrum of the retinylidene chromophore of a visual pigment in a digitonin extract of bovine rhodopsin (1). Subsequent work here (1–5) and elsewhere (6–8) has demonstrated the sensitivity of resonance Raman spectroscopy as a probe of the electronic and molecular structure of the retinylidene chromophore in rhodopsin and its photochemically produced thermal intermediates. The wealth of information obtained from these ground state spectra, when coupled with recent data on the excited states of the retinylidene chromophore (9–12), can provide insights into the primary photophysical and photochemical processes of vision.

The principal problem in this field is understanding the information present in the resonance Raman spectra of rhodopsin and its intermediates. Our approach has been to conduct parallel Raman studies of model compounds (13) with conformations and chemical environments that mimic those of rhodopsin and its intermediates. We have obtained the resonance Raman spectrum of a metarhodopsin, and we report here similarities between the spectrum of this rhodopsin intermediate and the spectra of several model compounds studied. This initial success not only has determined the conformation of the retinylidene chromophore in squid acid metarhodopsin, but also has provided experimental criteria for evaluating the credibility of proposed mechanisms for the absorption red shift observed in rhodopsin.

Various experimental techniques have been used to obtain the resonance Raman spectra of a number of different rhodopsins and their intermediates. The principal finding of these studies is that the Schiff base in rhodopsin is protonated and can be deuterated. In the spectrum reported by Lewis et al. (1) and in later studies on the eye of a live rabbit by Lewis (4, 5), a stationary sample was used together with pulses* of laser light to overcome the sensitivity of rhodopsin to visible excitation. Such a technique takes advantage of photochemically induced back reactions of the thermal intermediates of rhodopsin. Subsequently Mathies et al. (8) and Callender et al. (7) used continuous laser beams and therefore had to obtain their resonance

Abbreviation: N*RB-HCl, N-retinylidene-n-butylammonium hydrochloride.

* In the first instance continuous radiation from a rhodamine 6G dye laser was mechanically chopped and a solution at 4°C was used. In the second instance a pulse dye laser was used.

Raman spectra on flowing samples of rhodopsin. Alternatively, both Lewis et al. (2) and Oseroff and Callender (6) used continuous lasers with stationary samples of rhodopsin maintained at low temperature to overcome rhodopsin’s photolability.

On the other hand, use of invertebrate rhodopsin obviates the need for the more elaborate sample techniques that have been resorted to for vertebrate rhodopsin Raman work. The bleaching sequence of squid rhodopsin—unlike vertebrate rhodopsins—does not terminate with the detachment of the retinylidene chromophore from opsin (14). In squid rhodopsin the thermal intermediate sequence in an acidic medium terminates with acid metarhodopsin (15, 16). Because the absorption maxima of rhodopsin and acid metarhodopsin are nearly coincident, this not only makes standard absorption studies quite difficult, but also places severe limitations on flow or rotating cell techniques. However, this allows small stationary sample volumes of squid rhodopsin to be subjected to continuous laser illumination at near physiological temperatures. Under these conditions, using laser illumination of moderate power, we have found nearly indefinite stability of squid rhodopsin, with rhodopsin and metarhodopsin predominating in a photostationary “equilibrium.” The study of squid rhodopsin not only provides these experimental advantages, but it is also important because of the significant similarities and differences in invertebrate as compared to vertebrate visual excitation.

MATERIALS AND METHODS

The squid retinula suspensions were prepared by a method similar to that of Hubbard and St. George (16). The retinas of dark-adapted (Loligo pealei) squid were used in the procedure, and all manipulations were carried out under red light. The impure suspension collected from the retinas was layered on a sucrose step gradient of 40–20% (0.1 M sodium phosphate buffer, pH 6.5) and centrifuged at 2° C for 20 min at 17,000 rpm in a Sorvall RC2B with an SS34 head; the rods settled at the sucrose interface while the impure material sedimented to the bottom. Flotations were continued until pure material was obtained, which was then washed four times in doubly distilled water. The Raman spectra were taken with this material.

The suspensions were placed in 4 mm sealed tubes, which were held in a copper cooling block at 0°C. A focused laser beam was used in a glancing geometry off the suspension surface. The exciting source was the 457.9 nm line of an argon ion laser, with an incident power of about 10 mW. The scattered radiation was collected at 90° and analyzed by a J-Y Optical Systems Ramanor H.C.2 double monochromator, using spectral resolution of 3–4 cm⁻¹. Photon counting techniques were employed to record the signals digitally from an EMI 6256 photomultiplier.

All-trans N-retinylidene-n-butylamine was prepared in a dry box by dissolving all-trans-retinal in 3-methylpentane and
adding 2.1 excess of n-butylamine. The solvent was evaporated and the solid was redissolved in 3-methylpentane and left at −15°C overnight. The resulting unprotonated crystals were filtered and dried, and Raman spectra were taken of this material. To prepare the protonated form, the unprotonated Schiff base was dissolved in absolute ethanol. Subsequently absolute ethanol saturated with gaseous HCl was added drop-wise until the absorption spectrum of the solution showed its major band at 448 nm. The solution was kept for several weeks at −15°C until crystals formed; they were filtered and dried. These procedures were performed in dim red light or in total darkness under a nitrogen atmosphere. The crystals were placed in melting point capillaries for the Raman work.

The all-trans-retinal used in these experiments was obtained from Eastman Organic Co. and was used without further purification. High-pressure liquid chromatography was used to check the purity of both the liquid and solid samples before and after illumination. No detectable isomerization was found to have occurred during the experiments. The trans-retinal was dissolved in CCl₄ at 0.02 M and transferred to a 100 μl cylindrical cell. Crystalline trans-retinal was placed in a melting point capillary.

The Raman spectra of the Schiff bases and retinal samples were obtained with 637.3 nm radiation from a Coherent Radiation continuous wave dye laser. The laser beam was filtered before focusing with a tunable optical filter (17), and a 90° scattering geometry was employed. The scattered light was analyzed with a Spex 1401 double monochromator and photon counting electronics were used to collect digital data (18). The spectral resolution was 2 cm⁻¹. All of the spectra in this report showed excellent reproducibility and signal-to-noise ratio.

RESULTS AND DISCUSSION

A retinular, "outer segment," suspension of squid (Loligo pealei) rhodopsin under illumination at 0°C attains a photostationary "equilibrium" with rhodopsin (λₘₐₓ = 493 nm), acid metarhodopsin (λₘₐₓ = 500 nm), and alkaline metarhodopsin (λₘₐₓ = 380 nm) present. There is also a negligible amount of isorhodopsin (λₘₐₓ = 473 nm) in the mixture. The sample under these conditions has long-term photochemical stability. The laser illumination was at 457.9 nm, which is on the absorption shoulder of rhodopsin and acid metarhodopsin, thereby allowing for resonance enhancement of these components. The constituents in the neutral pH solutions used under this illumination are approximately as follows: acid meta 49%, rhodopsin 40%, alkaline meta 9%, and isorhodopsin 2% (19). A consideration of the relative resonance enhancement of each intermediate suggests that the actual net spectrum is principally composed of acid metarhodopsin and rhodopsin.

The spectrum of the photostationary mixture of squid rhodopsin seen in Fig. 1A is pictured immediately above that of the crystalline all-trans protonated Schiff base in Fig. 1B. The crystalline all-trans unprotonated Schiff base, Fig. 1C, is presented for comparison. Spectra of all-trans-retinal, crystalline and in CCl₄, Figs. 1D and E, also show some similarities to the spectrum of this rhodopsin. However, as can be seen in comparing Figs. 1B, C, D, and E, the spectra of the retinals are quite different from the protonated and unprotonated Schiff bases in spite of the fact that the conformation of each is all-trans. Thus any comparison between the spectra of retinals and rhodopsin must be made with great caution. The spectra pictured in the figure have been normalized so that the intensities of the bands at about 1550 cm⁻¹ (C=O) are equal.

In order to model the acid metarhodopsin spectrum it is important that the assignments of the spectral components made in Fig. 1A are correct. The basis for these assignments is the common presence of rhodopsin in the differently composed photostationary mixtures of squid rhodopsin intermediates at 273 K and 77 K. As was mentioned previously, the major constituents of the mixture studied at 273 K under 457.9 nm illumination are acid meta about 49%, rhodopsin about 40% and alkaline meta 9%. On the other hand, the 77 K mixture is composed of bathorhodopsin, rhodopsin, and isorhodopsin. With the 514.5 nm illumination used to obtain the 77 K spectrum in Fig. 2 the composition of the photostationary mixture is bathorhodopsin about 24%, rhodopsin about 18%, and isorhodopsin about 58%. (The same spectral features at 77 K are observed with 457.9 nm and 514.5 nm illumination.) Thus, the only common major constituent in each mixture is rhodopsin, and a comparison of the spectra at 273 K and 77 K is the basis
for the assignments made in Fig. 1A. The conclusions reached by comparing the spectra obtained at these two temperatures were further confirmed by altering the composition of the 77 K photostationary mixture in a predictable manner—by changing the exciting laser frequency (2, 6) and/or by using a second coincident laser beam to modulate the photochemistry (6).

To illustrate our method of identifying the bands in the photostationary mixture, we have chosen in Fig. 2 a region of the spectrum that is critical to the conformational analysis in this paper. The 273 K and 77 K spectra in this region are shown for comparison. (A complete discussion of the 77 K spectra—which are more directly related to the primary photochemistry than the 273 K spectra—along with additional low temperature experimental details will be given in a forthcoming paper.) The bands at 1147 and 1157 cm\(^{-1}\) are not observed in the 273 K spectrum (solid line), and thus must be due to bathorhodopsin and isorhodopsin. Altering the photostationary mixture at 77 K not only confirms this assignment but also suggests that the 1147 cm\(^{-1}\) band is due to bathorhodopsin and the 1157 cm\(^{-1}\) band to isorhodopsin. On the basis of its non-coincidence with the 77 K spectrum, we have assigned the 1160 cm\(^{-1}\) band in the 273 K spectrum to acid metarhodopsin. Similar methods have been used to assign the bands at 1208, 1227, and 1235 cm\(^{-1}\) in the 77 K spectrum to rhodopsin, bathorhodopsin, and isorhodopsin, and those at 1190 and 1200 cm\(^{-1}\) in the 273 K spectrum to acid metarhodopsin. All of the spectral features (see Fig. 1A) were identified in an analogous manner.

Bands in the 950–990 cm\(^{-1}\) region are usually attributed to bending modes of the protons on the backbone of the chromophore (20). Except in the protonated Schiff base spectrum, at least two bands can be seen in this region. The backbone protons should be sensitive to local changes in charge density, and this sensitivity may account for the differences observed in the spectra. In the spectrum of the photopigments in Fig. 1A, the carbon–methyl stretching region between 995 and 1033 cm\(^{-1}\) is composed of two major peaks, with shoulders at high and low frequencies. The high-frequency peak appears to correspond to rhodopsin while the lower-frequency peak and the two shoulders attributed to acid metarhodopsin have structural similarities to \(\text{N-retinylidene-}\text{N}-\text{butylammonium hydrochloride (N}^\text{R}\text{B-HCl).}

The fingerprint region from 1150 to 1300 cm\(^{-1}\) is characteristic of the isomerization of the chromophore (13, 20–22). If the chromophore of acid metarhodopsin is all-trans, similarities with all-trans model compounds would be expected. There is a band at 1160 cm\(^{-1}\) in acid meta which is also present in N\(^+\text{RB-HCl and the retinals. The unprotonated Schiff base, however, has a doublet structure centered about 1165 cm\(^{-1}\), with a separation of 20 cm\(^{-1}\) between the two peaks. There is a strong band at about 1190 cm\(^{-1}\) which occurs in all the model compounds and is characteristic of the trans isomer. A peak occurs between 1201 and 1206 cm\(^{-1}\); it appears only in the acid metarhodopsin and N\(^+\text{RB-HCl. A comparison with the liquid}

\[\text{Chemistry: Sulkes et al.}\]


\[\text{FIG. 2. A comparison of squid rhodopsin spectra at 77 K (dotted line) and 273 K (solid line). Assignments of the spectral features to rhodopsin (R), bathorhodopsin (B), isorhodopsin (I), and acid metarhodopsin (M) are made above the two spectra for the 77 K case and below them for the 273 K case.}\]
base is a crucial result of the photochemistry of rhodopsin (2). It is possible that this photochemically-induced deprotonation initiates the net protonation changes observed in all rhodopsins. However, the differences in overall stoichiometry of proton uptake and release make it seem likely that the deprotonation of the Schiff base is linked only indirectly to the net protonation changes observed.

The similarity of the frequencies of the stretching vibrations in the protonated Schiff base model compound and acid metarhodopsin (see Fig. 1A and B) suggests that the carbon–nitrogen bond order in these two compounds is similar. X-ray studies (26) have shown that this is a double bond in the protonated Schiff base model compound. This fact, together with the similarities we noted in the remaining structural features of the metarhodopsin and the trans model compounds, indicates that the ground state conformation of acid metarhodopsin is similar to those of the all-trans models. However, there is a 70 nm red shift in the absorption spectrum of the crystals of N+RB-HCl (430 nm) and acid metarhodopsin (500 nm). There have in general been two explanations for the red shift seen in rhodopsin absorption spectra (27–29; B. Honig, A. D. Greenberg, U. Dimer, and T. G. Ebrey, to be published). One involves the raising in energy of the ground electronic state relative (27, 28) to the excited state involved in the absorption, while the other involves the lowering in energy of the excited state relative to the ground state (ref. 29; B. Honig, A. D. Greenberg, U. Dimer, and T. G. Ebrey, to be published). Plausible realizations of these theories are outlined in Fig. 3B and 3C, respectively. The suggestion depicted in Fig. 3B focuses on the importance of the ground state in producing the red shift. In such a model the ground state could be destabilized by a single negative charge on the opsins moving away from the positive Schiff base nitrogen (see Fig. 3B, ii). This would destabilize the resonance structures where the positive charge is centered on the nitrogen. The net effect of this charge movement is a higher energy ground electronic state. A similar effect could occur by opsin-imposed constraints that result in twisting of the retinylidene chromophore into a higher energy conformation. By contrast, in Fig. 3C the charge movement is the result of absorption of a photon and occurs in the excited state. In this model two effective negative charges are used; one acts as a counter ion for the protonated Schiff base and the other is placed within the vicinity of carbon 5, which is a tertiary center capable of stabilizing positive charge density. The negative charge near the β-ionone ring would tend to stabilize this excited state structure and thus lower its energy relative to the ground state.

The Raman data presented in this paper tend to support excited state charge stabilization, e.g., Fig. 3C, rather than models that would seriously perturb the ground state to effect an absorption red shift in the retinylidene chromophore. The similarities in the stretching vibration in N+RB-HCl and acid metarhodopsin suggest that the ground state bond order of the
bond cannot be very different in these compounds. However, the model seen in Fig. 3B would imply large changes in the bond order as the red shift is produced. In addition, the similarities seen in the other vibrational modes of the model compounds and metarhodopsin cast doubt on models where the chromophore is seriously twisted in the opsin cavity. Excited state charge stabilization is supported by other experimental and theoretical approaches to understanding the red shift observed in all rhodopsins. Mathies and Stryer have detected large changes in the dipole moments of model retinals in going from the ground to the excited state (12). This is consistent with a photon-induced charge movement, which is a crucial aspect of the model seen in Fig. 3C.

There is internal support for a dual charge hypothesis in the resonance Raman spectrum of squid rhodopsin. This is seen when the similar C=\(N\) and fingerprint vibrational frequencies in the squid and model spectra are contrasted with the considerably different vibrational frequencies of the C=C stretching modes in acid metarhodopsin and \(N^+RB\cdot HCl\). To understand this difference it is first important to recall that the C=C stretching mode of retinals can be lowered in frequency simply by changing the environment without increasing the number of double bonds. For example, in Fig. 1C and D the C=C stretching mode of \(trans\)-retinal crystals is different from that of the \(trans\)-retinal dissolved in CCl\(_4\). In the squid spectrum the C=C stretching mode is lowered relative to \(N^+RB\cdot HCl\). This reflects in the case of squid acid metarhodopsin the availability of more resonance forms in the ground state of the chromophore due to the dipolar environment of the opsin. However, if this were the only effect, there would be a lowering of the ground state energy relative to the excited state, effecting a blue shift. Thus without excited state charge stabilization there would be no red shift! The fact that only the C=C vibration is affected while the C=N and fingerprint vibrations (which are generated principally in the isoprenoid chain) are not altered suggests that a second effective charge is present in the vicinity of the ionone ring. The double bond in the ring sensitizes the C=C vibration, a group vibration of all the C=C bonds, to this second charge, which acts to stabilize photon-induced charge movement in the excited state.

CONCLUSION

We have been able to model the resonance Raman spectrum of the retinylidene chromophore in a rhodopsin intermediate. Our results demonstrate that the conformation of the chromophore in squid acid metarhodopsin is all-\(trans\) and that the Schiff base is protonated. These experimental results have suggested that excited state charge stabilization is the mechanism responsible for the absorption red shift observed in rhodopsins.

A.T.L. is supported by a National Institutes of Health Postdoctoral Fellowship (no. GM 06437). A.L. is an Alfred P. Sloan Fellow; this work was supported by a grant from the National Institutes of Health (EY01377).