Kitagawa, T., Tanaka, K. I., & Nojima, S. (1977) *Biochim. Biophys. Acta 467*, 137-145.
Little, C. (1981) *Methods Enzymol. 71*, 725-730.
Nagle, J. F., & Scott, H. L. (1978) *Biochim. Biophys. Acta 513*, 236-243.
Op den Kamp, J. A. F., de Gier, J., & Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 345*, 253-256.
Op den Kamp, J. A. F., Kauerz, M. T., & Van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 169-177.
Papahadjopoulos, D., Jacobson, K., Nir, S., & Isacs, T. (1973) *Biochim. Biophys. Acta 311*, 330-348.
Pieterson, J. C., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455-1460.
Pluncknthun, A., & Dennis, E. A. (1982) *Biochemistry 21*, 1743-1750.
Reman, F. C., Demel, R. A., de Gier, J., Van Deenen, L. L. M., Eibl, H., & Westphal, O. (1969) *Chem. Phys. Lipids 3*, 221-233.
Roberts, M. F., Deems, R. A., & Dennis, E. A. (1977) *J. Biol. Chem. 252*, 6011-6017.
Roberts, M. F., Otnaess, A.-B., Kensil, C. A., & Dennis, E. A. (1978) *J. Biol. Chem. 253*, 1252-1257.
Tinker, D. O., & Wei, J. (1979) *Can. J. Biochem. 57*, 97-106.
Tinker, D. O., Purdon, A. D., Wei, J., & Mason, E. (1978) *Can. J. Biochem. 56*, 552-558.
Upreti, G. C., & Jain, M. K. (1978) *Arch. Biochem. Biophys. 188*, 364-375.
Upreti, G. C., & Jain, M. K. (1980) *J. Membr. Biol. 55*, 113-123.
Van Deenen, L. L. M., & de Haas, G. H. (1964) *Adv. Lipid Res. 2*, 167-234.
Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127-136.
Wells, M. A. (1974) *Biochemistry 13*, 2248-2257.
Wells, M. A. (1978) *Adv. Prostaglandin Thromboxane Res. 3*, 39-45.

A Study of the Schiff Base Mode in Bovine Rhodopsin and Bathorhodopsin†

H. Deng and R. H. Callender*

Physics Department, City College of The City University of New York, New York, New York 10031

Received February 20, 1987; Revised Manuscript Received June 26, 1987

ABSTRACT: We have obtained the resonance Raman spectra of bovine rhodopsin, bathorhodopsin, and isorhodopsin for a series of isotopically labeled retinal chromophores. The specific substitutions are at retinal's protonated Schiff base moiety and include —HC=NH^+—, —HC=ND^+—, —$H^{13}C$=NH^+—, and —$H^{13}C$=ND^+—. Apart from the doubly labeled retinal, we find that the protonated Schiff base frequency is the same, within experimental error, for both rhodopsin and bathorhodopsin for all the substitutions measured here and elsewhere. We develop a force field that accurately fits the observed ethylenic (C=C) and protonated Schiff base stretching frequencies of rhodopsin and labeled derivatives. Using MINDO/3 quantum mechanical procedures, we investigate the response of this force field, and the ethylenic and Schiff base stretching frequencies, to the placement of charges close to retinal's Schiff base moiety. Specifically, we find that the Schiff base frequency should be measurably affected by a 3.0–4.5-Å movement of a negatively charged counterion from the positively charged protonated Schiff base moiety. That there is no experimentally discernible difference in the Schiff base frequency between rhodopsin and bathorhodopsin suggests that models for the efficient conversion of light to chemical energy in the rhodopsin to bathorhodopsin photoconversion based solely on salt bridge separation of the protonated Schiff base and its counterion are probably incorrect. We discuss various alternative models and the role of electrostatics in the rhodopsin to bathorhodopsin primary process.

When rhodopsin, the visual pigment protein, absorbs a photon, a species called bathorhodopsin is formed photochemically. Some two-thirds of the photon's energy is converted to chemical energy in the rhodopsin to bathorhodopsin photoreaction (Cooper, 1979; Cooper et al., 1986; Schick et al., 1987). At least two events are believed to occur in the rhodopsin to bathorhodopsin transformation [reviewed in Birge (1981) and Ottolenghi (1980)]. There is a large body of evidence that rhodopsin's chromophore photoisomerizes from an 11-cis to a distorted all-trans configuration. Probably following this, one or more protons of the apoprotein appear to translocate to a new position.

A few years ago, a model was proposed (Honig et al., 1979) to understand how so much of the available light energy could be converted to chemical energy in the primary process. The essential feature of this model was the movement of the positively charged Schiff base through space against an electrostatic field. Specifically proposed was the separation of the positively charged protonated Schiff base moiety from a nearby and presumably hydrogen-bonded negatively charged counterion. This charge separation of a salt bridge was presumed to be the consequence of retinal's photoisomerization. Taking Coulomb's law and assuming reasonable values for the partial charges on the Schiff base and counterion and the separation distance that could occur between the protonated Schiff base and its counterion in an 11-cis to all-trans isomerization, it was shown that a (later observed; Cooper, 1979)

† Work supported by a grant from the National Institutes of Health, EYO 3142.

0006-2960/87/0426-7418$01.50/0

large fraction of the absorbed photon's energy could be converted to chemical energy in terms of this mechanism. There is a great deal of evidence supporting this type of model although no direct measurement, such as X-ray crystallographic studies, of the tertiary structure of rhodopsin and bathorhodopsin exists.

The results of resonance Raman spectroscopy, aside from confirming and characterizing the retinal 11-cis to all-trans photoisomerization, are that the stretching frequency of the protonated Schiff base, —HC=NH^+—, is essentially the same for rhodopsin and bathorhodopsin and their deuteriated derivatives. These results suggest, qualitatively, that the environment of the protonated Schiff base is similar in rhodopsin and bathorhodopsin. This appears to be inconsistent with a simple charge separation model since it might be expected that the protonated Schiff base–counterion charge separation would affect substantially the frequency of the protonated Schiff base stretching frequency.

The goal of this paper is to examine such qualitative concepts more thoroughly, both experimentally and theoretically. To probe whether or not rhodopsin's and bathorhodopsin's Schiff base mode is "accidentally" at the same frequency, we have obtained the resonance Raman spectra of bovine rhodopsin, bathorhodopsin, and isorhodopsin (an artificial chromophore based on the 9-cis isomer of retinal) for a series of isotopically labeled retinals in order to determine the Schiff base frequency. The retinals used in this study are labeled at retinal's protonated Schiff moiety and include —HC=NH^+—, —HC=ND^+—, —$H^{13}C$=NH^+—, and —$H^{13}C$=ND^+—. Apart from the doubly labeled retinal, we find that the protonated Schiff base frequency is the same for both rhodopsin and bathorhodopsin for all these substitutions and for others that have been previously reported. This is in agreement with previous Fourier transform infrared (FTIR) difference absorbance studies by Bagley et al. (1985). In the singly labeled rhodopsin – bathorhodopsin difference spectrum, they observed no bands in the Schiff base region and concluded that rhodopsin and bathorhodopsin have the same Schiff base frequency. These results strongly suggest that the Schiff base mode has the same essential character in both rhodopsin and bathorhodopsin.

In order to assess which factors play a role in determining the position of the protonated Schiff base frequency, we develop a force field that accurately fits the observed ethylenic (C=C) and protonated Schiff base stretching frequencies of rhodopsin and its labeled derivatives. Apart from the doubly labeled derivatives, the protonated Schiff base mode is shown to be fairly well localized to the Schiff base moiety. It is composed principally of C=N stretch and C=N—H bending motions. Thus, the protonated Schiff base frequency and changes upon isotopic labeling of the Schiff base moiety should be sensitive to changes in molecular parameters localized to this end of the retinal chromophore.

Next, using previously developed quantum mechanical procedures, we estimate the changes on our force field produced by moving a counterion away from the protonated Schiff base moiety from 3.0 to 4.5 Å. The 3.0-Å separation corresponds to the salt bridge separation in rhodopsin as given in Honig et al. (1979), and the 1.5-Å change has been calculated (Honig et al., 1976) to be capable of yielding the observed change in λ_{max} in the rhodopsin (λ_{max} = 500 nm) to bathorhodopsin (λ_{max} = 543 nm) phototransition. We find that a protonated Schiff base–counterion charge separation from 3.0 to 4.5 Å would have modest but measurable effects on the frequency of the Schiff base mode. That no change occurs in the Schiff base stretching frequency when rhodopsin transforms to bathorhodopsin thus suggests that the simplest electrostatic model for energy transformation in visual pigments, involving solely a salt bridge separation of the protonated Schiff base with its counterion, is probably incorrect. In view of these results, we discuss various alternative models.

Materials and Methods

Experimental. Bovine rod outer segments were prepared according to the method of Papermaster and Dreyer (1974), and the opsin was prepared according to the method of Ebrey (1982). The ^{13}C-substituted C15 of 11-*cis*-retinal and the regenerated pigment were prepared according to the method of Bagley et al. (1985). This labeled rhodopsin was the generous gift of Prof. K. Nakanishi. Deuteriation of N15 was achieved by concentrating the appropriate visual pigment to a thick paste by centrifugation and resuspension in D_2O, the procedure being twice repeated.

The concentrated pigment was deposited in a concave depression in a temperature-controlled copper sample holder arranged for 90° scattering from the face of the sample as described in Aton et al. (1980). The specimen was mounted in a homemade liquid nitrogen cold finger dewar, and the temperature was maintained at 120 K. We found that at this temperature the Raman spectra of the pigments were identical with spectra taken at 80 K. At the higher temperature, the signal to noise ratio was better because of a significantly decreased fluorescence background.

The resonance Raman spectra were recorded by a computerized optical multichannel analyzer detector and spectrometer system that has been described in Deng et al. (1985). As described in detail by Oseroff and Callender (1974) and Aton et al. (1980), the essential feature of the experimental procedure is to stimulate the Raman scattered light with a fixed single wavelength (the "probe" beam), while simultaneously irradiating the sample with a second spatially overlapping laser beam (the "pump" beam), whose wavelength and power can be varied. At these temperatures, irradiating with visible light drives the sample to a photostationary mixture of rhodopsin, bathorhodopsin, and isorhodopsin. The equilibrium composition is determined by the irradiating light frequency. The probe beam used was the 488-nm line from an argon ion laser (Spectra Physics Inc., Mountain View, CA, Model 165). The pump beams were 568.2-nm and 647.1-nm lines from a krypton ion laser (Coherent Radiation Inc., Palo Alto, CA, Model 2000-CR). For each sample, three Raman spectra were taken. The first was a probe only spectrum; the second was a probe with 568.2-nm pump with a pump/probe ratio of 30:1; the third was a probe with a 647.1-nm pump with a pump/probe ratio of 100:1. This results in a set of three spectra of varying photostationary state rhodopsin, bathorhodopsin, and isorhodopsin mixtures. We have shown previously how the individual Raman spectrum of each species can be obtained provided the sample compositions are known (Aton et al., 1980; Narva & Callender, 1980).

The sample composition of the three pump/probe experimental configurations was determined as follows. The rhodopsin, bathorhodopsin, and isorhodopsin composition has been determined previously by Suzuki and Callender (1981) for single-line excitation at 488.0 nm (and other frequencies). The bathorhodopsin spectrum contains unique marker bands at 856, 877, and 921 cm^{-1}. Thus, the relative amount of bathorhodopsin in the 488.0/568.2 and 488.0/647.1 experiments could be determined by measuring the relative sizes of the bands to the 488.0 nm probe only experiment. The rhodopsin spectrum contains a unique (but small) band at 848 cm^{-1} while

that of isorhodopsin contains one at 832 cm^{-1}. The relative intensity of those two bands was used to estimate the relative concentration of rhodopsin and isorhodopsin. With the approximate concentrations so obtained, the appropriate spectral additions and subtractions yielded spectra that were very close to but not identical with the previously published spectra of bovine rhodopsin, bathorhodopsin, and isorhodopsin. This is probably due to the slight inaccuracies, estimated to be ±5%, in the compositional results of Suzuki and Callender (1981) and in the difficulty of determining accurately the magnitude of the 848- and 832-cm^{-1} bands. However, using this set as a trial set of compositions, we than made small (up to 5%) adjustments in the composition until we obtained the previously published and quite distinctive rhodopsin, bathorhodopsin, and isorhodopsin spectra [see Oseroff and Callender (1974), Mathies et al. (1976), Callender et al. (1976), Erying and Mathies (1979), Aton et al. (1980), and Palings et al. (1987)]. These are given in Figures 1a, 2a, and 3a. The concentrations of the three species, respectively, were found to be 34% (rhodopsin)/50% (bathorhodopsin)/16% (isorhodopsin) in the 488.0-nm experiment, 31/8/63 in the 488.0/568.2 experiment, and 58/18/26 in the 488.0/647.1 experiment. We make the assumption that the photochemical quantum yields of interconversion between the three species are unaffected by isotopic substitution. We thus use the same mixture ratios in calculating the isotopically labeled species spectra. Suzuki and Callender (1981) have shown that Schiff base deuteriation has no effect on the quantum yields.

Computational. The procedures for calculating normal modes have been described previously (Kakitani et al., 1983). The model retinal used in the normal mode calculation was basically the same as before (Kakitani et al., 1983), except that the β-ionine ring was modeled by a ring where carbons 1, 2, 3, 4, and 18 were replaced by heavy atoms with atomic weight of 42, 14, 14, 14, and 15, respectively. The retinal was taken to have an 11-cis isomeric configuration [rather than all-trans, as in Kakitani et al. (1983)], and a 45° and 30° torsional angle was assumed for the C6–C7 and C12–C13 bonds. The set of force constants used here are essentially those of Kakitani et al. (1983), but some were changed to reproduce the experimental results as described below. Changes in force constant that brought about the separation of a counterion from the positively charged protonated Schiff base of retinal were calculated by MINDO/3 methods (Bingham et al., 1975) to obtain quantum mechanically derived force constants (Pulay et al., 1983). The approach here is adapted from the results of Gilson et al. (1988), as described below.

Results

The resonance Raman spectra of rhodopsin, bathorhodopsin, isorhodopsin, and their isotopically labeled derivatives are given in Figures 1–3, respectively. The pure species spectra have been obtained from multispecies spectra as described under Materials and Methods. There are a number of pronounced spectral changes induced by specific mass labeling. We discuss the so-called "fingerprint" region (ca. 1100–1400 cm^{-1}) first and then, of more interest in this paper, the ethylenic–Schiff base region (ca. 1500–1700 cm^{-1}). We then follow this with the results of our theoretical analysis of the ethylenic–Schiff base region.

Fingerprint Spectral Region. The ca. 1100–1400-cm^{-1} region has been called the fingerprint region because of its sensitivity of retinal configuration and terminal end group. The sensitivity to retinal configuration is very evident by contrasting the rhodopsin (Figure 1a, 11-cis isomer), bathorhodopsin

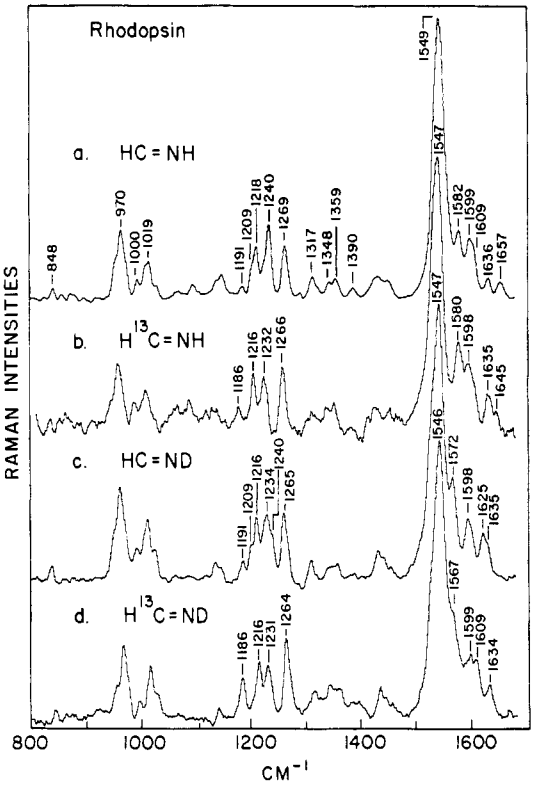

FIGURE 1: Resonance Raman spectra of rhodopsin (a) and its labeled Schiff base moiety derivatives: (b) —$H^{13}C$=NH^+—, (c) —HC=ND^+—, and (d) —$H^{13}C$=ND^+—. The spectra were obtained by "pump-probe" and subtraction procedures described in the text. The resolution was 8 cm^{-1}.

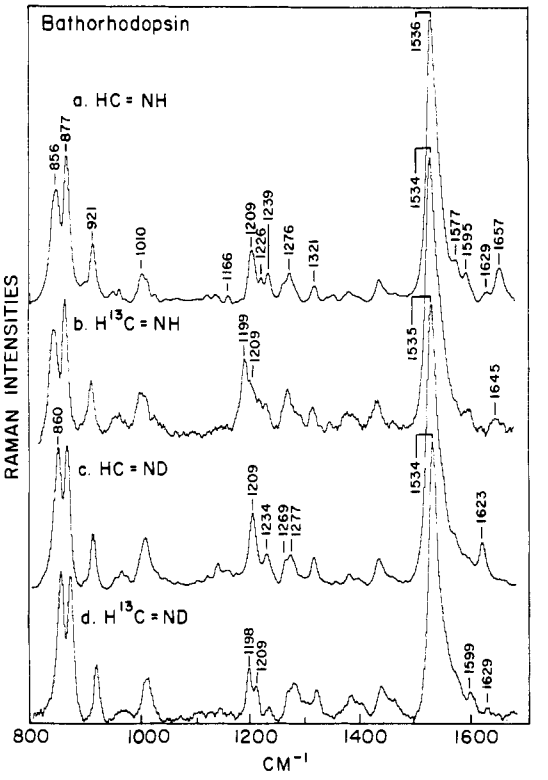

FIGURE 2: Resonance Raman spectra of bathorhodopsin (a) and its labeled Schiff base moiety derivatives: (b) —$H^{13}C$=NH^+—, (c) —HC=ND^+—, and (d) —$H^{13}C$=ND^+—. The spectra were obtained by "pump-probe" and subtraction procedures described in the text. The resolution was 8 cm^{-1}.

(Figure 2a, distorted trans isomer), and isorhodopsin (Figure 3a, 9-cis isomer) data. The bands in the fingerprint region are composed of C–C stretches and C–C–C and C–C–H bends

Table I: C=N Band Positions (cm^{-1}) for Various Protonated Schiff Bases

species	HC=NH	HC=ND	$H^{13}C$=NH	DC=NH	DC=ND	$H^{13}C$=ND
rhodopsin	1657	1625	1645	1648[a]	1610[b]	1609[c]
bathorhodopsin	1657	1623	1645	1648[a]	1602[b]	1599[c]
isorhodopsin	1657	1631	1647	1648[a]	1614[b]	
bacteriorhodopsin[d]	1640	1624	1623			1607
PSB[e] in methanol[b]	1656	1632	1637	1641	1618	1614
PSB[e] in CH_2Cl_2[b]	1653	1632	1632	1639	1618	1612

[a] Data from Raman spectra of Eyring et al. (1982). [b] Data from infrared spectra in Bagley et al. (1985). [c] Assignment unclear; see text for discussion. [d] Data from our unpublished results. [e] PSB stands for protonated Schiff base in retinal.

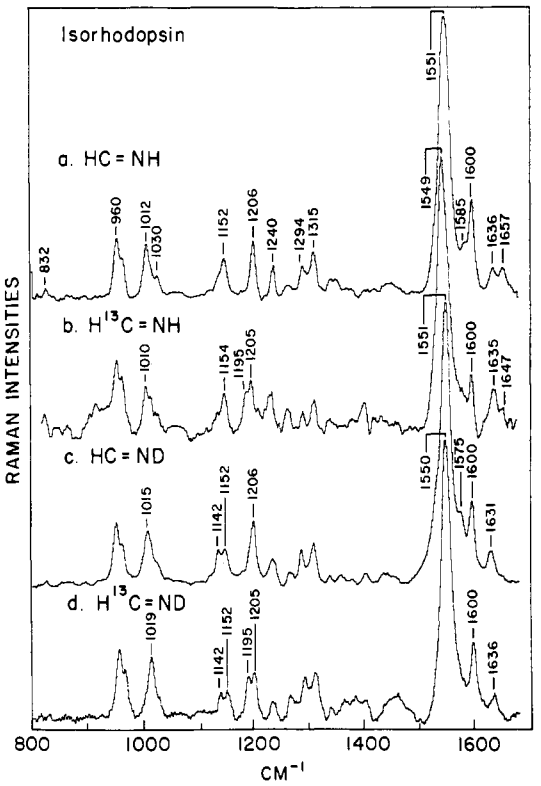

FIGURE 3: Resonance Raman spectra of isorhodopsin (a) and its labeled Schiff base moiety derivatives: (b) —$H^{13}C$=NH^+—, (c) —HC=ND^+—, and (d) —$H^{13}C$=NH^+—. The spectra were obtained by "pump-probe" and subtraction procedures described in the text. The resolution was 8 cm^{-1}.

[for a review, see Mathies et al., (1987)]. Some of the observed bands in this region evidence significant frequency shifts upon labeling of the C15 position and nitrogen deuteriation.

In rhodopsin, the 1191-cm^{-1} band shifts to 1186 cm^{-1}, and part of the 1240-cm^{-1} band appears to shift to 1234 cm^{-1} upon ^{13}C substitution of the C15 position. The shoulder at 1209 cm^{-1} appears to move to 1216 cm^{-1} when C15 is labeled by ^{13}C. A pure C–C stretching mode would shift around 24 cm^{-1} when one ^{12}C carbon is replaced by ^{13}C. Thus, the ca. 6-cm^{-1} shifts in these bands indicate that these modes are delocalized but contain significant motion of the C15 carbon. Likewise, part of the 1240-cm^{-1} band contains significant C–N–H bend as evidenced from its shift to 1234 cm^{-1} upon nitrogen deuteriation. In bathorhodopsin, part of the 1209-cm^{-1} band moves to 1199 cm^{-1} upon ^{13}C substitution, and the 1226-cm^{-1} band appears to disappear upon nitrogen deuteriation. This suggests significant involvement of C15 motion and C–N–H bend in these modes, respectively. In isorhodopsin, a new band apparently appears at 1195 cm^{-1} upon ^{13}C substitution while part of the 1152 cm^{-1} band intensity moves to 1142 cm^{-1} upon nitrogen substitution.

We find it very interesting that simple ^{13}C and D labeling can result in band disappearances or appearances. This suggests to us that retinal's normal modes can involve such a highly coupled pattern of internal coordinates that even a minor change in the mass of one atom may change substantially the character of the observed modes. Our theoretical work below on the ethylenic (C=C) and Schiff base (C=N) vibrational modes also suggests this possibility.

Ethylenic–Schiff Base Region. The C=C and C=N stretching vibrations make the largest contribution to these modes. There are five ethylenic C=C bonds and one C=N Schiff base bond in the conjugated polyene like moiety of retinal protonated Schiff bases so that a total of six bands may, in principle, be observed in the 1500–1700 cm^{-1} region. Six bands are, in fact, observed in the Raman spectrum of rhodopsin (Figure 1a). The 1657-cm^{-1} band has previously been assigned to the protonated Schiff base stretching mode on the basis of its shift to 1625 cm^{-1} upon deuteriation of the protonated Schiff base. This result is seen in Figure 2c. Similarly, the 1657-cm^{-1} band moves to 1645 cm^{-1} in the —$H^{13}C$=NH^+— data given in Figure 1b and to 1609 cm^{-1} in the —$H^{13}C$=ND^+— data of Figure 1d. It is of interest that rhodopsin's 1582-cm^{-1} band (Figure 1a) shifts to 1572 and 1567 cm^{-1} for the —HC=ND^+— and —$H^{13}C$=ND^+— substitutions, respectively. This indicates that this mode contains a substantial amount of C=N stretch or C=N—H bend.

Table I summarizes the position of the Schiff base mode in rhodopsin, bathorhodopsin, and isorhodopsin for the various isotopically labeled pigments that have been measured, both here and elsewhere. We have included results from other studies that have examined the —DC=NH^+— and the —DC=ND^+— pigments, bacteriorhodopsin, and the all-trans protonated Schiff base of retinal in two solvents, methyl alcohol and dichloromethane. The remarkable feature of the data in this table is the essentially identical position of the Schiff base frequency for rhodopsin and bathorhodopsin in the singly labeled derivatives. It is only the doubly labeled (—$H^{13}C$=N^+D— and —DC=ND^+—) pigments where rhodopsin and bathorhodopsin differ in labeling behavior. We shall show below that the band we have identified as the Schiff base mode is almost certainly very delocalized in these doubly labeled derivatives and is no longer appropriately called a "Schiff base" band. The reason for this appears to be that the frequency of the C=N moiety is reduced to such an extent upon double labeling that a great deal of mixing with the ethylenic C=C motions occurs. We presume that the most likely reason for the differences between rhodopsin and bathorhodopsin in the doubly labeled compound results from the different C=C force constants and couplings in the two pigments. The two pigments have quite different λ_{max}'s, for example, arising from a differing extent of π electron delocalization, which would imply a differing force field. Indeed, the positions of the ethylenic modes, at least some of them, are markedly affected by π electron delocalization.

We may contrast the data of rhodopsin and bathorhodopsin with that of the model compounds. All-trans protonated Schiff base in methanol, for example, has a Schiff base frequency

Table II: Calculated Ethylenic–Schiff Base Normal Modes for Rhodopsin and Assignments along Double Bonds[a]

exptl	calcd	C5=C6	C7=C8	C9=C10	C11=C12	C13=C14	C15=N
				HC=NH			
1549	1549		−0.24	−0.21	−0.15		
1582	1583		0.14		−0.10	−0.30	
1599	1598		−0.11	0.28	−0.20		
1609, sh	1606		0.22	−0.11	−0.20	0.17	
1636	1635	−0.38					
1657	1658				−0.09		0.31
				HC=ND			
1547	1548		−0.23	−0.20	−0.17		
1572	1577		0.15	0.11	−0.11	−0.22	−0.16
1598	1595		0.10	−0.24	0.17	−0.20	0.10
	1605		−0.23	0.17	0.13	−0.18	
1625	1625				−0.19		0.27
1635, sh	1637	−0.38					
				$H^{13}C$=NH			
1547	1549		−0.24	−0.21	−0.15		
1580	1582		0.13		−0.09	−0.29	
1598	1597		−0.10	0.28	−0.20	0.10	
1609, sh	1606		−0.23	0.12	0.19	−0.17	
1635	1636	−0.37					
1645	1640				−0.12		0.27
				DC=NH			
1537	1538		−0.13	−0.13	−0.16	−0.25	
	1559		−0.23	−0.18		0.24	
1585							
1599	1597		0.09	−0.28	0.22	−0.09	
	1605		−0.25	0.12	0.21	−0.12	
1634	1637	−0.39					
1548	1655				−0.10		0.30
				$H^{13}C$=ND			
1546	1546		−0.21	−0.19	−0.17		−0.11
1567, sh	1570		0.16	0.14		−0.15	−0.21
1599	1593		0.10	−0.19	0.12	−0.27	0.12
	1604		−0.21	0.22		−0.18	0.09
1609	1613		−0.10		0.27		−0.18
1634	1636	−0.38		0.09			

[a] Data from present Raman results (see Table I and Figures 1–3) and from FTIR data of Bagley et al. (1985). The contributions to each mode by the double bond internal coordinate is the coefficient ($\delta s/\delta Q$) of that coordinate. Only contributions equal to or greater than 0.09 (absolute value) are given. sh stands for shoulder.

at 1656 cm^{-1}, the same as rhodopsin and bathorhodopsin. However, the response of this mode for the protonated Schiff base in methanol in isotopic substitution is very different than those for rhodopsin and bathorhodopsin. Moreover, there are pronounced solvent effects on the Schiff base frequency and its response to labeling when we compare the all-trans protonated Schiff base in polar methanol and nonpolar dichloromethane. Apart from the —HC=ND^+— data, the frequency of the Schiff base mode is different for the two solutions for all the other isotopes.

Force Field Derivation. Our goal in this section is to obtain a reasonable force field that describes the rhodopsin data. We are mainly interested here in the response of the —HC=NH^+— stretching frequency to various isotopic substitutions. Since we expect this mode to couple with ethylenic —C=C— stretches, we obtain a force field that accurately describes these modes and their response to labeling.

The force constant field was obtained by adapting a set we have previously derived (Kakitani et al., 1983). This set of force constants very accurately calculates the frequency of the Schiff base in its change upon deuteriation. However, in order to reproduce the ethylenic frequencies and their changes upon various isotopic substitutions, the following modifications were made. The C=C and C–C stretching force constants were not kept all the same but were changed according to their bond orders obtained by the PPP method (Kakitani et al., 1983, 1985). The C=C,C=C and C–C,C–C coupling constants were decreased from −0.1 to −0.15 mdyn/Å; the C=C,C—C coupling constants were increased from 0.3 to 0.35 mdyn/Å; the C=C,C—C and next nearest stretch–stretch neighbor couplings were increased from 0.0 to 0.15 mdyn/Å. While these minor changes could reproduce the data for native rhodopsin and $^{13}C15$ in H_2O and D_2O very well, the rather large shifts in C=C frequencies observed in cases of deuteron labeling of retinal's carbons (Eyring et al., 1982; Bagley et al., 1985) were not observed. For example, the major rhodopsin ethylenic band shifts downward by 10 cm^{-1} for 15D-substituted rhodopsin; other shifts in the major ethylenic frequency are observed also in 11D, 12D, and 14D rhodopsins (Eyring et al., 1982). These shifts suggest that significant couplings exist between C=C stretching and C–H in-plane bending motions. We introduced these values into our force field and adjusted their values to fit the available data. The complete set of force constants and the normal mode frequencies and associated eigenvectors in the 1500–1700-cm^{-1} range are given in the supplementary material (see paragraph at end of paper regarding supplementary material). The root mean square deviation between the calculated and observed ethylenic and Schiff base frequencies is 2.5 cm^{-1}, and the maximum deviation is 7 cm^{-1}.

Table II shows the experimental band positions in the 1500–1700-cm^{-1} region for rhodopsin and labeled derivatives and the calculated normal mode frequencies. The table also shows the contributions to each mode by the five C=C ethylenic stretching internal coordinates and the C=N Schiff base internal coordinate. The 1636-cm^{-1} mode is little affected

by any of the substitutions and is quite localized to motion of the C5=C6 bond. The other four C=C modes are delocalized to varying degrees along retinal's polyene chain. The Schiff base mode is quite localized in rhodopsin to the Schiff base moiety. However, this mode becomes more delocalized in the isotopically labeled rhodopsins. Roughly, the mode becomes more delocalized as the frequency decreases. Intuitively, this behavior seems correct as coupling between a purely C=N stretch and C=C stretch would increase as their frequencies become more equal. This behavior is demonstrated by comparing the singly and doubly labeled rhodopsins. The "C=N mode" in the singly labeled rhodopsins has a contribution from motion extending to the C11=C12 stretch. In the doubly labeled $H^{13}C$=ND rhodopsin, the mode extends to C7=C8, very highly delocalized indeed. Moreover, all five C=C modes have a significant C=N contribution for the doubly labeled rhodopsins. Although not shown in Table II but contained in the supplementary material, all the ethylenic and C=N modes contain significant C–H or N–H bend.

We wish to emphasize that the force field derived above is not unique. There are a larger number of internal coordinates and related force constants than independent data used for the fitting. For example, we found that a force field differing from the field defined above in the C11=C12, C13=C4, and C15=N stretching force constants by about 10%, the C14–C15,C15=N and C15=N,C13=C14 stretch–stretch coupling by about ~75%, and the C15=N,C=N–H stretch–bend coupling by about 10% would equally well reproduce the experimentally observed frequencies. The exact values for this second force field are given in the supplementary material as are the contributions along the various internal coordinates. While these changes in the force field are not very large, the motions of the normal modes obtained from the second force field can differ substantially from the first force field in many cases. For example, the 1657-cm^{-1} Schiff base mode would be predicted to contain a significant contribution from C13=C14 (–0.13 for C13=C14 and 0.30 for C15=N) with this second force field.

Our purpose here is not to obtain an exact force field. It is unclear to us whether this is even feasible. Our central goal here is to understand how a force field responds to closeby point charge and how, specifically, the frequency of the Schiff base would respond, if at all, upon changes in the position of close point charges. This is accomplished by use of our approximate force field, derived above, in the next section.

Response of Schiff Base Mode to Counterion Position. Recently, Gilson et al. (1988) have applied the MINDO/3 method (Bingham et al., 1975) to calculate quantum mechanical force fields for simple polyenes and protonated Schiff bases. In general, quantum mechanical calculations of force constants are not accurate enough to reproduce observed frequencies to within something like 50%. However, it has been shown that scaling of calculated force constants can lead to accurate force fields for small molecules (Pulay et al., 1983). The approach used in Gilson et al. (1988) was to scale the force constants obtained from the MINDO/3 calculations so as to reproduce the spectra of butadiene. The same scaling factors were used for a number of polyenes, and very good fits were obtained.

Here, we use this approach in a somewhat different manner. The above empirically derived force field for rhodopsin is assumed to correspond to the 11-cis protonated Schiff base of retinal at rhodopsin's active site including any interaction between the chromophore and the apoprotein. We then calculated how this set of constants would respond to specific changes using the following recipe. The chromophore and active site are modeled, so that a force field can be calculated with the MINDO/3 methods of Gilson et al. (1988). Changes in the chromophore–protein interaction are assumed, and the corresponding changes in the MINDO/3-derived force field are calculated. The empirically derived force field is scaled in proportion to the calculated changes, and the new normal mode frequencies are calculated. In this way, we may assess how various changes in interaction between rhodopsin's chromophore and the surrounding protein influence the observed normal modes. We consider here how charges near retinal's Schiff base moiety affect the force field and the Schiff base frequency.

Table III: Effects on Frequency of the C=N Mode by Changing Various Force Constants or Coupling Constants

force constant[a]	change	position (from 1658)	change
total change in $\nu_{C=N}$	3.0 → 4.5 Å	1639	−19
C15=N	8.88 → 8.48	1640	−18
C15=N–H	0.5 → 0.41	1644	−14
C15=N, C15=N–H	−0.15 → −0.13	1654	−4
C15=N, C14–C15	0.5 → 0.6	1654	−4
C15=N, C14–C15=N	0.7 → 1.05	1663	+5
C15=N, C13=C14	−0.15 → −0.25	1660	+2
C15=N, C12–C13	0.15 → 0.25	1662	+4
C14–C15	6.7 → 6.9	1660	+2
C13=C14	8.7 → 8.35	1657	−1

[a] Stretching force constants in mdyn/Å, bending constants in mdyn·Å/rad^2, and stretch–bend cross-terms in mdyn/rad.

Table III shows the results for the following calculation. A positive point charge, presumed to be located 3.0 Å from the nitrogen (center to center) of an 11-cis protonated Schiff base along the direction of the protonating proton, is moved to 4.5 Å from the nitrogen. As discussed below, this molecular arrangement is close to a specific model for light to chemical energy conversion proposed in Honig et al. (1979). The table shows how each force constant is changed, how each change (keeping all the others constant) affects the Schiff base frequency, and how the entire 4.5-Å force field affects the Schiff base frequency.

A number of conclusions are evident from Table III. First, movement of charges close to the Schiff base are calculated to affect the Schiff base frequency in modest but clearly measurable amounts. Also, there are only two force constants that have a major effect on the Schiff base frequency in response to charge movement. Those are the stretching force constant of the Schiff base itself, $K_{C15=N}$, and the bending force constant, $K_{C15=N-H}$. Previous studies of the Schiff base frequency have emphasized the importance of the Schiff base bending force constant on the position of the Schiff base frequency through a strong interaction between the stretching and bending internal degrees of freedom (Aton et al., 1979; Kakitani et al., 1983). The large downward shift of the Schiff base frequency in rhodopsin upon deuteriation has been explained by a much weaker coupling between those two modes when the bending frequency decreases from ca. 1350 to ca. 950 cm^{-1} upon deuteriation. Indeed, the same calculations as performed above, but on a deuteriated Schiff base of retinal, yield only a 4-cm^{-1} shift in the deuteriated Schiff base stretching frequency when the point charge is moved from 3.0 to 4.5 Å due solely to the effect of the C15=N–H coordinate. This is in contrast to the 14-cm^{-1} change calculated for the protonated case. Finally, we find it interesting that, while both the Schiff base stretch and bending coordinates have a sizable negative effect, there is a great deal of cancellation from the

effects of all the remaining internal coordinates.

Discussion

We have measured the Schiff base frequency in rhodopsin and bathorhodopsin for a number of isotopically labeled pigments. Apart from doubly labeled analogues where the Schiff base mode would appear to be highly coupled to other internal coordinates, the Schiff base frequencies of both rhodopsin and bathorhodopsin are the same, within experimental error, for the analogues studies here and in other reports. This strongly suggests that the Schiff base mode is, to a large degree, unaffected in the rhodopsin to bathorhodopsin phototransition. This intuitive conclusion is reinforced by experiments (summarized in Table I) showing that the frequency of the C=N mode of various retinal protonated Schiff bases, for example, the retinal protonated Schiff base in isorhodopsin and in different solvent types, responds differently to isotopic labeling. In order to understand this behavior, we discuss various factors that might influence the frequency of the Schiff base.

Factors That Influence the Schiff Base Frequency. One factor that has little influence of the Schiff base frequency is chromophore isomerization. Various isomers of Schiff bases in solution all show essentially the same Schiff base frequency (Callender & Honig, 1976). This insensitivity to isomerization would appear to result from the localization of the Schiff base mode to the Schiff base moiety.

The delocalization of the chromophore polyene backbone π electrons, which give absorption maxima red shifts, is not sufficient by itself to affect the frequency of the Schiff base. This has been quite nicely demonstrated by the experimental results of Baasov and Sheves (1985). Point charges placed near model protonated Schiff bases but relatively far from the Schiff base moiety showed large red shifts in their λ_{max}, and concomitant changes in the dominant ethylenic C=C frequency but no measurable change in the Schiff base frequency. The shifts in λ_{max} and C=C frequency are clear indications of π electron delocalization. Theoretically, this behavior can be understood in terms of the recent MINDO/3 calculations of Gilson et al. (1988).

The effects on the frequency of the C=N mode of charges placed quite near the Schiff base moiety appear, however, to be significant. As summarized in Table II [and also obtained in the more extensive calculations of Gilson et al. (1988)], there are a number of calculated changes in the force field by moving a charge from 3.0 to 4.5 Å from the Schiff base nitrogen. The effects on the C=N stretch and C=N—H bend stand out, giving about equal contributions to the change in the Schiff base frequency. These calculations are intuitively reasonable. A charge so close to the Schiff base moiety should affect the π and σ electron density of the C=N bond, and one would expect this to result in a change in this force constant and Schiff base frequency for a mode reasonably localized to the C=N moiety. In fact, Baasov and Sheves (1985) have demonstrated this effect experimentally by measurements of a Schiff base analogue where a positive charge located 2 Å from the Schiff base induced a 6-cm^{-1} change in the Schiff base frequency and a concomitant 14-cm^{-1} shift in C=C stretch and 30-nm shift in λ_{max}.

The effect of the C=N—H bend on the Schiff base frequency is also reasonable since a negative charge close to the hydrogen of C=N—H will increase the energy of C=N—H bending compared to a more distant negative charge. This is true because much of the Schiff base positive charge is located on the Schiff base hydrogen. As discussed above, there is a substantial component of C=N—H bend in the Schiff base mode. This size of this component is related to the frequency difference between the C=N—H bending and C=N stretching frequencies (Aton et al., 1980). As the C=N—H frequency approaches the C=N stretch from below, the frequency of the Schiff base mode is increased. Weakening the interaction of a charge with the Schiff base proton by moving the charge away from the proton weakens the interaction energy, lowers the frequency of the C=N—H bend, and thus lowers the frequency of the Schiff base mode. This is the behavior shown in the calculations. This result is borne out by the experimental studies of Sheves et al. (1987) of retinal protonated Schiff bases, which show that the hydrogen-bonding environment of the Schiff base moiety and/or the interaction with nearby charges markedly affect the Schiff base frequency. In fact, a measure of hydrogen bonding appears to be the frequency shift of the Schiff base bond upon deuteriation of the nitrogen. The very large 31-cm^{-1} shift in rhodopsin and bathorhodopsin suggests both these species are strongly hydrogen bonded (Sheves et al., 1987).

Implications for Rhodopsin Photochemistry. It is commonly believed that electrostatic interactions between retinal and the apoprotein opsin are the primary determinants in both pigment color regulation and energy transduction. The simplest picture of energy transduction was given by Honig et al. (1979). The underlying assumption of the picture is that the positively charged chromophore in rhodopsin is stabilized by electrostatic interactions between the chromophore and apoprotein. The photoisomerization of the chromophore in the rhodopsin to bathorhodopsin transition disrupts these interactions, resulting in a high-energy bathorhodopsin species. The specific model included an unspecified opsin negative charge (or arrangement of one or more stabilizing dipoles) salt bridged to the positive Schiff base moiety. Isomerization of the retinal chromophore resulted in breaking the salt bridge and separating these charges. It was shown that resonable molecular parameters could result in sizable energy conversion. However, as we have shown above, it appears that the simple disruption of a salt bridge in the rhodopsin to bathorhodopsin photoreaction should result in a measurable effect on the Schiff base frequency, and none is observed.

In this regard, we should review the reasons to suppose that a through-space motion of the positively charged species against an electrostatic field is involved in the primary photochemistry. In the first place, all visual pigments known contain 11-cis chromophores of protonated Schiff bases of retinal (or a very close analogue like vitamin A_2). This is true across many different species including vertebrates and invertebrates [reviewed in Balogh-Nair and Nakanishi (1982) and Stavenger and Schwemer (1984)]. This is even true, apparently, in UV-absorbing insect pigments. We have recently observed (Pande et al., 1987) a band characteristic of a protonated Schiff base at 1662 cm^{-1} in the spectrum of *Ascalaphus macaronius* rhodopsin. This pigment has a λ_{max} = 345 nm. That the Schiff base linkage in *Ascalaphus* is protonated is remarkable. Color regulation in UV-absorbing pigments would be much more easily accomplished with an unprotonated Schiff base since solution λ_{max} values are around 360 nm while protonated Schiff bases have $\lambda_{max} \approx 440$ nm. This emphasizes the special charged nature of the protonated Schiff base chromophore–opsin linkage. Moreover, an 11-cis to all-trans photoisomerization accompanies the formation of batho products, again across many different species. The protonated Schiff base in rhodopsins would certainly be expected to be energy stabilized by electrostatic mechanisms. Chromophore isomerization would certainly disrupt this stabilization.

Moreover, it is difficult to rationalize how large amounts of light energy can be converted to chemical energy by other physical effects, such as steric interactions and/or strain effects, although it is likely that some, perhaps as much as half, of bathorhodopsin's 35 kcal/mol energy is of this form (Eyring & Methies, 1979; Birge & Hubbard, 1980, 1981; Warshel & Barboy, 1982; Birge et al., 1988). The reason for this is that, while visual pigment primary photochemistry is characterized by an extremely efficient and fast excited state process with little or no energy barrier (Doukas et al., 1984), the very opposite is true regarding electronic ground-state properties. The ground-state energy barrier between rhodopsin and bathorhodopsin is on the order of 45 kcal/mol (Honig et al., 1979; Cooper, 1979; Doukas et al., 1984), very large indeed. Strain and/or steric effects do not particularly discriminate between ground and excited electronic states (Warshel & Barboy, 1982). If anything, such factors would be expected to introduce somewhat larger barriers in the excited state than in the ground state. On the other hand, excitation of a retinal protonated Schiff base to its π^* excited state results in a very marked decrease in the Schiff base moiety positive charge found in the ground state (Birge & Hubbard, 1980; Birge et al., 1988). Thus, rotations about retinal's C11–C12 double bond, which would be hindered in the ground state if the positively charged protonated Schiff is electrostatically stabilized, are not particularly hindered in the excited state from such interactions (Birge & Hubbard, 1980).

Assuming that electrostatic factors play a dominant role in the energy transduction process, how may we rationalize that the Schiff base mode is not measurably affected by the rhodopsin to bathorhodopsin photoreaction. Lacking specific structural information concerning the local protein environment of the chromophore, as might be obtained in crystallographic studies, it is not possible to work out detailed models. There are, however, a number of possibilities. In the first place, the data strongly suggest that the Schiff bases of both rhodopsin and bathorhodopsin are strongly hydrogen bonded. The frequencies of both Schiff base bands are high, and more to the point, both exhibit large frequency shifts upon Schiff base deuteriation. As mentioned above, this behavior is strongly correlated with strong hydrogen bonding.

Given this, there are two possibilities, broadly speaking, for the electrostatic interaction between chromophore and apoprotein stabilizing rhodopsin relative to bathorhodopsin. In the first case, one can imagine that the Schiff bases of both rhodopsin and bathorhodopsin are hydrogen bonded by an unknown group or groups. The apoprotein superimposes an additional electrostatic field to stabilize rhodopsin's structure, which does not affect the Schiff base frequency in the rhodopsin to bathorhodopsin phototransition. For example, it has been proposed (Gilson et al., 1988; Honig, 1988) that a large relatively constant electric field in the vicinity of the Schiff base could be produced by α-helix dipoles. Models of rhodopsin's tertiary structure based on sequence data suggest that the helix containing the retinal may be disrupted, and this can lead to large local fields (Honig, 1988). Photoisomerization that occurs during the rhodopsin to bathorhodopsin photoreaction would then result in the motion of the positive Schiff base against a substantial, but relatively constant field. Motion through a relatively constant field would have little effect on the Schiff base frequency as is implied in the experimental and theoretical work described above. Another example within this framework has been suggested in Birge et al. (1988). Here a specific negatively charged counterion is envisioned that lies below the chromophore in such a way that the major electrostatic interactions between it and the positive charged chromophore have to do with retinal's C15 and C13 carbons and *not* the Schiff base moiety. Like the case of a relatively constant electric field, the effect on the Schiff base frequency when rhodopsin isomerizes to bathorhodopsin could be minimal. The hydrogen bonding that rhodopsin's Schiff base experiences may be broken and then re-formed with a new group when bathorhodopsin is formed; or only a single hydrogen-bonding group is involved, and this follows the Schiff base movement. This could happen if there are water molecules at the active site. In this view, the hydrogen-bonding patterns have little to do with energetics but are essential determinants in the Schiff base frequency.

Another model, consistent with the data, is that the counterion of rhodopsin's protonated Schiff base is some charged species that forms a salt bridge. Upon photoisomerization, the salt bridge is disrupted, and the Schiff base of bathorhodopsin forms a hydrogen bond with some neutral group. In both rhodopsin and bathorhodopsin, the C=N—H bending mode frequency would be increased because of the local interaction between the Schiff base proton and either the charged protein moiety in rhodopsin or say dipole groups in bathorhodopsin. As described above, this leads to a higher C=N frequency and large deuteriation effect, which could mask effects on the C=N frequency brought about by movement against an electrostatic field. It is clear that many specific possibilities exist and that further studies, particularly protein structural work, are needed before any detailed understanding of chromophore–apoprotein interaction is possible.

Supplementary Material Available

Three tables listing complete force field coordinates and constants and the calculated frequencies and assignments for the ethylenic modes for force fields I and II (5 pages). Ordering information is given on any current masthead page.

References

Aton, B., Doukas, A., Narva, D., Callender, R., Dinur, U., & Honig, B. (1980) *Biophys. J. 29*, 79–94.

Baasov, T., & Sheves, M. (1985) *J. Am. Chem. Soc. 107*, 7524–7533.

Bagley, V., Balogh-Nair, V., Croteau, A., Dollinger, T., Ebrey, L., Eisenstein, M., Hong, K., Nakanishi, K., & Vittitow, J. (1985) *Biochemistry 24*, 6055–6071.

Balogh-Nair, V., & Nakanishi, K. (1982) in *New Comprehensive Biochemistry* (Tamm, C., Ed.) Vol. 3, pp 283–334, Elsevier Biomedical, Amsterdam.

Bingham, R. C., Dewar, M. J. S., & Lo, D. H. (1975) *J. Am. Chem. Soc. 97*, 1285–1293.

Birge, R. R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 315–354.

Birge, R. R., & Hubbard, L. M. (1980) *J. Am. Chem. Soc. 102*, 2195–2204.

Birge, R. R., & Hubbard, L. M. (1981) *Biophys. J. 34*, 517–534.

Birge, R. R., Einterz, C. M., Knapp, H. M., & Murray, L. P. (1988) *Biophys. J.* (in press).

Callender, R., & Honig, B. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 33–55.

Callender, R., Doukas, R., Crouch, R., & Nakanishi, K. (1976) *Biochemistry 15*, 1621–1629.

Cooper, A. (1979) *Nature (London) 282*, 531–533.

Cooper, A., Dixon, S., & Tsuda, M. (1986) *Eur. Biophys. J. 13*, 195–201.

Deng, H., Pande, C., Callender, R., & Ebrey, T. (1985) *Photochem. Photobiol. 41*, 467–470.

Doukas, A. G., Junnarkar, M. R., Alfano, R. R., Callender,

R. H., Kakitani, T., & Honig, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4790-4794.
Ebrey, T. (1982) *Methods Enzymol. 88*, 516-528.
Eyring, G., & Mathies, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 33-37.
Eyring, G., Curry, B., Broek, A., Lugtenburg, J., & Mathies, R. (1982) *Biochemistry 21*, 384-393.
Gilson, H., Honig, B., Croteau, A., Zarrilli, G., & Nakanishi, K. (1988) *Biophys. J.* (in press).
Honig, B. (1988) in *Biophysical Studies of Retinal Proteins* (Ebrey, T., Fraunfelder, H., Honig, B., & Nakanishi, K., Eds.) University of Illinois Press, Urbana-Champaign, IL (in press).
Honig, B., Greenberg, A. D., Dinur, U., & Ebrey, T. G. (1976) *Biochemistry 15*, 4593-4599.
Honig, B., Ebrey, T., Callender, R., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503-2508.
Kakitani, H., Kakitani, T., Rodman, B., Honig, B., & Callender, R. (1983) *J. Phys. Chem. 87*, 3620-3628.
Mathies, R., Oseroff, A. R., & Stryer, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1-5.
Mathies, R., Smith, S. O., & Palings (1987) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 2, pp 59-109, Wiley, New York.
Narva, D., & Callender, R. (1980) *Photochem. Photobiol. 32*, 273-276.
Oseroff, A. R., & Callender, R. (1974) *Biochemistry 13*, 4243-4248.
Ottolenghi, M. (1980) *Adv. Photochem. 12*, 97-200.
Palings, I., Pardoen, J. A., Van der Berg, E., Winkel, C., Lugtenburg, J., & Mathies, R. A. (1987) *Biochemistry 26*, 2544-2556.
Pande, C., Deng, H., Rath, P., Callender, R. H., & Schwemer, J. (1987) *Biochemistry* (following paper in this issue).
Papermaster, S., & Dreyer, J. (1974) *Biochemistry 13*, 2438-2444.
Pulay, P., Fogorasi, G., Ponger, G., Boggs, J. E., & Vargha, A. (1983) *J. Am. Chem. Soc. 105*, 7037-7041.
Schick, G., Holloway, R. A., Cooper, T. M., Murry, L. P., & Birge, R. R. (1987) *Biochemistry 26*, 2556-2562.
Sheves, M., Baasov, T., & Friedman, N. (1987) *Biochemistry 26*, 3210-3217.
Stavenger, D. G., & Schwemer, J. (1984) in *Photoreception and Vision in Invertebrates* (Ali, M. A., Ed.) pp 11-61, Plenum, New York.
Suzuki, T., & Callender, R. (1981) *Biophys. J. 34*, 261-265.
Warshel, A., & Barboy, N. (1982) *J. Am. Chem. Soc. 104*, 1469-1476.

Resonance Raman Spectroscopy of an Ultraviolet-Sensitive Insect Rhodopsin†

C. Pande,‡ H. Deng,‡ P. Rath,‡ R. H. Callender,*,‡ and J. Schwemer§

Physics Department, City College of New York, New York, New York 10031, and Institute of Zoophysiology, Ruhr-Universität, 4630 Bochum 1, FRG

Received April 14, 1987; Revised Manuscript Received July 17, 1987

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

† This work was supported in part by National Institutes of Health Grant EYO 3142 (R.C.) and by the Deutsche Forschungsgemeinschaft through a Heisenberg Stipendium and SFB 114 (J.S.).
‡ City College of New York.
§ Ruhr-Universität.

0006-2960/87/0426-7426$01.50/0