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Resonance Raman Spectroscopy of an Ultraviolet-Sensitive Insect Rhodopsin†

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ABSTRACT: We present the first visual pigment resonance Raman spectra from the UV-sensitive eyes of an insect, *Ascalaphus macaronius* (owlfly). This pigment contains 11-*cis*-retinal as the chromophore. Raman data have been obtained for the acid metarhodopsin at 10 °C in both H_2O and D_2O. The C=N stretching mode at 1660 cm^{-1} in H_2O shifts to 1631 cm^{-1} upon deuteriation of the sample, clearly showing a protonated Schiff base linkage between the chromophore and the protein. The structure-sensitive fingerprint region shows similarities to the all-trans-protonated Schiff base of model retinal chromophores, as well as to the octopus acid metarhodopsin and bovine metarhodopsin I. Although spectra measured at −100 °C with 406.7-nm excitation, to enhance scattering from rhodopsin (λ_{max} 345 nm), contain a significant contribution from a small amount of contaminants [cytochrome(s) and/or accessory pigment] in the sample, the C=N stretch at 1664 cm^{-1} suggests a protonated Schiff base linkage between the chromophore and the protein in rhodopsin as well. For comparison, this mode also appears at ~1660 cm^{-1} in both the vertebrate (bovine) and the invertebrate (octopus) rhodopsins. These data are particularly interesting since the absorption maximum of 345 nm for rhodopsin might be expected to originate from an unprotonated Schiff base linkage. That the Schiff base linkage in the owlfly rhodopsin, like in bovine and in octopus, is protonated suggests that a charged chromophore is essential to visual transduction.

The visual pigment rhodopsin of invertebrates, like that of vertebrates, contains 11-*cis*-retinal as the chromophore [e.g., see Hubbard and St. George (1958)]. It is known that the chromophore is attached to the protein by a protonated Schiff base linkage in the bovine rhodopsin (Oseroff & Callender, 1974). While much is known about the vertebrate rhodopsin, relatively scarce data are available for invertebrate rhodopsins. Recently, it has been shown that a protonated Schiff base linkage also exists between the chromophore and the protein in octopus rhodopsin and its photoproducts (Kitagawa & Tsuda, 1980; Pande et al., 1984, 1987).

Calorimetric studies on both bovine and octopus rhodopsins (Cooper, 1979; Cooper et al., 1986; Schick et al., 1987) have shown that almost the same amount of photon energy (~35 kcal/mol) is converted into chemical energy during the primary step of vision, namely, the isomerization of the chromophore

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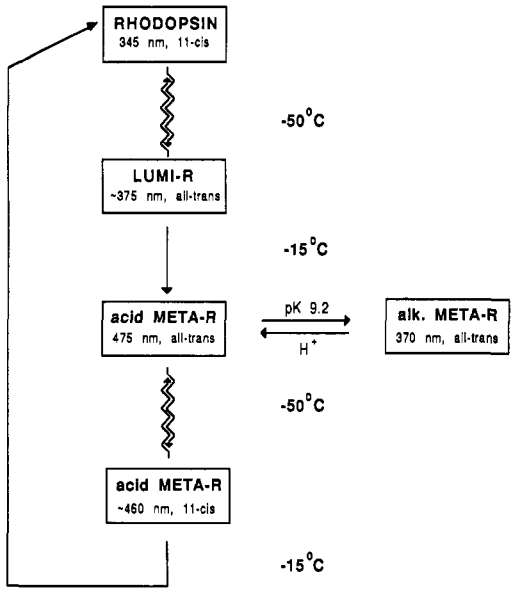

FIGURE 1: Light and dark reactions of owlfly rhodopsin. The photoreactions have been denoted by wavy lines, while the thermal (dark) reactions are represented by straight lines. The figure has been adapted from Hamdorf et al. (1973).

from the 11-cis to the all-trans form when bathorhodopsin is photochemically formed from rhodopsin. Some theoretical models for visual transduction (Honig et al., 1979b; Deng & Callender, 1987; Gilson et al., 1988; Birge et al., 1988) suggest that a substantial amount of this energy conversion is achieved by the movement, through space, of the positively charged protonated Schiff base against an electrostatic field due to opsin in the retinal binding pocket, upon chromophore photoisomerization. These models, therefore, require the Schiff base linkage between the chromophore and the protein to be protonated and that chromophore isomerization occurs during the primary photophysics of vision.

It was shown some time ago (Paulsen & Schwemer, 1972) that the rhodopsin in the UV-sensitive eyes of *Ascalaphus macaronius* (owlfly) contains an 11-*cis*-retinal chromophore. While the bovine and the octopus rhodopsins absorb at ~500 nm, the owlfly rhodopsin absorbs maximally at 345 nm. On the basis of studies with model compounds, the relative bathochromic shift in the former two with respect to the absorption maxima of retinal Schiff bases (λ_{max} ~360 nm) has been explained to arise from the increased π-electron delocalization as a result of protonation of the Schiff base linkage between the chromophore and the opsin. Protonated Schiff bases of retinal in solution have absorption maxima at ~440 nm. The bathochromic shift observed in pigments absorbing in the visible, from this solution value of ~440 nm, is thought to be modulated by the protein environment, probably involving charged groups [see, e.g., Honig and Ebrey (1982)]. It is, therefore, of particular interest to understand the nature of the chromophore–opsin linkage in owlfly rhodopsin, since its absorption maximum is blue-shifted (shifted to shorter wavelength) compared to even retinal Schiff bases in solution.

Upon irradiation, owlfly rhodopsin undergoes a series of dark reactions shown in Figure 1. Although extensive data on the photochemical intermediates of this system are not yet available, it appears to undergo reactions similar to the bovine (vertebrate) and cephalopod (invertebrate) rhodopsins. Absorption of photon converts rhodopsin to the supposed (yet unobserved) primary photoproduct, bathorhodopsin, which thermally decays to metarhodopsin via lumirhodopsin. Like the other invertebrates, squid and octopus, the owlfly metarhodopsin equilibrates between the acid–base pair, the acid metarhodopsin and the alkaline metarhodopsin, which differ in the protonation state of the Schiff base linkage between the chromophore and the protein. Under physiological conditions, the predominant photoproduct of owlfly rhodopsin is acid metarhodopsin. Unlike vertebrate (bovine) metarhodopsins which are unstable and hydrolyze to retinal and opsin, the metarhodopsin of owlfly as well as other invertebrates, namely, squid and octopus, is stable and can be photochemically pumped back to the primary pigment, rhodopsin (for example, see Figure 2), in a quantitative manner.

We present here the results of our resonance Raman study on owlfly acid metarhodopsin as well as preliminary results on the primary pigment rhodopsin. Our data show that the owlfly acid metarhodopsin contains a protonated Schiff base linkage between the chromophore and the protein, like its counterparts in the bovine and the octopus visual pigments. Our data on rhodopsin strongly suggest that it too contains a protonated Schiff base linkage. These data suggest that the protonated Schiff base linkage between retinal and opsin may be a property that is shared by all visual pigments, suggesting a general mechanism of visual transduction. In addition, there are marked similarities among the Raman spectra of the acid metarhodopsins of owlfly and octopus, bovine metarhodopsin I, and the protonated Schiff base of *all-trans*-retinal with model compounds in solution. This strongly suggests that owlfly acid metarhodopsin contains an *all-trans*-retinal isomer.

Materials and Methods

Retinas were isolated from *Ascalaphus* (owlfly) eyes, collected, and homogenized in phosphate buffer (0.1 M, pH 6.0). Following centrifugation, the pellet was resuspended in 35% sucrose (w/v; in phosphate buffer), layered under phosphate buffer, and centrifuged again. Rhabdomeric membranes were collected from the interface and washed several times with phosphate buffer. After the final centrifugation, the pellets were irradiated with blue light (472 nm; interference filter) in order to convert possible fractions of acid metarhodopsin to rhodopsin and stored at −80 °C until further use.

For experiments at 10 °C, the rhodopsin was solubilized in 15 mM CHAPSO (Calbiochem). For deuteriation experiments, the CHAPSO solution was made in D_2O, and the rhodopsin was deuteriated by spinning down twice in D_2O prior to solubilization. The sample in CHAPSO was sonicated briefly to facilitate the solubilization of rhodopsin in the detergent. It was then centrifuged at ~10000*g* for 10 min to remove the insoluble matter. The supernatant was used for the Raman as well as the absorption measurements.

The absorption spectra were measured either in a dual-beam spectrophotometer, Hitachi 356 (Bochum), or in a Perkin-Elmer Lambda 3b (New York) using quartz microcuvettes (volume 20 μL; Hellma). The typical rhodopsin extracts used for Raman measurements had an absorbance of 1 at 345 nm.

The Raman spectra of acid metarhodopsin were obtained at 10 °C in detergent-solubilized samples contained in a 3 mm × 3 mm cuvette in a thermostated cell holder which was cooled with a Lauda circulator bath. The rhodopsin spectra were obtained at −100 °C on an unsolubilized sample contained in the cold finger of the low-temperature probe described previously (Aton et al., 1977). The cuvette setup typically requires a 40-μL sample while the low-temperature setup needs a 30-μL sample. Due to its red-shifted absorption maximum (λ_{max} 475 nm) compared to rhodopsin, the acid metarhodopsin spectra were measured with 457.9-nm line from an Ar ion laser (Spectra Physics, Model 165). The rhodopsin (λ_{max} 345 nm) spectra were measured with the 406.7-nm line from a Kr ion

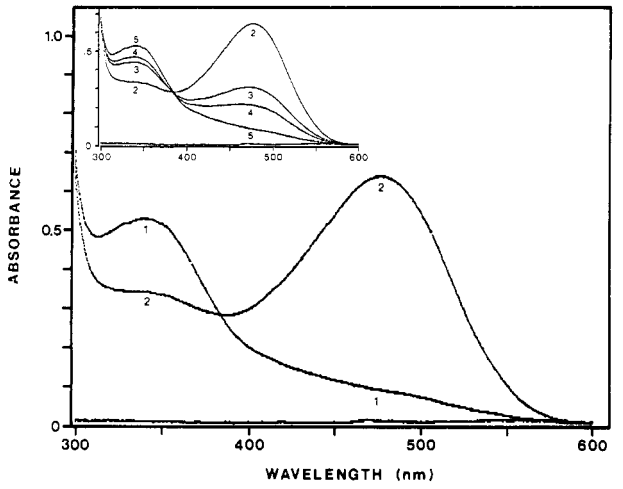

FIGURE 2: Spectral absorbance of rhodopsin (spectrum 1) and acid metarhodopsin (spectrum 2). UV light (350 nm, interference filter, Xenon arc 150 W) was used to set up the photo-steady-state between rhodopsin and metarhodopsin (30:70; curve 2). The inset shows the reconversion of acid metarhodopsin to rhodopsin by successive irradiation with blue light (472 nm, interference filter; curves 3, 4, and 5). Rhodopsin was extracted in 50 μL of 3% CHAPSO (pH 6.0) from membranes isolated from 20 retinas.

laser (Coherent, Model CR-2000). The 350.7-nm line from the Kr laser was also tried for rhodopsin but was found unsuitable due to an enormous background fluorescence.

The Raman spectra were measured in a triplemate spectrometer (Spex Industries, Model 1877) using 1200 g/mm grating. This spectrometer is connected to a cooled solid-state detector/controller system (EG&G Princeton Applied Research Model 1420-2/1218) and is interfaced to an LSI-11 (Digital Equipment Corp.) computer. The details of this setup have been discussed elsewhere (Pande et al., 1986). The spectral resolution in these experiments was 8 cm^{-1}.

Results and Discussion

The absorption spectrum of rhodopsin is shown in Figure 2. Besides its absorbance in the UV region, there is some tailing in the visible spectral region which is due to small contaminations [cytochrome(s) and/or accessory pigments]. Prolonged irradiation of this extract with light of 350 nm (interference filter; Xenon arc 150 W) converts a maximal fraction of approximately 70% to acid metarhodopsin (curve 2, Figure 2) which, in turn, is completely reconverted to rhodopsin by light which is only absorbed by acid metarhodopsin (e.g., 472 nm). It should be mentioned that while similar photoreversibility is known to exist in all invertebrate visual pigment systems studied so far (Stavenga & Schwemer, 1984), the vertebrate visual pigments hydrolyze to retinal and opsin upon irradiation. Like in cephalopods, the acid metarhodopsin of owlfly is converted to an alkaline form upon raising the pH (pK = 9.2). This has prompted the suggestion that the binding of the chromophore to the opsin in acid metarhodopsin occurs via a protonated Schiff base linkage which deprotonates upon the formation of alkaline metarhodopsin (Schwemer et al., 1971).

The acid metarhodopsin resonance Rama spectra in H_2O and D_2O are shown in Figure 3a and Figure 3b, respectively. The spectra were measured with a 457.9-nm probe beam from an Ar laser (15 mW), while a coaxial 350.7-nm beam (200 mW) was pumping the rhodopsin formed by the probe beam back to acid metarhodopsin (see Figure 1). The details of the spectra are discussed below.

Ethylenic Region. The dominant, Raman-active, ethylenic band (C=C stretch) of acid metarhodopsin appears at 1556

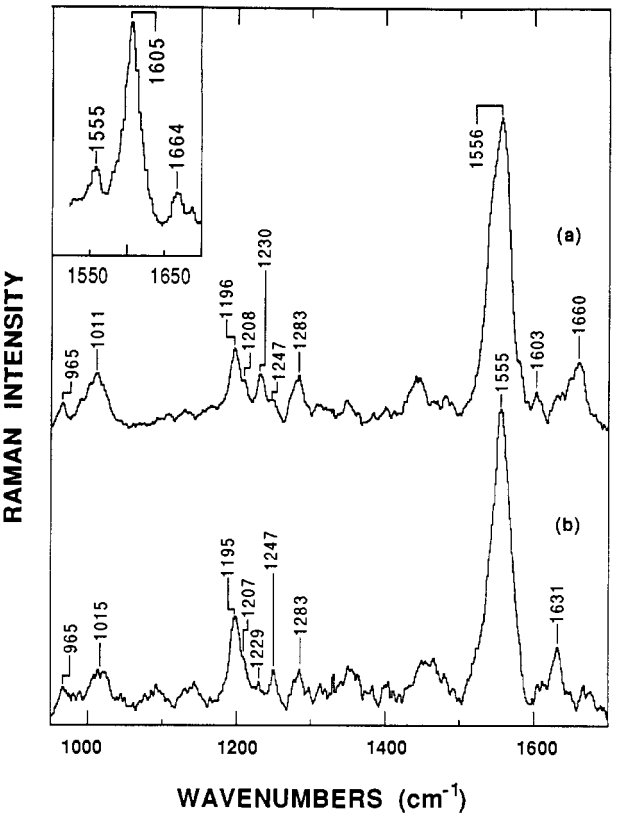

FIGURE 3: (a) Resonance Raman spectrum of *Ascalaphus* (owlfly) acid metarhodopsin in 15 mM CHAPSO at 10 °C. (b) The same as (a) except the sample was deuteriated (see text). The inset shows the resonance Raman spectrum of unsolubilized owlfly rhodopsin at −100 °C. The acid metarhodopsin spectra were measured with 457.9-nm light from an Ar^+ laser, while the rhodopsin spectrum was measured with 406.7-nm light from a Kr^+ laser. The sample concentration was ~1 ODU at 345 nm.

cm^{-1} (Figure 3a). It has been observed that the position of the ethylenic frequency is inversely correlated to the absorption maximum of the pigment (Aton et al., 1977; Doukas et al., 1978). The acid metarhodopsin absorption maximum at 475 nm would suggest an ethylenic band at 1555 cm^{-1}, in excellent agreement with the observed position. There is also a marked shoulder at the lower frequency side of the strong 1556 cm^{-1} band. This presumably arises due to the other intermediate(s) which precede(s) the acid meta, when the pump beam pumps the rhodopsin to acid meta, e.g., the bathorhodopsin. The observed correlation between the ethylenic stretching frequency and the absorption maximum of visual pigments and bacteriorhodopsin would suggest that the absorption maximum of this (these) specie(s) be red-shifted compared to the acid metarhodopsin. The predominant band, however, is clearly due to acid metarhodopsin, and, therefore, it is reasonable to believe that the other spectral features are representative of the acid metarhodopsin (also see below). These spectra were collected in about 1 h, since by this time the intensity of the Raman signal from the sample had decreased appreciably, presumably due to the sample bleaching under these light intensities.

While measuring the spectra in D_2O, shown in Figure 3b, we slightly modified the procedure to avoid contributions from the other intermediate(s). Instead of using the simultaneous pump probe technique, we first illuminated the sample with the pump beam, converting the sample to acid metarhodopsin, blocked the pump beam, and then collected the Raman scattering for ~5 min with the probe beam. During this time, the acid metarhodopsin spectrum was observed to progressively

decrease in intensity with time due to its reconversion to rhodopsin. This whole procedure was repeated several times, and the data were collected for ~1 h. As can be seen, although the ethylenic peak position appears at the same position (1555 cm^{-1}) as in Figure 3a, the shoulder observed in Figure 3a has almost disappeared, and the ethylenic band is a lot sharper.

Schiff Base Region. The Schiff base (C=N) stretching mode in the acid metarhodopsin appears at 1660 cm^{-1} as seen in Figure 3a. Deuteriation of the sample results in the shift of this mode to 1631 cm^{-1}. These data clearly show that the Schiff base linkage in owlfly acid metarhodopsin is protonated. For comparison, the Schiff base vibrational modes in bovine metarhodopsin I and octopus acid metarhodopsin occur at 1657 and 1656 cm^{-1}, respectively (Doukas et al., 1978; Kitagawa & Tsuda, 1980; Pande et al., 1987). For the octopus acid metarhodopsin, this mode was shown to shift to 1625 cm^{-1} upon deuteriation (Kitagawa & Tsuda, 1980).

Fingerprint Region. The fingerprint region (1100–1350 cm^{-1}) mainly originates from the C–C stretch and the C–C–H bending motions (Callender & Honig, 1977) and is quite sensitive to the isomeric state of the chromophore, as well as to the chromophore terminal end group and, perhaps, other factors such as electrostatic or steric chromophore–protein interactions. The owlfly acid metarhodopsin spectrum (Figure 3a) shows a predominant band at 1196 cm^{-1} in this region and two, relatively less intense, bands at 1230 and 1283 cm^{-1}. The octopus acid metarhodopsin spectrum (Pande et al., 1987), for comparison, shows remarkable similarity to this spectrum with peaks at 1197, 1227, and 1274 cm^{-1}. The general features for the metarhodopsin I intermediate of bovine rhodopsin (Doukas et al., 1978) in this region, though similar, show slightly different peak positions. It is interesting to note that all these metarhodopsin species show marked similarity to the *all-trans*-retinal protonated Schiff base of model compounds and are quite different from the other isomers. These data, in complete agreement with the earlier suggestions (Hamdorf et al., 1973), reveal that, like in the bovine and octopus systems, the acid metarhodopsin of owlfly contains an all-trans chromophore. The significance, if any, of the minor differences in this region of the spectra between the acid metarhodopsin spectrum in water and in deuteriated samples (Figure 3a,b) will have to await further studies with selectively isotopically labeled retinals.

We tried various experimental configurations in order to obtain the spectrum of the primary pigment rhodopsin. Unfortunately, as mentioned above, the samples were gradually bleached under our illumination conditions. We were able to obtain only preliminary data before all our available samples were bleached. The following conditions yielded the best results. Unsolubilized rhabdomeric membranes were used, and measurements were made at −100 °C to maximize the amount of rhodopsin by blocking its thermal (dark) decay pathway (Figure 1). At this temperature, the metarhodopsins cannot form (Hamdorf et al., 1973; Stavenga & Schwemer, 1987), and even the formation of lumirhodopsin is significantly reduced. Although we would have preferred to make the measurements at liquid nitrogen temperature (−190 °C), as has been the practice in previous Raman studies of vertebrate and invertebrate rhodopsins, the overwhelming fluorescence at that temperature proved prohibitive. The 406.7-nm line from a Kr^+ laser was used to selectively enhance the Raman signal from rhodopsin (λ_{max} 345 nm). As mentioned above, all attempts to measure the scattering with 350.7-nm light resulted in overwhelming fluorescence background. It should be noted that in the same run, but with 457.9-nm light, we could not obtain any Raman signal. Unfortunately, spectra obtained with the 406.7-nm light also result in a significant contribution from small contaminations in the sample due to cytochromes which, although do not scatter at 457.9 nm (Figure 3a), show significant Raman scattering due to resonance enhancement at 406.7 nm. Raman measurements with 406.7-nm light at 10 °C served as the necessary control for the above experiment, since these data showed Raman scattering from the impurities but not from rhodopsin or its stable photoproduct, acid metarhodopsin, as judged by the absence of significant Raman bands in both the ethylenic and the Schiff base regions (1550–1670 cm^{-1}). This is not surprising, since illumination of rhodopsin (λ_{max} 345 nm) at this temperature with 406.7-nm light will produce a significant amount of acid metarhodopsin (λ_{max} 475 nm), and the Raman scattering from this species is not expected to be as resonance enhanced at this excitation wavelength as at 457.9 nm (see Materials and Methods also).

The inset to Figure 3 shows the spectral features in the 1500–1700 cm^{-1} region in the data obtained with 406.7-nm excitation at −100 °C. There is no significant contribution from cytochromes in this region; however, there appears to be a small amount of contamination from the acid metarhodopsin, as seen by the presence of a small apparent ethylenic mode at 1555 cm^{-1} (see Figure 3a). This band is very small compared to the the main band at 1605 cm^{-1}, so we expect negligible contribution to the Schiff base region from acid metarhodopsin. The shift toward higher frequency of the ethylenic mode of rhodopsin (λ_{max} 345 nm) at 1605 cm^{-1}, compared to that of acid metarhodopsin (λ_{max} 475 nm) at 1555 cm^{-1}, is consistent with the emperically observed inverse correlation between the λ_{max} and the ethylenic mode frequency for retinal-based pigments (Aton et al., 1977; Doukas et al., 1978). However, a quantitative extrapolation of the previously derived correlation (Doukas et al., 1978) would predict a value of ~1585 cm^{-1} for the ethylenic mode of rhodopsin using the absorption maximum of 345 nm. It should be pointed out that the correlation is theoretically not well understood. Thus, the quantitative discrepancy in this study is very interesting and may be useful to further the theoretical understanding of this empirical relationship.

The most interesting result of this experiment is the observed position of the Schiff base mode at 1664 cm^{-1} which is close to the position of protonated Schiff base modes observed in a large variety of pigments. For example, in the owlfly acid metarhodopsin spectrum, this mode appears at 1660 cm^{-1}, and in the bovine and octopus rhodopsins, it appears at 1657 and 1656 cm^{-1}, respectively [see, e.g., Pande et al. (1987)]. This frequency is quite far from the observed frequencies of the unprotonated Schiff base bands which are found to lie in the region of 1620–1635 cm^{-1}. This suggests that the owlfly rhodopsin contains a protonated Schiff base linkage between the retinal chromophore and the opsin. Unfortunately, all our samples bleached before we could obtain a rhodopsin spectrum in D_2O. A deuteriation effect, like that observed for the owlfly acid metarhodopsin, would be considered definite proof.

Conclusions

There are two central attributes that characterize the primary photochemistry of visual pigments in higher animals. The first is that the retinal chromophore undergoes an 11-cis to all-trans isomerization upon the absorption of a photon. The previous results of Paulsen and Schwemer (1972) showing that owlfly contains an 11-cis chromophore and the present resonance Raman results suggesting that the acid metarhodopsin

form contains an all-trans chromophore show that this attribute extends to the insect visual pigments.

The second chemical characteristic of visual pigments is that the chromophore is linked to the protein via a protonated Schiff base. Our Raman measurements on the owlfly visual pigments show that the Schiff base linkage between the chromophore and the protein in acid metarhodopsin is protonated and strongly suggest that it is protonated in rhodopsin as well. We discuss below the important implications of a protonated Schiff base for rhodopsin but caution that the definitive proof of the state of protonation must await a deuteriation experiment as discussed above. We shall perform such measurements when new samples are available, a once a year occurrence.

That the primary pigment rhodopsin is likely protonated is particularly remarkable since it would seem far easier for nature to achieve ultraviolet sensitivity by choosing an unprotonated Schiff base. Protonated Schiff bases of retinal in solution absorb near 440 nm while unprotonated Schiff bases absorb near 360 nm. This suggests that the chromophore is charged for some special reason. The reason would probably have to do with the conversion of light energy to chemical energy which occurs in visual transduction. We have proposed (e.g., Honig et al., 1979b; Deng & Callender, 1987; Gilson et al., 1988) that much of this energy conversion is achieved upon photoisomerization of the chromophore which results in the movement, through space, of the positively charged protonated Schiff base moiety against an electrostatic field due to opsin at the retinal binding site. The simplest imaginable mechanism involves the breaking of a salt bridge between the protonated Schiff base of rhodopsin and a negatively charged counterion due to the surrounding protein. Since the apoprotein (opsin) structure of rhodopsin is unknown, it is unclear how precisely the chromophore interacts with the surrounding protein. However, the positively charged chromophore is certainly electrostatically stabilized. Simple electrostatic considerations (Honig et al., 1979b; Deng & Callender, 1987; Gilson et al., 1988) and sophisticated molecular orbital calculations (Birge et al., 1988) show that considerable chemical energy can be created when this stabilization is disrupted by chromophore photoisomerization. Thus, it would appear that nature has selected a common mechanism of visual transduction across the diverse species which have been studied to date.

Experimental and theoretical work (Honig et al., 1979a) suggests that color regulation in visual pigments is achieved through chromophore–protein electrostatic interaction. The specific λ_{max} of visual pigments is sometimes quantified by the difference between the λ_{max} of a retinal protonated Schiff base in solution (440 nm) and the λ_{max} of the visual pigments expressed in wavenumber (energy) units. This is the so-called "opsin" shift which must result from specific chromophore–protein interactions. For example, the opsin shift in bovine rhodopsin (λ_{max} 498 nm) is +2700 cm^{-1} while that in bacteriorhodopsin (λ_{max} 568 nm) is +5100 cm^{-1}. On the same basis, one observes an opsin shift of −6000 cm^{-1} (note the negative sign) in the case of owlfly rhodopsin. The specific models for color (λ_{max}) regulation in bovine rhodopsin [e.g., see Honig et al. (1979a)] and bacteriorhodopsin [e.g., see Nakanishi et al. (1980) and Harbison et al. (1985)] are based on the placement of strategically located charges or dipoles along the polyene chain of retinal. This substantially modifies the π-electron configuration and can easily result in significant red-shifts. Qualitatively, it seems very plausible that similar placement of charges (or dipoles), either in different positions and/or of opposite sign, could result in the significant blue-shifts observed in UV-absorbing pigments like the one present in owlfly.

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