The Schiff Base Bond Configuration in Bacteriorhodopsin and in Model Compounds†

N. Livnah and M. Sheves*

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: The Schiff base linkage bond configuration of bacteriorhodopsin was studied using model compounds consisting of *all-trans*- and 13-*cis*-retinal-protonated Schiff bases bearing C=N anti and syn bond configurations. The C=N configuration was analyzed using a combination of Fourier transform infrared spectroscopy and isotopically labeled chromophores. It was found that, in the model compounds, the coupling between the C_{14}—C_{15} stretching frequency and the N—H rock is weak in the *all-trans*-retinal-protonated Schiff base in both the anti and syn C=N configurations. However, this coupling is relatively strong in the 13-*cis*-retinal-protonated Schiff base in both the anti and syn C=N configurations. Thus, it is concluded that, in model compounds, the C_{14}—C_{15} mode can serve as a marker for the C_{13}=C_{14} bond configuration but not for the C=N. A different situation may prevail in bacteriorhodopsin due to different conformations of the retinal chromophore in the protein binding site and in solution. This difference suggests that the C_{14}—C_{15}/NH coupling in retinal-protonated Schiff bases is affected by the retinal conformation.

All retinylidene proteins discovered to date are composed of a retinal chromophore bound via a protonated Schiff base to the ϵ-amino group of a lysine residue. These proteins can be divided into two major classes. First, those associated with the process of vision that are characterized as the rhodopsins, and second, the proteins called the bacterial rhodopsins, which were initially discovered in the bacterium *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971). The bacterial pigment, called bacteriorhodopsin (bR[1]), found in the purple membrane of *Halobacterium halobium* contains an all-trans retinal chromophore, whereas the configuration of the retinal in visual pigments is 11-cis.

The photocycle of bacteriorhodopsin is associated with a series of spectroscopically distinguishable ground-state intermediates denoted as J, K, L, M, N, and O. Following light absorption, the retinal chromophore undergoes a primary, all-trans → 13-cis isomerization (Tsuda et al., 1980; Pande et al., 1981; Braiman & Mathies, 1982; Itsieh et al., 1983), inducing protein conformational changes which lead to proton translocation across the membrane. In the dark, bR_{568} (light-adapted bR) converts to a dark-adapted form, which consists of a 60:40 mixture of 13-cis and all-trans protonated Schiff base chromophores (bR_{548} and bR_{568}, respectively) (Scherrer et al., 1989). Isomerization around the C=N bond of the protonated Schiff base linkage can be a key factor in determining the Schiff base proton orientation in the pigment and in the photocycle and might be involved in the direction of the proton movement.

Using resonance Raman spectroscopy, it was suggested (Smith et al., 1984) that bR_{568}, as well as the photochemically induced intermediates, consists of the anti C=N configuration. The suggestion was based on normal mode calculations which demonstrated that, when the retinal Schiff base is in the syn C=N configuration, the C_{14}—C_{15} stretch and the NH rock are strongly coupled. Thus, large upshifts of the C_{14}—C_{15} stretch should be observed following deuteration of the Schiff base linkage. The coupling is much weaker in the anti C=N configuration, and therefore, a much smaller upshift of the C_{14}—C_{15} stretching frequency is predicted following deuteration. The anti configuration in bR_{568} and the syn in bR_{548} were further supported by ^{13}C NMR based mainly on analysis of the chemical shift of C_{14} (Harbison et al., 1984).

In the work for this article, we studied model compounds bearing syn and anti C=N configurations of retinal-protonated Schiff bases and examined the coupling between the C_{14}—C_{15} stretching frequency and the NH rock. It was found that, in the model compounds, the coupling between the C_{14}—C_{15} stretching frequency and the NH rock is very weak in the all-trans isomer, both in the syn and anti configurations. However, the coupling is significant in the 13-cis isomer in both the anti and syn C=N configurations. Thus, in the model compound the C_{14}—C_{15} frequency band can serve as a marker band for the all-trans and 13-cis configurations, rather than for the C=N configurations.

MATERIALS AND METHODS

The ^{13}C-labeled retinal and the 1,1-desdimethylretinal derivatives were synthesized according to previously described methods (Lugtenburg, 1985; Friedman et al., 1989). All-trans and 13-cis retinals (Sigma) were used without further purification. Butylamine, ethanolamine, and methylamine were distilled prior to use. The chromophores were condensed with the corresponding amine in trifluoroethanol. The solvent was evaporated and the residue (Schiff base) was redissolved in chloroform. All of the FTIR measurements were carried out in chloroform solutions using a 0.6×10^{-2} M chromophore concentration. Schiff base protonation was achieved by titration with HCl fumes until the absorption was fully shifted from 360 to 450 nm. Similar FTIR spectra were obtained using an HCl solution (37% in H_2O). The similar results indicate that water does not interact with the chromophore or affect the spectra. The PSB deuteration was performed by addition of DCl (37% in D_2O, Sigma) to the protonated sample

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* Author to whom correspondence should be addressed.

[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; RPSB, retinal-protonated Schiff base; PSB, protonated Schiff base.

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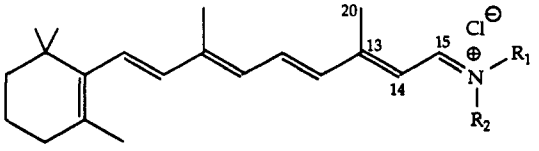
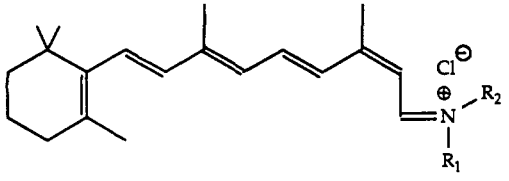
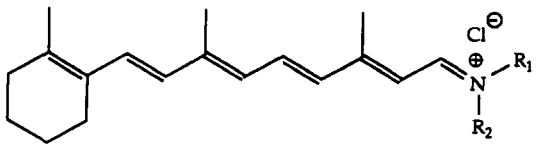
Chart I



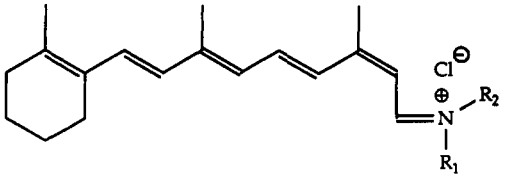
1a R_1 = n-butyl R_2 = H
1b R_1 = H R_2 = n-butyl
2a R_1 = CH_3 R_2 = H
2b R_1 = H R_2 = CH_3
3a R_1 = CH_2CH_2OH R_2 = H
3b R_1 = H R_2 = CH_2CH_2OH



4a R_1 = n-butyl R_2 = H
4b R_1 = H R_2 = n-butyl
5a R_1 = CH_3 R_2 = H
5b R_1 = H R_2 = CH_3
6a R_1 = CH_2CH_2OH R_2 = H
6b R_1 = H R_2 = CH_2CH_2OH



7a R_1 = n-butyl R_2 = H
7b R_1 = H R_2 = n-butyl
8a R_1 = CH_3 R_2 = H
8b R_1 = H R_2 = CH_3



9a R_1 = n-butyl R_2 = H
9b R_1 = H R_2 = n-butyl
10a R_1 = CH_3 R_2 = H
10b R_1 = H R_2 = CH_3

until full deuteration was detected by FTIR. FTIR spectra were obtained using a Nicolet 510 spectrometer. NMR spectra were measured on a Bruker AMX 400-MHz instrument. Absorption measurements were performed using an HP 8452A diode array spectrophotometer.

RESULTS AND DISCUSSION

To evaluate the extent of C_{14}—C_{15} stretching frequency coupling with the NH rock in the anti and syn C=N configurations, we looked for systems that would adopt these configurations. *all-trans*-Retinal was condensed with *n*-butylamine, methylamine, and ethanolamine, and the corresponding Schiff bases were protonated with HCl to give retinal-protonated Schiff bases (RPSB) **1a,b**, **2a,b**, and **3a,b**, respectively.

The syn and anti configurations of a retinal PSB can be analyzed by ^{1}H NMR and clearly distinguished by their different chemical shifts and *J*-splittings, as previously suggested (Pattaroni & Lauterwein, 1981). The two isomers differ mainly in the chemical shift of $C_{15}H$, in its *J*-splitting with the N-H, and in the chemical shifts of $C_{20}H$. It was found (Figure 1) that both **1a,b** (butylamine derivative) and **3a,b** (ethanolamine derivative) RPSB consist of 90% anti configuration (**1a** and **3a**), while in **2a,b** (methylamine derivative) a 1:1 mixture of the two isomers is formed (Figure 2). This difference in isomer ratio allows for the analysis of both anti and syn isomers. The coupling between the C_{14}–C_{15} stretching frequency and the NH rock was evaluated by exchanging the proton attached to the Schiff base linkage by a deuteron and comparing the C_{14}–C_{15} stretching frequency in both cases.

Figure 3 presents the FTIR spectra of RPSB **1a,b** (Figure

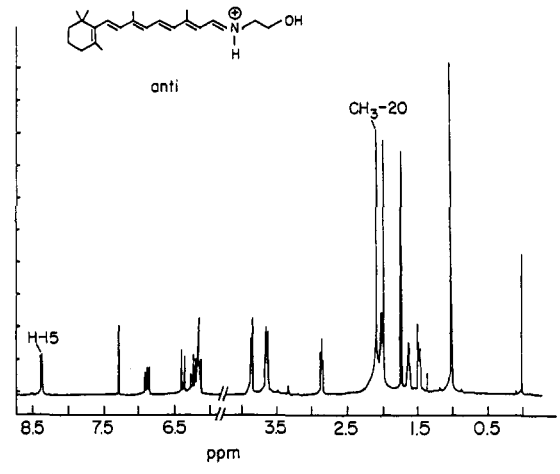


FIGURE 1: ^{1}H NMR spectrum in chloroform of *all-trans*-retinal-protonated Schiff base derivative **3a,b** (ethanolamine).

3A) and **2a,b**, (Figure 3C) and the same compounds with exchange of the Schiff base proton for a deuteron (Figure 3B,D, respectively). The fingerprint region of a RPSB in resonance Raman spectroscopy was assigned using selective labeling with carbon-13 (Smith et al., 1984, 1987). According to this assignment, in all-trans RPSB, the band at 1193 cm^{-1} consists of a combination of the C_{14}–C_{15} single bond, whose frequency is 1191 cm^{-1}, and C_8–C_9, whose frequency is 1204 cm^{-1}. Other single bonds were assigned at 1238 (C_{12}–C_{13}) and 1160 cm^{-1} (C_{10}–C_{11}). It is clearly seen from Figure 3 that no significant shifts are observed in the 1193 cm^{-1} band upon deuteration of the all-trans RPSB for neither the anti nor the syn isomer (Table I).

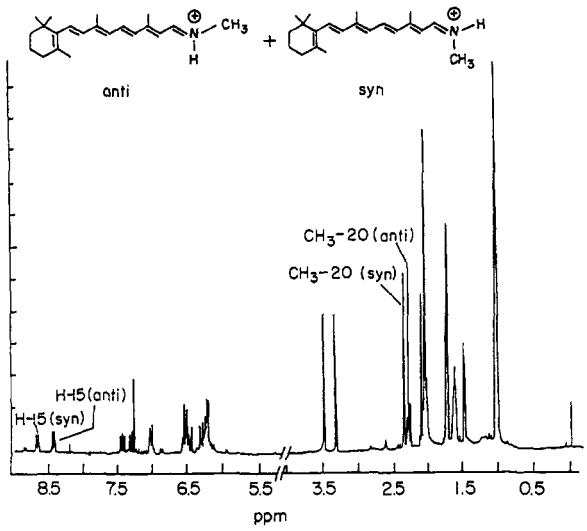


FIGURE 2: ^{1}H NMR spectrum of all-trans RPSB **2a,b** (in chloroform) consisting of a mixture of C=N anti and syn isomers.

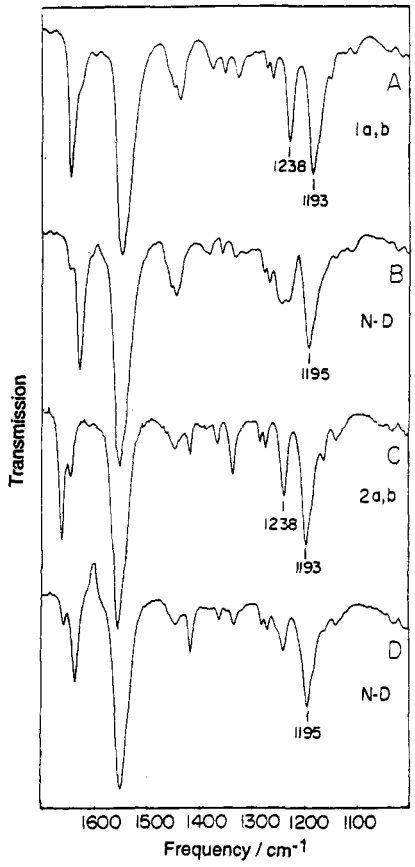


FIGURE 3: FTIR spectra of *all-trans*-retinal-protonated Schiff base derivatives: (A) **1a,b**; (B) N–D derivative of A; (C) **2a,b**; (D) N–D derivative of C.

To further support the assignment of the 1193 cm^{-1} band, we prepared an *all-trans*-retinal in which the C_{14}—C_{15} bond was labeled with ^{13}C at both the C_{14} and C_{15} carbons. Figure 4 presents the FTIR spectra of labeled RPSB derived from ethanolamine (**3a,b**) and methylamine (**2a,b**). It is clearly evident that the band at 1193 cm^{-1} (Figure 3A–C), which was assigned to consist of a combination of C_{14}—C_{15} and C_8—C_9, splits. Due to the ^{13}C labeling, the main component shifted down to 1170 cm^{-1} for the ethanolamine (which consists mainly of anti isomer; Figure 4A) and to 1177 cm^{-1} for the methylamine PSB (which consists of a 1:1 mixture syn and anti isomers; Figure 4C), leaving a small peak at 1199 cm^{-1}.

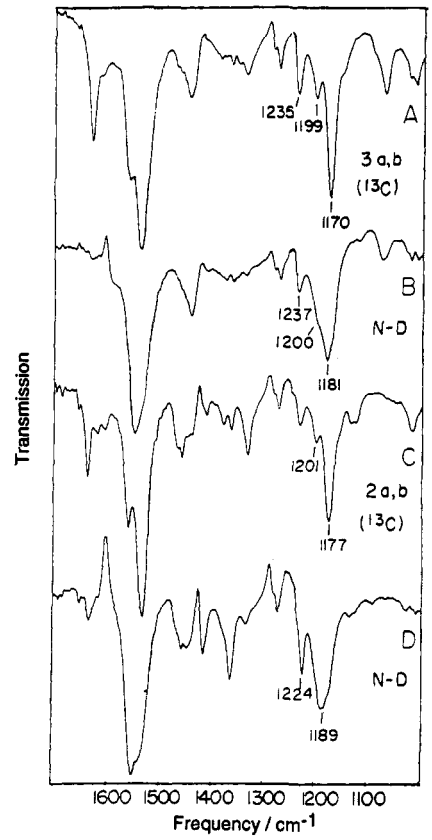


FIGURE 4: FTIR spectra of *all-trans*-retinal-protonated Schiff base derivatives doubly labeled with ^{13}C at the C_{14} and C_{15} carbons: (A) **3a,b**; (B) N–D derivative of A; (C) **2a,b**; (D) N–D derivative of C.

Following deuteration, the 1170 and 1177 cm^{-1} peaks move up by about 11 cm^{-1} to 1181 and 1189 cm^{-1}, respectively (Figure 4B,D). The ^{13}C labeling effect clearly shows that the band at 1193 cm^{-1} (Figure 3A,C) mainly consists of C_{14}—C_{15} stretching frequency. The insensitivity of the 1193 cm^{-1} band to the exchange of the Schiff base proton for a deuteron in both **1a,b** and **2a,b** indicates that the coupling between the C_{14}—C_{15} stretching frequency and the NH rock is very small in both the anti and syn configurations in all-trans RPSB (Table I). We note that substitution of the C_{14}—C_{15} by ^{13}C increases the C_{14}—C_{15}/NH coupling, as reflected in the upshift movement of the 1170 cm^{-1} band (Figure 4A) by 11 cm^{-1} (Figure 4B) following deuteration of the Schiff base linkage. A similar shift occurs in the syn C=N configuration, as is evident from the movement of the 1177 cm^{-1} band (consisting of both anti and syn isomers; $^{13}C_{14}$—$^{13}C_{15}$ stretching frequency) to 1189 cm^{-1} following deuteration, due to a similar upfield shift of both superimposed peaks of the anti and syn isomers.

To evaluate the coupling between the C_{14}–C_{15} stretching frequency and the NH rock of the 13-cis isomer of RPSB, we condensed 13-*cis*-retinal with butylamine, methylamine, and ethanolamine and protonated the resulting Schiff bases to obtain **4a,b**, **5a,b**, and **6a,b**, respectively. ^{1}H NMR spectra indicate that, similar to all-trans RPSB, butylamine and ethanolamine derivatives of 13-cis RPSB (**4** and **6**) consist of 90% anti C=N configuration, whereas the methylamine derivative **5** consists of a 1:1 mixture. Figure 5 presents the FTIR spectra of **6a,b** and **5a,b** (Figure 5A,C) and their Schiff base deuterated species (Figure 5B,D).

The band at 1168 cm^{-1} was assigned, using resonance Raman spectroscopy (Smith et al., 1984, 1987), as a combination of C_{14}—C_{15} stretching, whose frequency is 1176 cm^{-1}, and

Table I: C_{14}—C_{15} Stretching Frequencies (cm^{-1}) of Protonated Schiff Bases

compound	C_{14}—C_{15} (NH)	C_{14}—C_{15} (ND)	$^{13}C_{14}$—$^{13}C_{15}$ (NH)	$^{13}C_{14}$—$^{13}C_{15}$ (ND)
retinal				
all-trans, C=N anti (**1a**)	1193	1195	1170	1181
all-trans, C=N syn (**2b**)	1193	1195	1177	1189
13-cis, C=N anti (**6a**)	1168	1200	1146	1181
13-cis, C=N syn (**5b**)	1168	1184	1154	*a*
1,1-dedimethylretinal				
all-trans, C=N anti (**7a**)	1190	1192		
all-trans, C=N syn (**8b**)	1191	1195		
13-cis, C=N anti (**9a**)	1168	1194		
13-cis, C=N syn (**10b**)	1170	*a*		

[a] Compounds **5a,b** and **10a,b** consist of a 1:1 mixture of anti and syn isomers, each of which undergoes a different C_{14}—C_{15} band shift upon deuteration. Therefore, the C_{14}—C_{15} stretching frequency in the ND form of those compounds is a broad band, centered at 1181 and 1200 cm^{-1} for **5a,b** and **10a,b**, respectively.

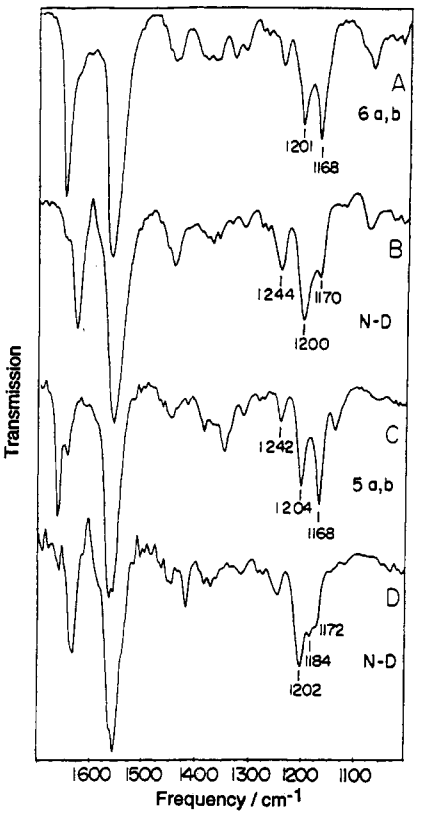


FIGURE 5: FTIR spectra of 13-*cis*-retinal-protonated Schiff base derivatives: (A) **6a,b**; (B) N–D derivative of A; (C) **5a,b**; (D) N–D derivative of C.

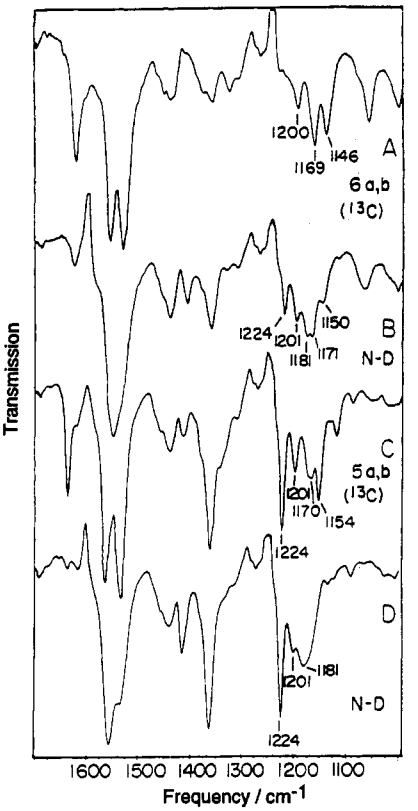


FIGURE 6: FTIR spectra of 13-*cis*-retinal-protonated Schiff base derivatives doubly labeled with ^{13}C at the C_{14} and C_{15} carbons: (A) **6a,b**; (B) N–D derivative of A; (C) **5a,b**; (D) N–D derivative of C.

C_{10}—C_{11} at 1167 cm^{-1}. C_{12}—C_{13} was assigned at 1137 cm^{-1} and C_8—C_9 at 1201 cm^{-1}. Therefore, the effect of exchanging the Schiff base proton for a deuteron should be observed at the 1168 cm^{-1} band for the 13-cis RPSB. Figure 5 reveals that in the 13-cis RPSB the C_{14}—C_{15} stretching shifts are observed in both the anti and syn isomers. The anti isomer in the 13-cis RPSB (**6a**) shifts from 1168 to 1200 cm^{-1} upon deuteration (Figure 5A,B), indicating that the C_{14}—C_{15} and the NH rock are coupled in the anti C=N configuration (Table I). As indicated in Figure 5C,D, in methylamine RPSB (**5a,b**), which contains a 1:1 mixture of syn and anti isomers, several changes occur in the 1168 cm^{-1} band following deuteration of the Schiff base linkage. A new band appears at 1184 cm^{-1}, while the band at 1168 cm^{-1} loses intensity and the band at 1204 cm^{-1} gains intensity (Figure 5D). We conclude that the syn component of the 1168 cm^{-1} band has shifted to 1184 cm^{-1}, while the anti component, as we have seen previously for the butylamine RPSB (Figure 5A,B), has shifted to 1200 cm^{-1}. The intensity left at 1168 cm^{-1} is due to the C_{10}—C_{11} bond stretching frequency. These results indicate that the C_{14}—C_{15} mode is coupled to the NH rock in both the anti and syn configurations of the 13-cis RPSB. To ascertain that exchange of the Schiff proton for a deuteron in **5a,b** and **6a,b** did not cause isomerization from 13-cis to all-trans, we measured the ^1H NMR spectra of **5a,b** and **6a,b** following the FTIR measurements. The spectra did not show any significant isomerization.

To further confirm the assignment of the C_{14}—C_{15} stretching frequency in 13-cis RPSB, we double-labeled the C_{14}—C_{15} bond with carbon-13. Comparison of Figures 5A and 6A indicates that the band at 1168 cm^{-1} consists of the C_{14}—C_{15} and C_{10}—C_{11} stretching frequencies of 13-cis RPSB. The 1168 cm^{-1} band of **6a,b** (Figure 5A) splits into two bands in the labeled 13-cis RPSB (Figure 6A). A new band appeared at 1146 cm^{-1}, while the band at 1168 cm^{-1} lost intensity. This shift induced by the carbon 13 labeling at the C_{14}—C_{15} bond confirms the assignment that part of the 1168 cm^{-1} band consists of C_{14}—C_{15} stretching frequency. Following deu-

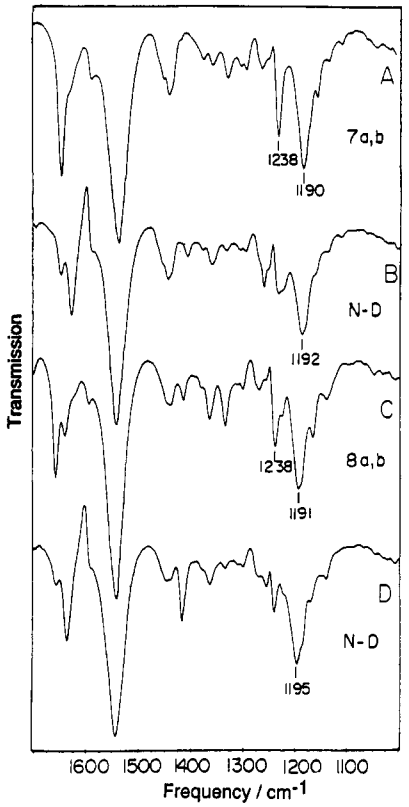


FIGURE 7: FTIR spectra of *all-trans*-1,1-desdimethylretinal-protonated Schiff base derivatives: (A) **7a,b**; (B) N–D derivative of A; (C) **8a,b**; (D) N–D derivative of C.

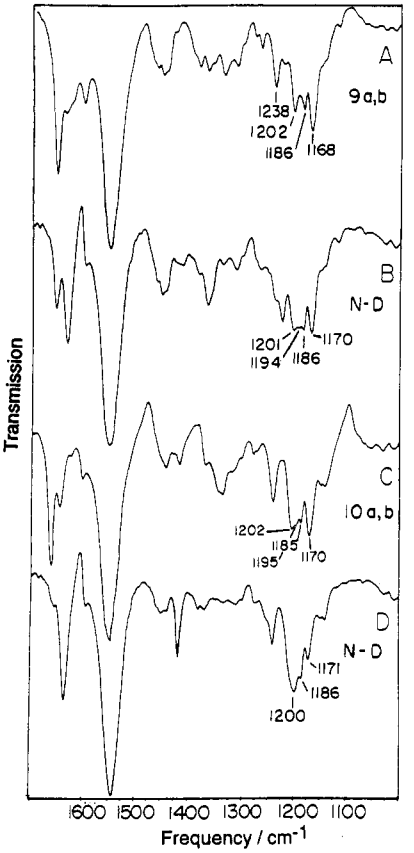


FIGURE 8: FTIR spectra of 13-*cis*-1,1-desdimethylretinal-protonated Schiff base derivatives doubly labeled with ^{13}C at the C_{14} and C_{15} carbons: (A) **9a,b**; (B) N–D derivative of A; (C) **10a,b**; (D) N–D derivative of C.

teration (Figure 6B), the band at 1146 cm^{-1} moves up by 35 cm^{-1} to 1181 cm^{-1}, in keeping with the shift observed for the unlabeled compound (Figure 5A,B). Methylamine RPSB (**5a,b**) consists of a 1:1 mixture of syn and anti C=N isomers. The band at 1154 cm^{-1} (Figure 6C), which corresponds to the C_{14}—C_{15} stretching frequency of ^{13}C-labeled **5a,b**, also moves up following deuteration (Figure 6D) and a new broad band appears, centered at 1181 cm^{-1}. This band is probably broadened due to the different shifts of the two isomers.

The experiments with the labeled retinals support our previous conclusions that in the 13-cis RPSB both the syn and the anti C=N isomers experience a significant coupling between the C_{14}—C_{15} mode and the NH rock. The coupling with the anti isomer is stronger, since the C_{14}—C_{15} frequency shifts up by 33 cm^{-1} upon RPSB deuteration, while in the syn isomer it shifts only by 16 cm^{-1}. In the all-trans RPSB, none of the isomers show this coupling.

Our results imply that in model compounds the coupling between the C_{14}–C_{15} and the NH rock cannot serve as a marker for the C=N bond configuration. In an attempt to mimic the chromophore structure in bR better, we modified the ring–chain conformation of the model compound. The retinal chromophore in bR adopts the s-trans ring–chain planar conformation (Schreckenbach et al., 1978; Harbison et al., 1985; Lutgenburg et al., 1986; Albeck et al., 1992), which induces a red shift in the absorption maximum relative to solution, in which the retinal chromophore mainly adopts a twisted s-cis ring–chain conformation.

To obtain further insight into the nature of C_{14}—C_{15}/NH coupling and to examine whether the altered ring–chain conformation in bR can affect the coupling, we prepared *n*-butylamine and methylamine RPSB derivatives of 1,1-desdimethylretinals **7** and **8**. It was found previously (Friedman et al., 1989; Albeck et al., 1992) that 1,1-desdimethylretinal adopts an s-trans ring–chain planar conformation in solution and its protonated Schiff base (Cl^- serves as a counterion) absorbs at 490 nm in methylene chloride, relative to 455 nm of native RPSB. ^1H NMR spectra of **7** and **8** reveal that, similar to *all-trans*-retinal, the ratio of **7a** to **7b** (anti to syn C=N configurations) is 9:1, whereas the methylamine derivative consists of an anti/syn mixture of 1:1. Figure 7 shows the FTIR spectra of **7** and **8**. The C_{14}—C_{15} band at 1190 cm^{-1} does not shift significantly following deuteration of the Schiff base linkage in both **7** and **8**. These results indicate that, similar to the all-trans RPSB, the coupling between the C_{14}—C_{15} stretch and the NH in **7** and **8** is minor in both the syn and anti isomers.

An experiment which was carried out for the 13-cis derivative of the desdimethylretinals **9** and **10** is presented in Figure 8. For the butylamine derivative **9a,b**, the 1168 cm^{-1} band which was assigned to C_{10}—C_{11} + C_{14}—C_{15} loses intensity following RPSB deuteration while a new peak appears at 1194 cm^{-1} (Figure 8A,B), which is an almost analogous shift to the one observed for the native RPSB (Figure 5A,B).

In the methylamine derivative, **10a,b** (similar to the native C=N RPSB), a new broad peak appears due to the different shifts of the syn and anti isomeres (Figure 8C,D). A conclusion comparable to that for 13-*cis*-retinal can be made in the case of 13-*cis*-1,1-desdimethylretinal. Namely, in both the syn and anti isomers the C_{14}—C_{15} mode is coupled to the NH rock in this retinal configuration. This similarity between native and 1,1-desdimethylretinals implies that the ring–chain conformation does not affect the nature of the C_{14}—C_{15}/NH coupling.

The anti and syn C=N configurations in bR_{568} and bR_{548} were also detected by ^{13}C NMR studies (Harbison et al.,

1984). The analysis was based on the $^{13}C_{14}$ chemical shift, which was upfield shifted by ~11 ppm in bR_{548} vs bR_{568}. This difference was attributed to the γ-effect associated with a syn C=N configuration in bR_{548}. This γ-effect, which affects the C_{14} chemical shift, originates from interaction with the ϵ-CH_2 lysine group. To obtain further insight into the proposed γ-effect, we measured the ^{13}C NMR spectra of compound **2** (bearing a 1:1 mixture of anti and syn C=N configurations) labeled with ^{13}C at C_{14} and C_{15}. It was found that the chemical shift of C_{14} was 120.64 ppm for the anti C=N configuration and 115.63 ppm for the syn isomer. This 5 ppm upfield shift is in keeping with the suggestion for the γ-effect in the syn configuration. However, the larger effect (~11 ppm) observed in bR_{548} suggests a very specific conformation of the retinal chromophore and the lysine chain in bR_{548}, which induces a very intense γ-effect. In this respect we note that a strong HOOP mode was observed at 800 cm^{-1} in bR_{548} and was assigned to $C_{14}H$. The strong HOOP may support a special retinal geometry that introduces a very large γ-effect. In our model compound, a small upfield shift was also detected for C_{15} in the syn isomer relative to the anti (162.32 vs 164.13 ppm).

The results described in this article indicate that, in model compounds in solution, the coupling between the C_{14}—C_{15} stretching frequency and the NH rock occurs only in the 13-cis isomer in both the anti and syn C=N configurations and not in the all-trans isomer. Therefore, the C_{14}—C_{15} mode can actually serve as a marker for the C_{13}—C_{14} bond configuration. In light-adapted bR_{568}, no significant C_{14}—C_{15}/NH coupling was observed, in contrast to bR_{548} (dark-adapted) where a significant coupling emerged. These results are in keeping with the model compound studies since bR_{568} and bR_{548} bear all-trans and 13-cis configurations, respectively. In the photochemically induced intermediates in the bR photocycle, a C_{14}—C_{15}/NH coupling was not detected despite the fact that K, L, and N intermediates bear the 13-cis configuration. The model compounds predict a coupling in these intermediates as well. This discrepancy is probaly due to different conformations of the retinal chromophore in the protein binding site and in solution, indicating that the C_{14}—C_{15}/NH coupling is affected by the retinal conformation. We note that FTIR linear dichroism and photoselection measurements combined with quantum chemical theoretical analysis indicated a twisting around of the retinal single bonds, especially in the photocycle intermediates (Fahmy et al., 1989).

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