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Structure of the Retinal Chromophore in 7,9-*dicis*-Rhodopsin†

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ABSTRACT: Bovine rhodopsin was bleached and regenerated with 7,9-*dicis*-retinal to form 7,9-*dicis*-rhodopsin, which was purified on a concanavalin A affinity column. The absorption maximum of the 7,9-dicis pigment is 453 nm, giving an opsin shift of 1600 cm^{-1} compared to 2500 cm^{-1} for 11-*cis*-rhodopsin and 2400 cm^{-1} for 9-*cis*-rhodopsin. Rapid-flow resonance Raman spectra have been obtained of 7,9-*dicis*-rhodopsin in H_2O and D_2O at room temperature. The shift of the 1654-cm^{-1} C=N stretch to 1627 cm^{-1} in D_2O demonstrates that the Schiff base nitrogen is protonated. The absence of any shift in the 1201-cm^{-1} mode, which is assigned as the C_{14}–C_{15} stretch, or of any other C–C stretching modes in D_2O indicates that the Schiff base C=N configuration is trans (anti). Assuming that the cyclohexenyl ring binds with the same orientation in 7,9-*dicis*-, 9-*cis*-, and 11-*cis*-rhodopsins, the presence of two cis bonds requires that the N–H bond of the 7,9-dicis chromophore points in the opposite direction from that in the 9-cis or 11-cis pigment. However, the Schiff base $C=NH^+$ stretching frequency and its D_2O shift in 7,9-*dicis*-rhodopsin are very similar to those in 11-*cis*- and 9-*cis*-rhodopsin, indicating that the Schiff base electrostatic/hydrogen-bonding environments are effectively the same. The C=N trans (anti) Schiff base geometry of 7,9-*dicis*-rhodopsin and the insensitivity of its Schiff base vibrational properties to orientation are rationalized by examining the binding site specificity with molecular modeling.

Vertebrate visual pigments contain an 11-*cis*-retinal chromophore bound via a protonated Schiff base linkage to a specific lysine residue of the ~41 000-dalton apoprotein opsin (Birge, 1981). The absorption maxima of these pigments range from 440 to 580 nm (Lythgoe, 1972). The amino acid sequences of a number of opsins (Hargrave et al., 1983; Nathans et al., 1986; Ovchinnikov, 1982) have made it possible to identify protein perturbations that may be responsible for this broad range of absorption maxima (Kosower, 1988; Loppnow et al., 1989; Nathans et al., 1986). The primary event in vision is an isomerization around the C_{11}=C_{12} bond of the chromophore to form a twisted all-trans photoproduct (Eyring et al., 1980; Hubbard & Kropf, 1958; Kandori et al., 1989b; Yoshizawa & Wald, 1963). Although bathorhodopsin had been thought to be the first intermediate, several reports have indicated that there is an intermediate prior to bathorhodopsin (Kobayashi, 1980; Peters et al., 1977; Shichida et al., 1984), and Kandori et al. (1989a) and Shichida et al. (1984) have identified photorhodopsin as the first one-photon photoproduct. The specific protein–chromophore interactions which dictate

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the absorption maximum and the fast photochemistry are still poorly understood.

The "opsin shift" is the shift of the absorption maximum of the 11-*cis*-retinal protonated Schiff base (PSB)[1] chromophore from ~440 nm in methanol to 498 nm in the protein. Chemical analogue studies argued that the opsin shift of the 11-*cis*-retinal chromophore in rhodopsin is due to interaction with a negatively charged amino acid residue near C_{13} (Arnaboldi et al., 1979; Honig et al., 1979a; Koutalos et al., 1989). This idea has been supported by recent solid-state NMR studies of pigments containing ^{13}C-labeled retinals (Lugtenburg et al., 1988; Smith et al., 1990) that show chemical shift perturbations of the chromophore at C_{13}. Site-directed mutagenesis studies of bovine rhodopsin have indicated that the protonated Schiff base counterion is Glu-113 but have been unable to identify a second protein charge perturbation responsible for the opsin shift (Nathans, 1990; Sakmar et al., 1989; Zhukovsky & Oprian, 1989). Interestingly, the strength of the protonated Schiff base–counterion interaction does not change with the absorption maximum of the pigments that have been studied to date (Barry & Mathies, 1987; Barry et al., 1987; Loppnow et al., 1989; Palings et al., 1987), although a weak Schiff base–counterion interaction has been found to account for most of the opsin shift in an analogous retinal-containing protein, bacteriorhodopsin (de Groot et al., 1989; Lugtenburg et al., 1986; Spudich et al., 1986).

The bathorhodopsin photoproduct has an enthalpy content 32–36 kcal/mol higher than rhodopsin (Cooper, 1979; Schick et al., 1987). This is nearly 60% of the energy of the absorbed photon. Theoretical studies have investigated the possibility of electrostatic energy storage through changes in the protonated Schiff base–counterion interaction and of energy storage via conformational distortion of the chromophore (Birge et al., 1988; Honig et al., 1979b; Warshel & Barboy, 1982). Experimentally, the resonance Raman spectrum of bathorhodopsin has unusually intense hydrogen out-of-plane wagging vibrations in the 800–920-cm^{-1} region which indicate that the chromophore is conformationally distorted (Eyring et al., 1980) and perturbed near C_{12} (Eyring et al., 1982; Palings et al., 1989). The Schiff base configuration and environment in bathorhodopsin are effectively identical with those in rhodopsin (Eyring & Mathies, 1979; Palings et al., 1987), indicating that a significant fraction of the ~34 kcal/mol is not stored through electrostatic interactions between the protonated Schiff base and its counterion.

The goal of this study is to use dicis chromophores to probe the binding site of rhodopsin and learn more about the mechanism of energy storage and of the opsin shift. Resonance Raman (Palings et al., 1987) and FTIR (Bagley et al., 1985) spectroscopies have been used to show that 11-*cis*-rhodopsin, 9-*cis*-rhodopsin, and *all-trans*-bathorhodopsin all contain a C=N trans (anti), protonated Schiff base linkage to the protein. If the *dicis*-retinal binds with retention of configuration and if the cyclohexenyl ring binds in the same orientation as in 11-*cis*- and 9-*cis*-rhodopsin, a C=N syn (cis) configuration is expected. Additionally, if there is a stereochemically specific interaction involved in protonation and hydrogen bonding with the Schiff base, the C=N syn chromophore should exhibit little or no perturbations in its Schiff base properties from those of 11-*cis*-rhodopsin. Of the four *dicis*-rhodopsin analogues (7,9-dicis, 7,13-dicis, 9,11-dicis, and 9,13-dicis) whose retinal configuration has been rigorously established through chromophore extraction experiments (Shichida et al., 1988a; Trehan et al., 1990a), only the 7,9-dicis analogue was found to have completely retained the original retinal configuration. This isomer has, therefore, been selected for this study of the configuration of the imine bond in a dicis pigment.

Previous studies have shown the utility of resonance Raman spectroscopy for determining protein–chromophore interactions and the C=N configuration in visual pigments (Loppnow et al., 1989; Palings et al., 1987, 1989) and in bacteriorhodopsin (Mathies et al., 1987; Smith et al., 1984). In this paper, we present resonance Raman spectra of 7,9-*dicis*-rhodopsin in H_2O and D_2O which indicate that the Schiff base configuration and environment are the same as those found in the monocis pigments. These results are understood by comparison with molecular graphics models and binding site specificity results.

Experimental Procedures

Preparation of 7,9-dicis-Retinal. The *dicis*-retinal used in this study was obtained as a byproduct in a modified C_{15} + C_5 route designed for stereoselective synthesis of the bis-hindered retinal isomers (Trehan et al., 1990b). The isomer was isolated by preparative HPLC (column, 10 × 25 mm 5μ-Lichrosorb; solvent, 5% diethyl ether in hexane) and characterized by comparison of the ^1H NMR spectrum with that published by Liu and Asato (1984).

Preparation of Protonated Schiff Base. The 7,9-*dicis*-retinal Schiff base was synthesized by reaction of ~0.25 mg of retinal with a 30-fold excess of *n*-butylamine in 1 mL of methanol at room temperature for 1.5 h. The solvent and excess *n*-butylamine were evaporated under a stream of nitrogen, and the residue was dissolved in 9.5 mL of methanol. The solution was titrated with ~50 μL of a HCl-acidified methanol solution until a stable 7,9-dicis PSB was formed as assayed by absorption spectroscopy.

Preparation of Bovine Rod Outer Segments. Bovine rod outer segments (ROS) were isolated from fresh retinas by a sucrose flotation method (Shichida et al., 1988b) and suspended in 10 mM HEPES buffer (pH 7.0). The ROS suspension was irradiated in the presence of 100 mM hydroxylamine with yellow light from a 1-kW projector lamp (Sanko) passing through a Toshiba VO-54 cutoff filter, resulting in complete bleaching of rhodopsin to retinal oxime and opsin. The ROS thus obtained were washed eight times with HEPES buffer to remove unreacted hydroxylamine and peripheral protein. After lyophilization, the ROS were washed seven times with petroleum ether to remove retinal oxime and lipids and then resuspended in HEPES buffer.

Preparation of 7,9-dicis-Rhodopsin. Unlike the other dicis isomers, incubation of 7,9-*dicis*-retinal with bovine opsin produces only 7,9-*dicis*-rhodopsin (Shichida et al., 1988a; Trehan et al., 1990a). The ROS suspension obtained above was mixed with an ~2-fold excess of 7,9-*dicis*-retinal dissolved in ethanol and incubated for 24 h at 20 °C to produce 7,9-*dicis*-rhodopsin. After being washed eight times with 10 mM HEPES buffer (pH 7.0), the ROS were lyophilized, treated with petroleum ether, and solubilized in a 2% digitonin buffer. The solubilized 7,9-*dicis*-rhodopsin sample was divided into two parts for preparation of deuterated and undeuterated samples. In order to purify the 7,9-*dicis*-rhodopsin sample completely, the 2% digitonin extract containing the 7,9-*dicis*-rhodopsin was applied to a concanavalin A–Sepharose 4B affinity column that had been equilibrated with 10 mM HEPES buffer (pH 7.0) supplemented with 2% digitonin, 140 mM NaCl, and 1 mM $CaCl_2$. After thorough washing of the column bed with the buffer and with buffer supplemented with

[1] Abbreviations: FTIR, Fourier transform infrared; PSB, protonated Schiff base; HOOP, hydrogen out-of-plane; ROS, rod outer segments; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

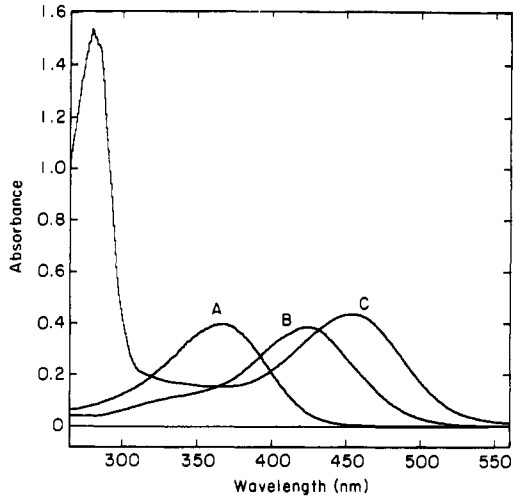


FIGURE 1: Room-temperature absorption spectra of 7,9-*dicis*-retinal in methanol (A), 7,9-dicis protonated Schiff base in methanol (B), and 7,9-*dicis*-rhodopsin (C). The λ_{max} of the 7,9-dicis aldehyde was at 366 nm. The shoulder at ~340 nm in the 7,9-dicis protonated Schiff base spectrum did not decrease upon further titration with acidified methanol and may be a higher lying cis-band electronic transition.

10 mM hydroxylamine, the 7,9-*dicis*-rhodopsin was eluted with buffer supplemented with 100 mM methyl α-mannoside. The 7,9-*dicis*-rhodopsin sample in D_2O was obtained by a similar method with a deuterated 10 mM HEPES buffer.

Resonance Raman Spectroscopy. Room-temperature, rapid-flow resonance Raman spectra of 7,9-*dicis*-rhodopsin were obtained with 10-mL samples having an absorbance of 0.4–1.0 OD/cm at 453 nm (100 mM methyl α-mannoside, 140 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, <10 mM NH_2OH·HCl, 2% digitonin, pH 7.0). Raman scattering was excited by spherically focusing (focal length 60 mm) the argon ion laser beam in the 0.8 mm diameter capillary containing the flowing rhodopsin solution. Multichannel detection of the scattering was accomplished with a cooled intensified vidicon detector coupled to a double spectrograph as described previously (Eyring et al., 1980). Spectral slit widths were 5.0–6.0 cm^{-1}. The laser power (300–400 μW), flow rate (300 cm/s), and beam waist (15 μm) were chosen to minimize the effects of photolysis on the Raman spectra (photoalteration parameter $F < 0.1$; Mathies et al., 1976). Rapid-flow Raman spectra of the 7,9-*dicis*-PSB were similarly obtained using a 488-nm probe. The spectrometer was calibrated with cyclohexene and dicyclopentadiene as external standards. Frequencies are accurate to ±1 cm^{-1}.

Computational Methods. The geometries and normal modes of the 7,9-dicis PSB, 9-cis PSB, and 11-cis PSB were obtained from a calculation according to the QCFF/PI method (Warshel & Karplus, 1974). The chromophore was truncated by replacing the δ-carbon of the lysine group with a group that has a mass of 15, a valence of 1, and the potential parameters of an sp^3 carbon.

Results

Absorption spectra of the 7,9-*dicis*-retinal, 7,9-dicis protonated Schiff base (PSB), and 7,9-*dicis*-rhodopsin are shown in Figure 1, and the absorption maxima and opsin shifts for the 9-cis, 11-cis, and 7,9-dicis chromophores are summarized in Table I. The absorption maximum of 7,9-*dicis*-rhodopsin is significantly blue-shifted (λ_{max} 453 nm) compared to those of 9-*cis*-rhodopsin (483 nm) and 11-*cis*-rhodopsin (498 nm). However, the 7,9-dicis protonated Schiff base (λ_{max} 423 nm) is only slightly blue-shifted compared to the 9-cis PSB (433 nm) in methanol. Thus, the opsin shift for 7,9-*dicis*-rhodopsin

Table I: Absorption Maxima and Opsin Shifts[a]

isomer	absorption max (nm) PSB	pigment	opsin shift (cm^{-1})
11-cis[b]	442	498	2500
9-cis[b]	433	483	2400
7,9-dicis	423	453	1600

[a] Absorption maxima (nm) and opsin shifts (cm^{-1}) for the 11-cis, 9-cis, and 7,9-dicis PSB and pigment. The absorption maxima are for the PSB in methanol and the pigment in aqueous buffer (100 mM methyl α-mannoside, 140 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, <10 mM NH_2OH·HCl, 2% digitonin, pH 7.0). [b] These data are from Fukada et al. (1990).

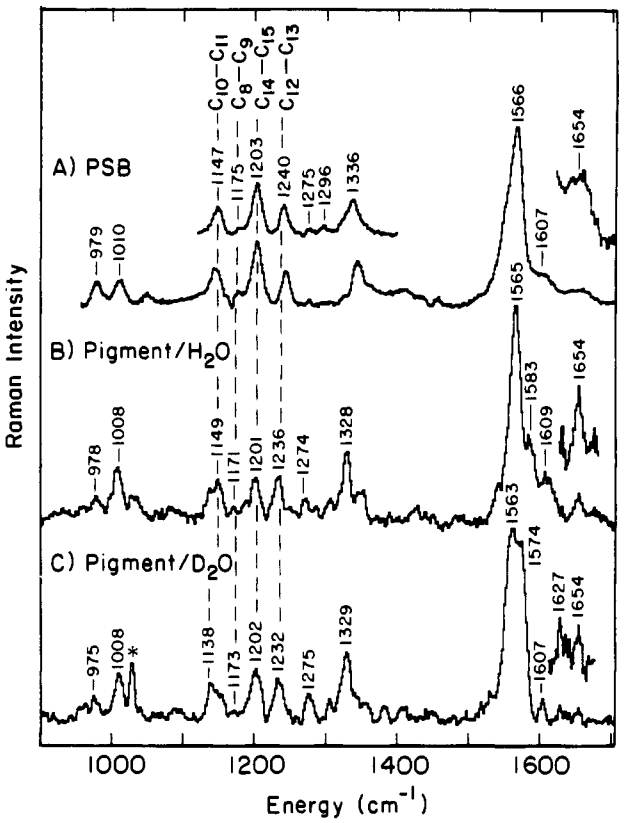


FIGURE 2: Rapid-flow resonance Raman spectra of the 7,9-dicis protonated Schiff base in methanol (A) and 7,9-*dicis*-rhodopsin in H_2O (B) and D_2O (C). The PSB spectrum was excited with 600 μW at 488 nm. Resonance Raman spectra of 7,9-*dicis*-rhodopsin were obtained with 360–550 μW of 457.9-nm excitation. The inset in (A) shows another spectrum of the fingerprint region with new spectrograph settings to shift the detector artifact seen at ~1160 cm^{-1} in the full spectrum. The Schiff base region of each spectrum has also been blown up in the insets. The asterisk indicates a laser plasma line.

is less (~1600 cm^{-1}) than the opsin shift for either 11-*cis*-rhodopsin (2500 cm^{-1}) or 9-*cis*-rhodopsin (2400 cm^{-1}).

Resonance Raman spectra of the 7,9-dicis PSB in methanol and the 7,9-*dicis*-rhodopsin in H_2O and D_2O are shown in Figure 2. The similarity of the PSB and pigment spectra indicates that we are indeed forming 7,9-*dicis*-rhodopsin. If the sterically crowded C_7=C_8 cis bond had isomerized upon formation of the pigment, the 1328-cm^{-1} Raman band should shift down to 1316 cm^{-1}, which is the frequency of a strong line in 9-*cis*-rhodopsin. Additionally, the Raman spectrum of 9-*cis*-rhodopsin has a hydrogen out-of-plane (HOOP) mode at 961 cm^{-1} (Mathies et al., 1976), whereas in 7,9-*dicis*-rhodopsin and in the 7,9-dicis PSB a HOOP mode appears at 978 cm^{-1}.

We can use these Raman data to characterize the Schiff base of the 7,9-dicis pigment. The shift of the 1654-cm^{-1}

Table II: Calculated Ethylenic Mode Frequencies and Intensities

	all-trans		7,9-dicis		9-cis	
mode	H_2O	D_2O	H_2O	D_2O	H_2O	D_2O
+−000	1651 (0.01)	1651 (0.01)	1647 (0.02)	1647 (0.02)	1650 (0.03)	1650 (0.03)
C=N	1636 (0.23)	1621 (0.15)	1638 (0.25)	1623 (0.15)	1637 (0.18)	1622 (0.12)
++−−0	1592 (0.06)	1592 (0.00)	1592 (0.21)	1592 (0.30)	1589 (0.04)	1588 (0.09)
++++0	1590 (1.0)	1590 (1.0)	1602 (1.0)	1602 (1.0)	1596 (1.0)	1595 (1.0)
++−++	1548 (0.10)	1547 (0.05)	1551 (0.09)	1551 (0.06)	1550 (0.12)	1549 (0.08)
00+−+	1525 (0.12)	1520 (0.07)	1527 (0.20)	1521 (0.12)	1525 (0.14)	1519 (0.09)

[a] Calculated with the QCFF/PI method for *N*-retinylidenemethylamine. Frequencies are in cm^{-1}. Numbers in parentheses are the intensities relative to the in-phase stretch combination and are obtained from the calculated Δ's by assuming intensity $\propto \Delta^2$. The modes are categorized according to the phases of the $C_5{=}C_6$, $C_7{=}C_8$, $C_9{=}C_{10}$, $C_{11}{=}C_{12}$, and $C_{13}{=}C_{14}$ stretches in the normal modes, and they are listed in this order. "0" indicates no significant projection of the normal mode on that particular double-bond stretch.

C=N stretch to 1627 cm^{-1} in D_2O shows that the pigment contains a protonated Schiff base linkage. The residual intensity at 1654 cm^{-1} in the D_2O spectrum is probably an additional C=C stretching mode. This band was not present in the spectrum of bleached rhodopsin in the same buffer solution, suggesting that it is not due to laser plasma lines or scattering from the opsin protein or buffer. We tested the extent of deuteration by recording the Raman spectra in the 2000–3800-cm^{-1} region. The OH and OD symmetric stretches appear at ~3450 and ~2700 cm^{-1}, respectively. By comparing the Raman spectra of the bleached D_2O and bleached H_2O samples, we determined that the buffer was 90–95% deuterated, ruling out the possibility that the line at 1654 cm^{-1} in the D_2O spectrum is caused by H_2O contamination. The rapid bleaching of the 7,9-dicis pigment by hydroxylamine, indicating that small molecules can easily get to the Schiff base, argues that there was sufficient time and accessibility for complete exchange of the Schiff base proton.

The single-bond stretching modes in 7,9-*dicis*-rhodopsin can be assigned on the basis of previously determined 9-*cis*-rhodopsin assignments (Palings et al., 1987). In 9-*cis*-rhodopsin, the C_{10}–C_{11}, C_8–C_9, C_{14}–C_{15}, and C_{12}–C_{13} single-bond stretches are assigned to the 1154-, 1206-, 1206-, and 1242-cm^{-1} Raman bands, respectively; the C_8–C_9 and C_{14}–C_{15} stretches are degenerate at 1206 cm^{-1}. In 7,9-*dicis*-rhodopsin, the only difference in chromophore structure is the change from a 7-trans to a 7-cis configuration. A 20–30-cm^{-1} downshift is expected in C–C stretch modes that are adjacent to a cis double bond (Curry et al., 1985). Thus, most of the modes in the 7,9-dicis pigment should be identical with those of 9-*cis*-rhodopsin. However, the C_8–C_9 stretch should shift down 20–30 cm^{-1} from its position in 9-*cis*-rhodopsin (Palings et al., 1987). The 1149, 1201, and 1236-cm^{-1} Raman bands of the 7,9-dicis pigment exhibit frequencies and intensities similar to those of the strong fingerprint bands of 9-*cis*-rhodopsin and are thus assigned as the C_{10}–C_{11}, C_{14}–C_{15}, and C_{12}–C_{13} stretching modes, respectively. In all of the spectra in Figure 2 a weak line is observed at ~1173 cm^{-1} that is not observed in 9-*cis*-rhodopsin (Palings et al., 1987). Since this band is ~30 cm^{-1} lower than the 1206-cm^{-1} C_8–C_9 stretch in 9-*cis*-rhodopsin, we assign the 1173-cm^{-1} band to the C_8–C_9 stretch of the 7,9-dicis pigment.

Previous Raman studies have shown that the sensitivity of the C_{14}–C_{15} stretching frequency to D_2O exchange can be used to determine the C=N configuration (Palings et al., 1987; Smith et al., 1984). In the C=N cis (syn) geometry, coupling between the N–H rock and the C_{14}–C_{15} stretch is removed upon deuteration of the protonated Schiff base nitrogen, and the C_{14}–C_{15} stretch shifts up by ~60 cm^{-1}. In the C=N trans (anti) geometry this coupling is absent, and the C_{14}–C_{15} stretching frequency is insensitive to deuteration of the protonated Schiff base nitrogen. There is no shift in the 1201-cm^{-1} C_{14}–C_{15} stretch nor any large shift of *any* fingerprint modes upon deuteration of the protonated Schiff base nitrogen in 7,9-*dicis*-rhodopsin (compare spectra B and C in Figure 2). Thus, the C=N configuration in 7,9-*dicis*-rhodopsin is trans (anti).

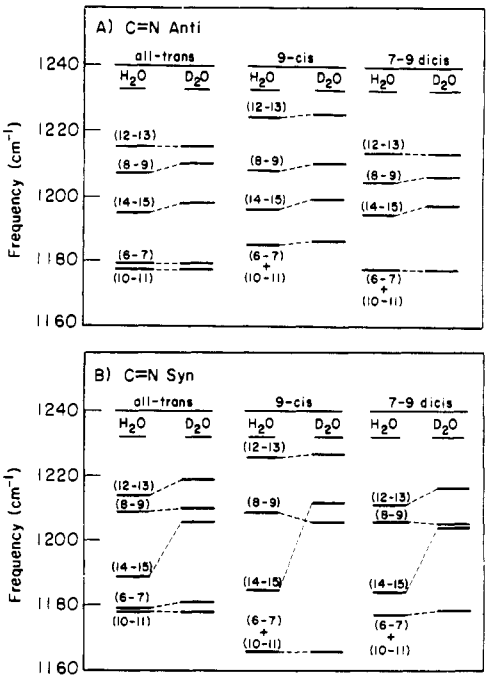
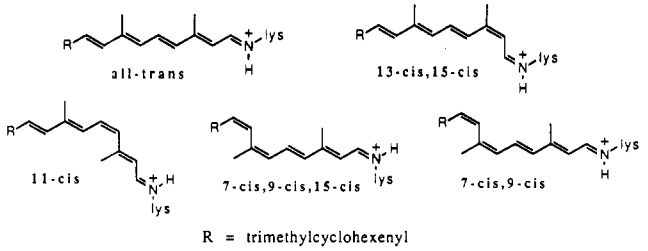


FIGURE 3: Calculated single-bond stretching modes in *all-trans*-, 9-*cis*-, and 7,9-*dicis*-*N*-retinylidenemethylamine for the C=N syn and anti configurations in H_2O and D_2O.

The intensity of the 1275-cm^{-1} mode increases in the D_2O spectrum, raising the possibility that this is an upshifted C_{14}–C_{15} stretch that has little intensity in the H_2O spectrum. This possibility is unlikely because of the following: (1) the C_{14}–C_{15} stretching mode at ~1200 cm^{-1} in 9-*cis*-rhodopsin is very strong (Palings et al., 1987), and its intensity and frequency should not change significantly in the 7,9-dicis isomer; (2) as discussed above, the intense 1201-cm^{-1} mode in 7,9-*dicis*-rhodopsin is at exactly the frequency expected for the C_{14}–C_{15} stretch; (3) in the 7,9-dicis pigment no other skeletal stretching mode is expected at this frequency. Thus, we believe that the majority of the intensity at 1201 cm^{-1} is due to the C_{14}–C_{15} stretching mode and that the lack of a shift of this mode in D_2O indicates a C=N anti chromophore. The increase of the 1275-cm^{-1} mode intensity in D_2O may be due to a shift of the rocking modes upon deuteration.

To test these assignments, QCFF/PI calculations were performed on the 9-*cis*-, *all-trans*-, and 7,9-*dicis*-retinal-*n*-butylamine protonated Schiff bases. The results are presented in Table II and Figure 3. QCFF/PI calculations predict the

Chart I



antisymmetric combination of the C_5=C_6 and C_7=C_8 stretches at a higher frequency than that of the C=N stretch. This mode is insensitive to deuteration of the protonated Schiff base and may account for the residual intensity at 1654 cm^{-1} in the D_2O spectrum of 7,9-*dicis*-rhodopsin. The unusual observation of these high-wavenumber ethylenic modes probably results from a combination of the blue excitation wavelength and the novel electronic properties of the dicis chromophores. Blue excitation will result in better resonance enhancement of the higher wavenumber out-of-phase C=C stretch combination modes, which derive more of their intensity from the higher lying "cis band" electronic transitions. The enhancement of these high-wavenumber C=C modes with blue excitation has been seen in studies on rhodopsin and isorhodopsin (Palings et al., 1987) and in resonance Raman microscope experiments on 440-nm absorbing pigments (Loppnow et al., 1989). This effect will be even more important in the dicis pigments because the two cis bends will shift more oscillator strength into the cis-band transitions.

In the single-bond stretching region (see Figure 3), QCFF/PI predicts that the C_{14}–C_{15} stretch will shift upon deuteration of the protonated Schiff base nitrogen only if the C=N configuration is cis (syn) and that this shift is independent of the configuration around the 7- and 9-double bonds. Furthermore, these calculations show that the C_{14}–C_{15} stretch is the only band which shifts significantly when the chromophore has the C=N cis (syn) configuration. Thus, the QCFF/PI calculations support our conclusion that the 7,9-*dicis*-retinal chromophore is connected to the protein by a C=N trans (anti) linkage.

Discussion

The primary purpose of this study is to characterize the structure and protein–chromophore interactions in 7,9-*dicis*-rhodopsin, a representative isomeric rhodopsin analogue containing a stable dicis geometry (Shichida et al., 1988a; Trehan et al., 1990b). Of particular interest is the C=N geometry. For the bacteriorhodopsin system, it has been shown by resonance Raman (Smith et al., 1984) and by ^{13}C NMR (Harbison et al., 1984) that the 13-cis isomer (BR-548) contains the C=N syn rather than the C=N anti geometry found in the trans isomer (BR-568). An obvious advantage of the doubly bent dicis chromophore in BR-548 is the identical orientation of the iminium nitrogen with that found in the all-trans BR-568 chromophore, making possible interaction of the two chromophores with an identical counterion from the protein (see Chart I). By analogy, one would expect that a dicis isomeric pigment of rhodopsin might have a triply bent C=N syn geometry with the orientation of the iminium nitrogen identical with that in the singly bent C=N anti chromophore of 11-*cis*- and 9-*cis*-rhodopsin. The present study shows the unanticipated result that the Schiff base geometry in 7,9-*dicis*-rhodopsin is anti.

One possible rationalization for the observed results can be found in a recent molecular modeling study of anchored

FIGURE 4: Structures of the C=N trans (anti) (—) and C=N cis (syn) (– –) anchored chromophores of 7,9-*dicis*-rhodopsin overlaid with that of 11-*cis*-rhodopsin (···). The C_α carbons, corresponding to the last atom of the appended pentyl groups, are rigidly superimposed. The rings and the remaining atoms were fitted according to the procedure described in Liu and Mirzadegan (1988). The ring of the longer C=N anti chromophore of 7,9-*dicis*-rhodopsin can be readily overlapped with that of the 11-cis chromophore, but the same cannot be achieved for the shorter C=N syn chromophore. The positions of the iminium nitrogen atoms are marked by dark circles.

isomeric rhodopsin chromophores (Liu & Mirzadegan, 1988). In this study, structures of the 16 geometric isomers of the retinyl iminium chromophore, appended with a butyl side chain and an additional carbon corresponding to C_α of Lys-296, were constructed by utilization of minimized structures of retinal isomers from MMP2-85 calculations. The distances between C_α's and the centers of cyclohexenyl rings of the isomeric pigments were calculated. The triply bent C=N syn isomer of the 7,9-dicis analogue has a longitudinal distance (16.04 Å) significantly shorter than that of rhodopsin (17.75 Å) while that of the C=N anti isomer is slightly longer (18.39 Å). Conformational adjustment of the butyl group shortened this critical distance for the C=N anti structure, thus allowing near total overlap of its cyclohexenyl ring with that of the 11-*cis*-rhodopsin while that of the C=N syn structure is considerably displaced (see Figure 4). Also, the position of the iminium nitrogen of the C=N syn structure is more displaced from that of the 11-cis isomer. The implication is that the C=N syn isomer is incompatible with the position of either the hydrophobic pocket for the cyclohexenyl ring or the counterion.

This molecular modeling analysis as well as the earlier work of Liu and Mirzadegan (1988) implicitly assumes that the cyclohexenyl ring always binds in the same hydrophobic pocket. While this cannot be proven, it is justified by the following observations. First, competitive binding experiments show that there is a selective binding site for the trimethylcyclohexenyl ring, and β-ionone is a competitive inhibitor for rhodopsin regeneration (Matsumoto & Yoshizawa, 1975; Maeda et al., 1979). Second, low-temperature spectroscopy shows that the similarly sterically crowded 7-*cis*-rhodopsin has native-like intermediates with normal transition temperatures (Kawamura et al., 1980). These experiments indicate that there is a specific cyclohexenyl ring binding pocket which binds the ring in a common orientation even when it is sterically crowded.

It is of interest to speculate on the exact nature of the interaction of the iminium nitrogen with the counterion, which has been recently identified as Glu-113 (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). The Hargrave and related models suggested that the counterion is likely to be oriented perpendicularly to the plane of the chromophore, thus relatively insensitive to the exact orientation of the iminium ion (Dratz & Hargrave, 1983). Recent measurements and molecular orbital simulations of the low-temperature photochemistry of 9-*cis*-rhodopsin suggest that the counterion sits underneath the C=NH^+ plane (Birge et al., 1988), and it has also been

suggested that water acts as the hydrogen-bond acceptor for the protonated Schiff base (Birge et al., 1988; Rafferty & Shichi, 1981). This picture allows involvement of a specific hydrogen-bond acceptor with the iminium hydrogen oriented in either direction. Compare, for example, the N–H orientations of the 11-cis, C=N anti and the 7,9-dicis, C=N anti chromophores in Figure 4. This picture further implies that the environment at the Schiff base end of the chromophore in rhodopsin, 7,9-*dicis*-rhodopsin, or even 9-*cis*-rhodopsin is similar, in agreement with the observation that the C=N stretching frequency and its shift in D_2O for the three isomeric rhodopsins are within 4 cm^{-1} of each other. This may also explain why the Schiff base properties of bathorhodopsin are so unperturbed (Eyring & Mathies, 1979; Palings et al., 1989).

The fingerprint region of the Raman spectra of 7,9-*dicis*-rhodopsin may be indicative of protein–chromophore interactions that are different from those in 9-*cis*-rhodopsin. In 9-*cis*-rhodopsin, the C_{10}–C_{11} and C_{14}–C_{15} single-bond stretching frequencies are observed at 1154 and 1206 cm^{-1}, respectively, shifted from the 9-cis protonated Schiff base frequencies seen at 1137 and 1189 cm^{-1}, respectively (Palings et al., 1987). This shift was interpreted as evidence for a protein perturbation near C_{13}. Since the vibrational intensities and frequencies of the 7,9-dicis pigment are similar to those of the 9-cis pigment, a charge perturbation near C_{13} may be expected to produce analogous band shifts. However, much smaller shifts are observed in the 1149-cm^{-1} C_{10}–C_{11} and 1203-cm^{-1} C_{14}–C_{15} stretching frequencies for 7,9-*dicis*-rhodopsin (see Figure 2A,B). The similarity of the 7,9-dicis PSB and 7,9-*dicis*-rhodopsin single-bond stretching frequencies is analogous to what was seen for the 9-cis isomer of a 440 nm absorbing pigment in the toad green rod (Loppnow et al., 1989). This suggests that the postulated interaction between C_{13} and a charged group is reduced or missing in 7,9-*dicis*-rhodopsin, possibly accounting for its lower opsin shift.

Chromophore extraction after irradiation of another *di-cis*-rhodopsin, 9,13-*dicis*-rhodopsin, has shown that it undergoes a one-photon, one double bond isomerization (Shichida et al., 1988b). Whether the same process occurs for the more hindered 7,9-dicis isomer remains to be answered. The 7-cis geometry is known to be sterically crowded with a C_5–C_8 dihedral angle estimated to be about 40° for molecules in solution (Liu et al., 1983). On the other hand, the photosensitivity of the hindered 7-*cis*-rhodopsin has been shown to be lower than those of the less crowded 11-cis and 9-cis isomers (Kawamura et al., 1980), possibly reflecting the greater importance of protein structure over the intrinsic chromophore property of steric crowding in affecting efficiency of photoisomerization. Nevertheless, it will be of interest to examine the dynamics of 7,9-*dicis*-rhodopsin following photoexcitation by low-temperature and time-resolved absorption and resonance Raman spectroscopies.

In conclusion, the resonance Raman spectra of 7,9-*dicis*-rhodopsin in H_2O and D_2O have been obtained. The spectra demonstate that the chromophore is a protonated Schiff base with a C=N trans Schiff base configuration. Preference for the latter configuration can be rationalized by binding site specificity in terms of the position of the hydrophobic binding pocket for the cyclohexenyl ring and/or the position of the counterion.

Registry No. 7,9-*dicis*-Retinal, 56085-53-1; *all-trans-N*-retinylidenemethylamine (anti), 27898-36-8; 9-*cis-N*-retinylidenemethylamine (anti), 27898-38-0; *all-trans-N*-retinylidenemethylamine (syn), 128657-30-7; 9-*cis-N*-retinylidenemethylamine (syn), 128657-31-8; 7,9-*dicis-N*-retinylidenemethylamine (anti), 128657-32-9; 7,9-*dicis-N*-retinylidenemethylamine (syn), 128657-33-0.

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