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Chemistry

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

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

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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 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 pealii*) 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.G.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

Abbreviation: $N^+RB{\cdot}HCl$, *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.

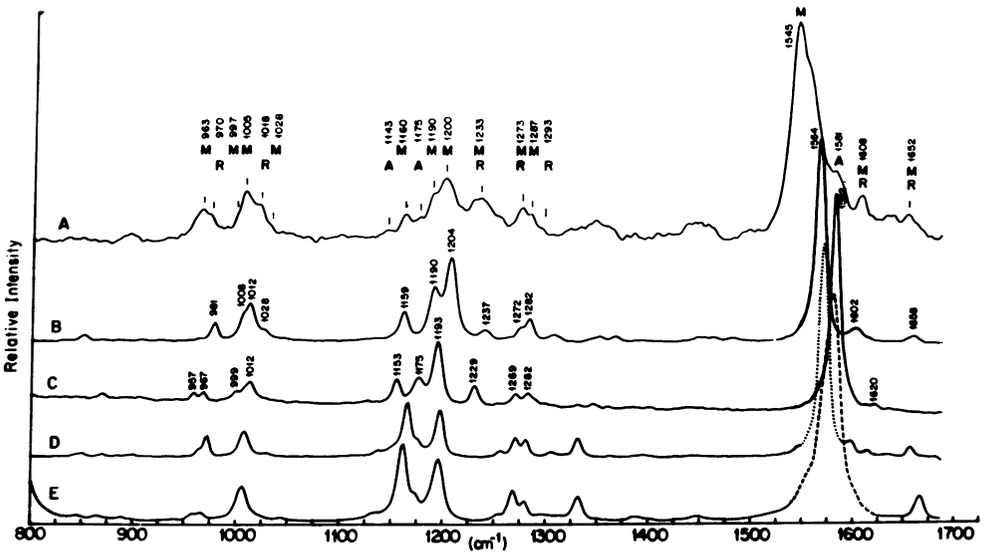


FIG. 1. Raman spectra of (A) squid rhodopsin, pH 7, 0°C; (B) crystalline all-*trans*-*N*-retinylidene-*n*-butylammonium hydrochloride; (C) crystalline all-*trans*-*N*-retinylidene-*n*-butylamine; (D) crystalline all-*trans*-retinal; and (E) all-*trans*-retinal in CCl_4. In the squid rhodopsin spectrum (A) peaks have been assigned to acid metarhodopsin, alkaline metarhodopsin, and rhodopsin, designated respectively by M, A, and R. Band positions are given for (A), (B), and (C) to ±2 cm^{-1}. Frequency assignments for (D) were reported in ref. 13.

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 CCl_4 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 pealii*) rhodopsin under illumination at 0°C attains a photostationary "equilibrium" with rhodopsin (λ_{max} = 493 nm), acid metarhodopsin (λ_{max} = 500 nm), and alkaline metarhodopsin (λ_{max} = 380 nm) present. There is also a negligible amount of isorhodopsin (λ_{max} = 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 CCl_4, 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=C) 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

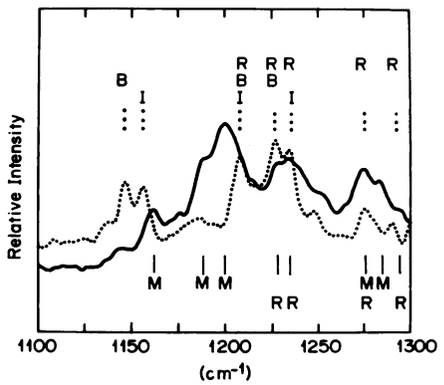


FIG. 2. A comparison of squid rhodopsin spectra at 77 K (dotted line) and 273 K (solid line). Assignments of the spectral features to rhodopsin (R), bathorhodopsin (B), isorhodopsin (I), and acid metarhodopsin (M) are made above the two spectra for the 77 K case and below them for the 273 K case.

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 noncoincidence 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 950–980 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 *N*-retinylidene-*n*-butylammonium hydrochloride (N^+RB·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^+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^+RB·HCl. A comparison with the liquid nitrogen spectrum of squid rhodopsin indicates that the major contribution to the triple peak structure seen between 1225 and 1260 cm^{-1} in spectrum A is due to rhodopsin. The doublet structure found in all of the spectra between 1265 and 1290 cm^{-1} is present in all of the *trans* isomers and in the acid metarhodopsin. Work in progress suggests that the C-9 and C-13 groups must be present for this structure to appear. Thus, there are remarkable similarities between the spectra of the all-*trans* model compounds and acid metarhodopsin. This strongly suggests that the conformation of the chromophore of acid metarhodopsin is all-*trans*.

The remainder of the spectrum contains bands in two other regions. The most intense band in each spectrum occurs between 1540 and 1580 cm^{-1}. This band, generally assigned to te C=C stretching vibration of the polyene (20–22), appears to be the most sensitive to changes in electron delocalization and/or changes in bond order of the double bonds within the polyene portion of the molecule; this is evidenced by the large changes in its vibrational frequency, with corollary changes in the λ_{max} of absorption. In spectrum A the shape of the C=C peak arises from the superposition of contributions from the intermediates of squid rhodopsin present in the photostationary mixture. The band at 1608 ± 3 cm^{-1} can be assigned to squid rhodopsin based on comparison with the liquid nitrogen data. The band at 1545 ± 1 cm^{-1} can be attributed to acid metarhodopsin while the shoulder at 1581 ± 2 cm^{-1} is due to the small fraction of alkaline metarhodopsin present. Similarly, the minor peaks on either side of the acid metarhodopsin band at 1160 cm^{-1} correspond to bands in the all-*trans* unprotonated Schiff base spectrum C and probably also arise from alkaline metarhodopsin.

Vibrations assigned to the C=N or C=O stretching modes occur in the 1620–1670 cm^{-1} region (20–22). The band at 1652 cm^{-1} has been assigned to both rhodopsin and acid metarhodopsin. This assignment was based on a comparison of the peak heights of the C=C stretching bands with the C=N band at 273 K and 77 K. At both these temperatures the intensities of the C=C bands for all the components in the photostationary mixtures relative to the C=N bands were very similar. This indicates that C=N intensity for all species is present in the 1652 cm^{-1} band. The 1652 cm^{-1} band in the squid spectrum, Fig. 1A, compares favorably with the 1658 cm^{-1} protonated Schiff base band, Fig. 1B. The position of this band is in agreement with its observation in other model compounds (22) and rhodopsins that are protonated (1–8). In addition, deuteration experiments on squid rhodopsin support the conclusion that the Schiff base in this invertebrate rhodopsin is also protonated (work to be published from this lab).

The observation that the Schiff base is protonated is interesting in view of the fact that the net stoichiometry of the proton changes in the squid cycle is opposite to that of bovine rhodopsin (16, 23), where a protonated Schiff base is also observed (1, 6–8). [In this connection it is perhaps significant that a hyperpolarization occurs in bovine rod cells (24) while a depolarization is observed in squid retinula cells (25).] A previous study from this laboratory has suggested that the deprotonation of the Schiff

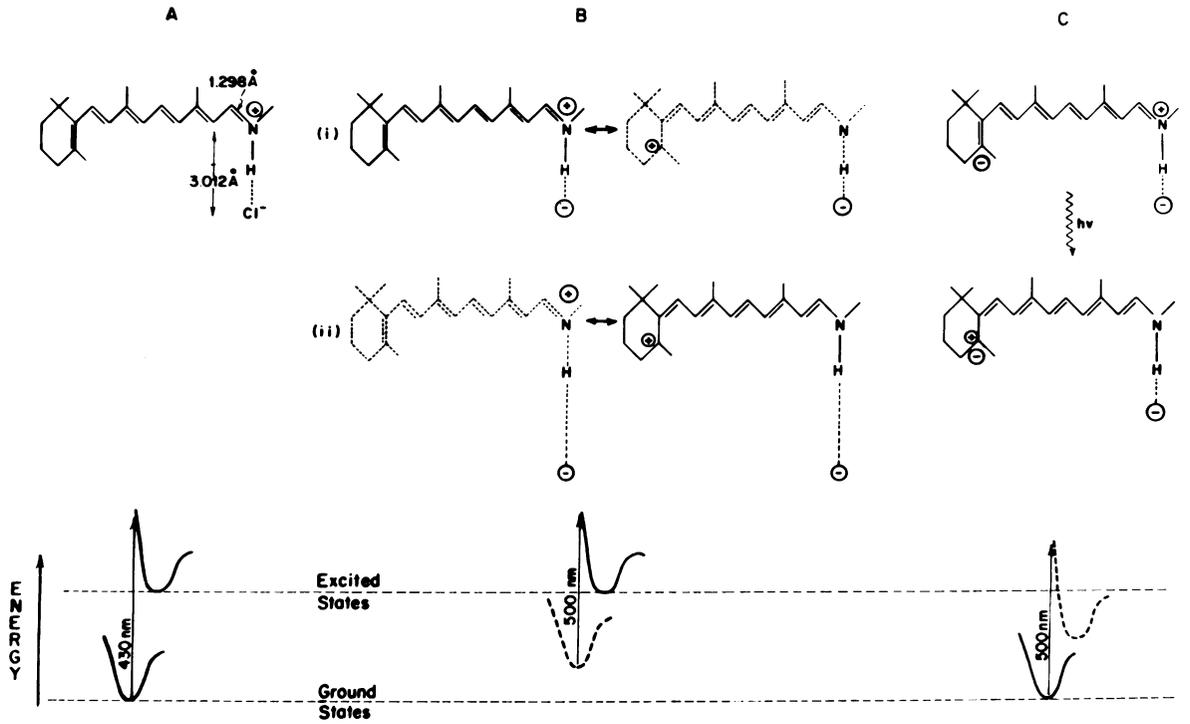
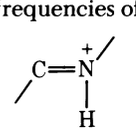


FIG. 3. Schematic representations of possible mechanisms for the red shift in the absorption of the chromophore of rhodopsin. (A) The absorption of a model protonated Schiff base (λ_{max} = 430 nm). (B) A mechanism for the red shift, suggesting that the ground state energy is raised by movement of the Schiff base counter ion. Resonance structures for two positions of the counter ion are shown in (i) and (ii). As the single negative charge is moved away from the Schiff base nitrogen a greater contribution from resonance structures with the positive charge near the ionone ring [see solid line structure B(ii)] has to be included. (C) A mechanism for the red shift in which the excited state is lowered relative to the ground state by a second negative charge on the protein, stabilizing photon-induced charge redistribution in the chromophore.

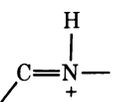
base is a crucial result of the photochemistry of rhodopsin (2). It is possible that this photochemically-induced deprotonation initiates the net protonation changes observed in all rhodopsins. However, the differences in overall stoichiometry of proton uptake and release make it seem likely that the deprotonation of the Schiff base is linked only indirectly to the net protonation changes observed.

The similarity of the frequencies of the

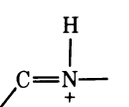


stretching vibrations in the protonated Schiff base model compound and acid metarhodopsin (see Fig. 1A and B) suggests that the carbon–nitrogen bond order in these two compounds is similar. X-ray studies (26) have shown that this is a double bond in the protonated Schiff base model compound. This fact, together with the similarities we have noted in the remaining spectral 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 70nm 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 opsin 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.

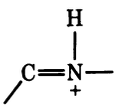
The Raman data presented in this paper tend to support excited state charge stabilization, e.g., Fig. 3C, rather than models that would seriously perturb the ground state to effect an absorption red shift in the retinylidene chromophore. The similarities in the



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.

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† In examples where the number of double bonds is increased (i.e., the sequence ethylene → hexatriene) the C=C stretch is also lowered; the red shift observed in this sequence is due again to stabilization of the excited state through the availability of more carbon sites. This permits a greater number of excited state resonance structures to be drawn, thus lowering the excited state energy.

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