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Factors Affecting the C=N Stretching in Protonated Retinal Schiff Base: A Model Study for Bacteriorhodopsin and Visual Pigments†

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ABSTRACT: Factors affecting the C=N stretching frequency of protonated retinal Schiff base ($RSBH^+$) were studied with a series of synthetic chromophores and measured under different conditions. Interaction of $RSBH^+$ with nonconjugated positive charges in the vicinity of the ring moiety or a planar polyene conformation (in contrast to the twisted retinal conformation in solution) shifted the absorption maxima but did not affect the C=N stretching frequency. The latter, however, was affected by environmental perturbations in the vicinity of the Schiff base linkage. Diminished ion pairing (i.e., of the positively charged nitrogen to its anion) achieved either by substituting a more bulky counteranion or by designing models with a homoconjugation effect lowered the C=N stretch energy. Decreasing solvation of the positively charged nitrogen leads to a similar trend. These effects in the vicinity of the Schiff base linkage also perturb the deuterium isotope effect observed upon deuteriation of the Schiff base. The results are interpreted by considering the mixing of the C=N stretching and C=N—H bending vibration. The C=N mode is shifted due to electrostatic interaction with nonconjugated positive charges in the vicinity of the Schiff base linkage, an interaction that does not influence the isotope effect. Weak hydrogen bonding between the Schiff base linkage in bacteriorhodopsin (bR) and its counteranion or, alternatively, poor solvation of the positively charged Schiff base nitrogen can account for the C=N stretching frequency of 1640 cm^{-1} and the deuterium isotope effect of 17 cm^{-1} observed in this pigment. Conversion of bR to the photochemically induced intermediate K_{610} involves environmental perturbation in the vicinity of the C=N linkage, lowering the C=N stretch energy. The C=N stretching frequency (1660 cm^{-1}) observed for rhodopsin indicates very effective hydrogen bonding with the Schiff base counteranion and/or effective solvation by protein dipoles or residual water.

Both bacteriorhodopsin (bR)[1] [the protein pigment of the purple membrane of the halophilic microorganism *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971)] and visual rhodopsins consist of a retinal chromophore (all-trans in bR and 11-cis in visual pigments) that is covalently bound to a membrane protein (opsin) via a protonated Schiff base linkage at a lysine residue. Excitation of visual pigments leads to changes in the electrical potential of the photoreceptor cell membrane that are transmitted to the brain through appropriate synaptic processes. In bR, light energy is converted into a proton gradient across the membrane that is subsequently used via a chemiosmotic mechanism to synthesize ATP [see Ottolenghi (1980), Stoeckenius et al. (1979), and Birge (1981) for reviews].

Light absorption by visual pigments or bR is followed by a sequence of structural transformations involving both the retinal polyene and the protein. The primary event includes conversion to a red-shifted intermediate (K_{610} in bR and bathorhodopsin in visual pigment) followed by thermal processes (bathorhodopsin → lumirhodopsin → metarhodopsin I ↔ metarhodopsin II → metarhodopsin III → *all-trans*-retinal + opsin in rhodopsin and $K_{610} \rightarrow L_{550} \rightarrow M_{410} \rightarrow O_{640}$ in bR). Obtaining further information on the molecular mechanisms associated with the above transformations is a prerequisite for correlating these processes with the corresponding biological functions, i.e., release of the defusable transmitter in photoreceptor cells and pumping of H^+ ions in *H. halobium*.

It was demonstrated by several groups that resonance Raman spectroscopy [see Warshel (1977), Callender and Honig (1977), Mathies (1979), and Lewis (1982) for reviews] and Fourier-transform infrared (FTIR) spectroscopy (Bagley et

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[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier-transform infrared; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; $LiAlD_4$, lithium aluminum hydride (deuteriated); $RSBH^+$, protonated retinal Schiff base; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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al., 1982; Siebert et al., 1982; Rothschild et al., 1983) might be useful for studying several important properties of these pigments and their photochemically induced intermediates (Oseroff & Callender, 1974; Stockburger et al., 1979; Hayward et al., 1981; Braiman & Mathies, 1982; Smith et al., 1983; Terner & El-Sayed, 1985; Bagley et al., 1985). The C=N stretching mode in the range 1620–1650 cm^{-1} has been used to identify the state of protonation of the Schiff base, mainly through band shifts, following deuteriation (Oseroff & Calender, 1974). Further information on the pigments and intermediates may also be gained from measuring their different C=N frequencies. Limited use has been made of the intense line between 1500 and 1600 cm^{-1} in the Raman spectrum, which has been assigned to the C=C stretching mode, a frequency that is inversely correlated to the absorption maximum of the various pigments and intermediates (Callender & Honig, 1977). Recently, we showed (Baasov & Sheves, 1985) that this inverse correlation also applies to synthetic retinal pyrrolidinium perchlorate salts, whose absorption maxima are shifted by interaction with nonconjugated positive charges. The inverse correlation was interpreted as a change in π-electron delocalization along the polyene chain, which also produces the observed changes in absorption maxima. Alteration in π-electron delocalization also changes the order of the double bonds, which is reflected in shifted C=C stretching frequencies (Heyde et al., 1971).

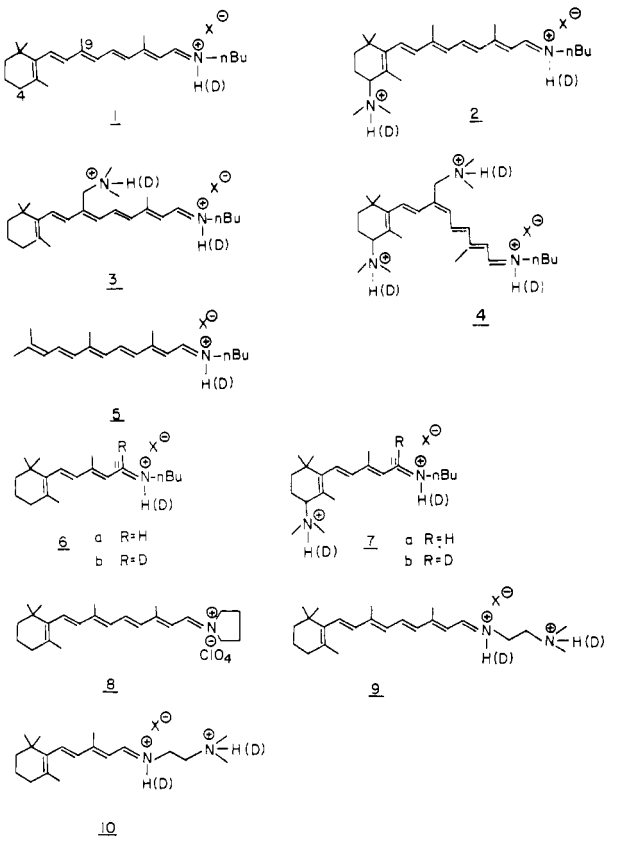
Unexpectedly, no correlation was found between the absorption maxima and the C=N stretching frequency. Bovine rhodopsin and its photochemically induced intermediates all exhibit the same C=N frequency (1660 cm^{-1}) despite their different absorption maxima (Narva & Callender, 1980; Bagley et al., 1985). In contrast, the C=N stretching frequency of 1640 cm^{-1} in bR changes during the photocycle. Similarly, there is no correlation between C=N stretching and λ_{max} of pyrrolidinium perchlorate salts of retinal analogues bearing nonconjugated positive charges in solution (Bassov & Sheves, 1985). The isotope effects in bR and rhodopsin also differ. Following deuteriation, C=N stretching in rhodopsin shifts to lower energy by ca. 30 cm^{-1} but only by ca. 17 cm^{-1} in bR (Aton et al., 1977; Terner et al., 1979; Alshuth et al., 1981; Lewis et al., 1982; Bagley et al., 1985).

The C=N stretching frequency can serve as a tool for probing its immediate environment, as well as processes taking place following light absorption by the various pigments. However, understanding of the factors influencing this mode is still scanty (Kakitani et al., 1983). In this investigation, we have studied the C=N stretching frequency using various protonated retinal Schiff base analogues in different solvents. The results lead to the conclusion that interactions of the retinal chromophore with nonconjugated positive charges in the vicinity of the ionone ring, as well as conformational changes in the retinal polyene, shift the absorption maximum of the protonated retinal Schiff base but not its C=N stretching frequency. The latter is influenced, however, by introducing a nonconjugated positive charge in the vicinity of the Schiff base linkage. Significant shifts in C=N stretching are also induced by changing hydrogen bonding at the N–H linkage. Moreover, the deuterium isotope effect on that system is smaller when the hydrogen bonding is weaker.

Materials and Methods

Preparation of Schiff Bases and Pyrrolidinium Perchlorate Salts. Aldehydes carrying nonconjugated positive charges along the polyene were synthesized as previously described (Baasov & Sheves, 1985). 11-^{2}H-Labeled derivatives (**6b** and **7b**, Chart I) were prepared by reduction of the corresponding

Chart I



ester (Baasov & Sheves, 1985) with $LiAlD_4$ (Et_2O, 0 °C), followed by oxidation with MnO_2. The Schiff bases derived from chromophores **1–7** were prepared by reaction of the corresponding aldehydes with 1.5 equiv of *n*-$BuNH_2$ at 25 °C for 30 min in dry ethanol. Evaporation of solvent and excess *n*-$BuNH_2$ afforded the required Schiff base. The Schiff bases derived from **9** and **10** were prepared similarly by condensing the aldehyde with 1.2 equiv of *unsym*-dimethylethylenediamine for 30 min at 25 °C, followed by solvent evaporation under high vacuum. Pyrrolidinium perchlorate salt **8** was prepared by condensation of *all-trans*-retinal with pyrrolidine perchlorate in ethanol at 25 °C for 6 h.

FTIR Measurements. The Schiff base was dissolved in the appropriate solvent and protonated by the identical solvent saturated with the required acid. Complete protonation was confirmed by absorption spectrometry.

Deuteriation of the Schiff base nitrogen was carried out either by using deuteriated reagents (trifluoroethanol or trifluoroacetic acid) or by dissolving the corresponding protonated Schiff base in deuteriated methanol, evaporating the solvent under high vacuum, and dissolving the residue in the desired solvent. FTIR measurements were carried out on a Nicolet MX-I instrument. Fluorinated alcohols (spectroscopic grade) were purified by elution through a basic alumina column, followed by distillation. TFA was distilled before use. The chromophores concentration in the experiments carried out with excess TFA was 10^{-4} M.

Results

Chromophores **2–4** bear nonconjugated positive charges at different loci: close to carbon 4 in **2**, close to carbon 9 in **3**, and two positive charges near carbons 4 and 9 in **4**. As outlined in Table I and Figure 1, C=N stretching hardly changes as a result of these nonconjugated charges, although a significant blue shift is observed in the absorption maxima.

Table I: C=N Stretching Frequencies and Absorption Maxima (in Chloroform) of Chromophores Carrying Nonconjugated Positive Charges in the Vicinity of C_5–C_{10} and of a Chromophore with s-Trans Conformation

chromophore	$\nu_{C=NH}$ (cm^{-1})	$\nu_{C=ND}$ (cm^{-1})	isotope shift (cm^{-1})	λ_{max} (nm)	$\Delta\nu$ (cm^{-1})[a]
1	1652	1632	20	456	
2	1653	1632	21	432	1200
3	1654	1632	22	425	1700
4	1655	1633	22	398	3200
5	1651	1631	20	482	−2000
6a	1656	1635	21	390	
7a	1660	1639	21	330	4900[b]
6b	1641	1619	22	390	
7b	1643	1620	23	330	4600[b]

[a] Difference between absorption maxima of the corresponding chromophore and that of retinal protonated Schiff base (in cm^{-1}). [b] Difference between the absorption maxima of **7** and **6** (in cm^{-1}).

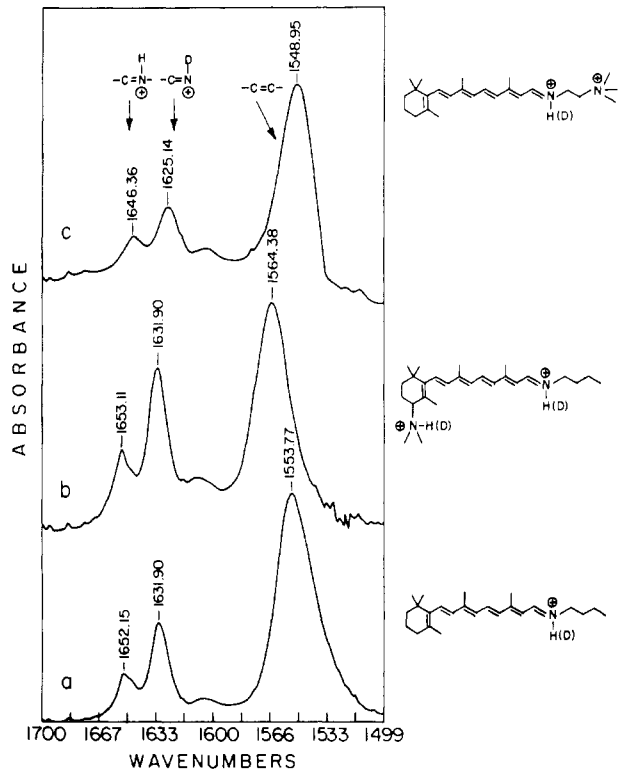


FIGURE 1: C=N and C=C stretching frequencies of mixtures of protonated and deuteriated retinal Schiff base chromophores bearing nonconjugated positive charges.

The opsin shift of bR [defined as the energy difference between the absorption of a protonated Schiff base of the chromophore in methanol and that of the corresponding pigments (Nakanishi et al., 1980)] was attributed in part to s-trans ring–chain planarity that contrasts with the twisted conformation existing in solution (Schreckenbach et al., 1978; Harbison et al., 1985; Spudich et al., 1986; Lugtenburg et al., 1986). To study the influence of the planar conformation, we compared the C=N mode of chromophore **5** to that of **1**. The former adopts a planar conformation in solution that is reflected in its red-shifted absorption maximum. However, there is no effect on the C=N mode (Table I). The lack of correlation between the C=N stretching and λ_{max} is quite surprising as the C=N bond is conjugated with the chain, and it was previously found that a correlation does exist between the corresponding λ_{max} and the C=C stretching frequencies in model compounds with nonconjugated positive charges (Baasov & Sheves, 1984).

It was suggested (Aton et al., 1980; Kakitani et al., 1983; Smith et al., 1983) that the C=N stretching frequency in the protonated retinal Schiff base is pushed to higher frequency by coupling with the C=N—H bending vibration. Deuteriation of the C=N bond essentially removes this coupling due to the lower frequency of the N–D band, leading to a C=N frequency that is closer to its "pure" $\nu_{C=N}$. Therefore, one possible explanation for the insensitivity of C=N stretching to interaction of the polyene with nonconjugated positive charges in the vicinity of the ring or to planar ring-chain conformation may result from coupling between the C=N and the N–H rock. Alteration of π-electron delocalization along the polyene chain due to the nonconjugated charges or the planar conformation, which could alter C=N stretching, may also perturb the C=N/N--H interaction, which might tend to counterbalance the aforementioned effects. To clarify this point, we examined the influence of nonconjugated positive charges on deuteriated Schiff bases of **2**–**5**. The results (summarized in Table I) reveal that C=N stretching is practically unchanged in the deuteriated chromophores. Thus, the insensitivity of the latter does not originate from counterbalancing C=N/N—H rock coupling. Further support of this observation was gained by comparing chromophores **6a** and **7a**. Here, the absorption maximum is markedly influenced by the nonconjugated positive charge (a shift of 4600 cm^{-1}). However, only a minor change is detected in C=N stretching frequency. Still another possible coupling exists between the in-plane rock of C_{15}–H and the C=N stretch (Braiman & Mathies, 1980). However, removal of the latter coupling by deuteriation (chromophores **6b** and **7b**) also reveals insensitivity of the C=N stretch to the presence of a nonconjugated positive charge in the vicinity of the ionone ring.

We next considered influences on the C=N mode due to factors operating in its vicinity. Substituting the counteranion of the positively charged nitrogen by a more bulky one produces a lowering of C=N stretch energy, as the interaction between the nitrogen and the counteranion is weakened. This was previously observed by Lewis (1982), who measured the Raman spectra of various protonated retinal Schiff bases ($RSBH^+$) in the crystalline state. As outlined in Table II and Figure 2, the C=N stretching frequency of $RSBH^+$ with Cl^- as the counteranion was observed at 1652 cm^{-1} (in $CHCl_3$), whereas excess TFA induces a shift to 1643 cm^{-1}. Excess TFA weakens in pairing between the trifluoroacetate counteranion and the positively charged nitrogen due to a homoconjugation effect (Kolthoff et al., 1966; Baasov & sheves, 1985), which modifies absorption maximum as well. A significant alteration of the C=N mode is observed in different solvents. Thus, the C=N stretching in protic or polar solvents, such as methanol, acetonitrile, or tetrahydrofuran, is observed at ca. 1655 cm^{-1}, while in hexafluoro-2-propanol (HFIP) it appears at 1644 cm^{-1}. Similar shifts in fluorinated alcohols were observed recently in resonance Raman studies (Sugihara & Kitagawa, 1986).

Different solvents and counterions also perturb the deuterium isotope shift observed following deuteriation of the nitrogen, presumably due to environmental factors. In

Table II: C=N Stretching Frequencies of Protonated Schiff Bases with Various Counterions and Solvents

chromophore and conditions (counterions and solvents)	$\nu_{C=NH}$ (cm^{-1})	$\nu_{C=ND}$ (cm^{-1})	isotope shift (cm^{-1})	λ_{max} (nm)	$\Delta\nu$ (cm^{-1})[a]
1, MeOH (Cl^-)[b]	1656	1633	23	440	
1, CH_3CN (Cl^-)[b]	1655	1633	22	440	
1, THF (Cl^-)[b]	1655	1633	22	440	
1, TFE (Cl^-)[b]	1650	1632	18	467	1300
1, HFIP (Cl^-)[b]	1644			492	2400
1, $CHCl_3$ (Cl^-)	1652	1633	19	456	800
1, $CHCl_3$ (ClO_4^-)	1649	1632	17	469	1400
1, $CHCl_3$ + 1 M TFA (CF_3COO^-)	1643	1627	16	495	2500
1, CH_2Cl_2 + 1 M TFA (CF_3COO^-)	1641	1627	14	510	3100
6a, MeOH (Cl^-)[b]	1659	1637	22	380	
6a, CH_3CN (Cl^-)[b]	1658	1637	21	380	
6a, $CHCl_3$ (Cl^-)	1656	1635	21	390	700
6a, CH_2Cl_2 (ClO_4^-)	1654	1635	19	410	1650
6a, CH_2Cl_2 + 1 M TFA (CF_3COO^-)	1648	1632	16	430	3000

[a] Difference between absorption maxima of the chromophore in methanol solution and that of the corresponding conditions (in cm^{-1}). [b] In leveling solvents, the counterionic character does not influence C=N stretching frequencies.

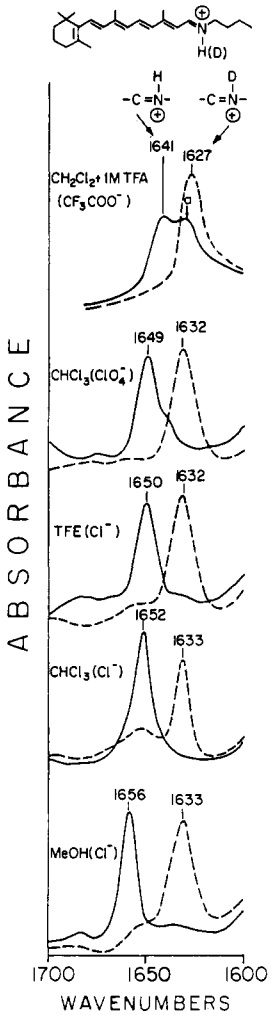


FIGURE 2: C=N stretching frequencies of protonated retinal Schiff base (—) and its deuteriated form (--) under various conditions (as indicated). (a) Trifluorocarboxylate band.

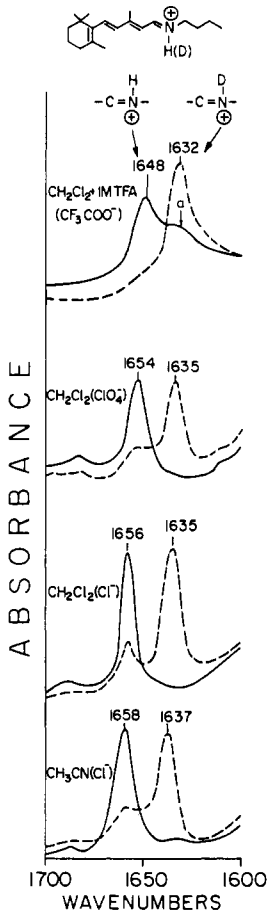


FIGURE 3: C=N stretching frequencies of chromophore **6a** [protonated (—) and deuteriated (--) forms] under various conditions as indicated. (a) Trifluorocarboxylate band.

methanol, a shift of ca. 23 cm^{-1} is detected (Table II), whereas an isotope shift of 14 cm^{-1} is observed with excess TFA in methylene chloride and 17 cm^{-1} with perchlorate in chloroform. Similar trends are observed with the shorter polyene **6a** (Table II and Figure 3).

The C=N stretching of the various deuteriated chromophores is very similar, contrasting with behavior of the protonated forms. For example, in methanol, the protonated Schiff base of retinal absorbs at 440 nm and exhibits C=N stretching at 1656 cm^{-1}. With excess TFA in methylene chloride, the same chromophore exhibits absorption of 510 nm and stretching at 1641 cm^{-1}. In the deuteriated analogue,

Table III: C=N Stretching Frequencies of Retinal Pyrrolidinium Perchlorate Salt **8** in Various Solvents

solvent	$\nu_{C=N}$ (cm^{-1})	λ_{max} (nm)
MeOH	1630	447
THF	1631	445
CH_3CN	1631	447
TFE	1629	472
CH_2Cl_2	1626	482
1 M TFA-CH_2Cl_2	1625	495

Table IV: C=N Stretching Frequencies and Absorption Maxima of Chromophores **9** and **10** Carrying Nonconjugated Positive Charges in the Vicinity of the Schiff Base Linkage and Their Mother Chromophores

chromophore and conditions (counterions and solvents)	$\nu_{C=NH}$ (cm^{-1})[a]	$\nu_{C=ND}$ (cm^{-1})[a]	isotope shift (cm^{-1})	λ_{max} (nm)[a]	$\Delta\nu$ (cm^{-1})[b]
9, CH_3CN (Cl^-)	1651 (1655)	1630 (1633)	21 (22)	454 (440)	700
9, $CHCl_3$ (Cl^-)	1646 (1652)	1625 (1632)	21 (20)	480 (456)	1100
9, TFE (Cl^-)	1639 (1650)	1623 (1632)	16 (18)	496 (467)	1250
9, CH_2Cl_2 + 1 M TFA (CF_3COO^-)	1637 (1641)	1622 (1627)	15 (14)	538 (513)	900
10, CH_3CN (Cl^-)	1656 (1658)	1633 (1637)	23 (21)	390 (380)	700
10, $CHCl_3$ (Cl^-)	1652 (1656)	1631 (1635)	21 (21)	402 (390)	750
10, $CHCl_3$ (ClO_4^-)	1648 (1654)	1630 (1635)	18 (19)	426 (410)	900
10, CH_2Cl_2 + 1 M TFA (CF_3COO^-)	1644 (1646)	1627 (1632)	17 (14)	442 (430)	650

[a] Value for mother chromophore of corresponding compounds (**1** for **9** and **6a** for **10**) are given in parentheses. [b] Difference between the absorption maxima of the mother chromophore and the given chromophore.

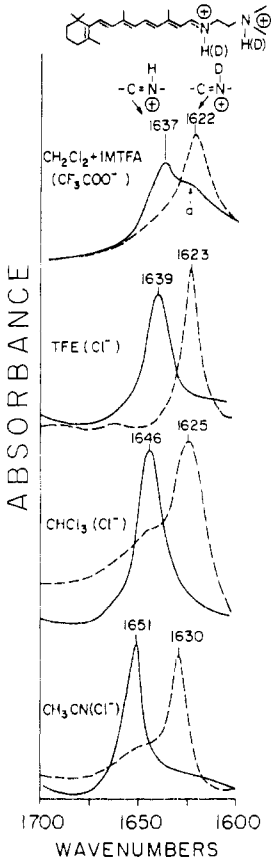


FIGURE 4: C=N stretching frequencies of chromophore **9** under various conditions: (—) protonated form; (– –) deuteriated form. (a) Trifluorocarboxylate band.

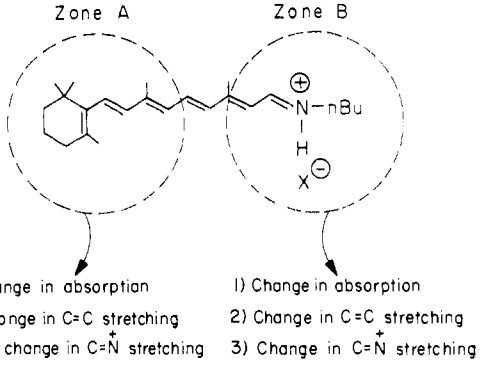


FIGURE 5: Schematic representation of changes occurring in the spectra of protonated retinal Schiff base in response to perturbations introduced in its vicinity. These perturbations include (zone A) addition of nonconjugated charges or ring-chain planarity structure and (zone B) changes in counterion, solvent, and nonconjugated charges.

stretching was detected at 1633 cm^{-1} in methanol and 1627 cm^{-1} in TFA/CH_2Cl_2. Thus, removal of the coupling between C=N stretching and N–H rock significantly reduces the sensitivity of the C=N mode to environmental perturbations.

A similar conclusion may be drawn by looking at the C=N mode of the pyrrolidinium perchlorate salt of *all-trans*-retinal (**8**), a molecule that lacks an interaction between C=N and N–H rock. Table III reveals that environments leading to significant perturbances in absorption maxima do not significantly change C=N stretching.

An environmental perturbation may also be achieved by introducing a nonconjugated positive charge in the vicinity of the C=N bond (Table IV and Figure 4). Thus, chromophore **9** exhibits (in $CHCl_3$) a C=N stretching frequency (1646 cm^{-1}) shifted to lower energy by ca. 6 cm^{-1} relative to the analogous chromophore lacking the nonconjugated charge. In trifluoroethanol (TFE), in which the interaction between positive charges is enhanced due to their poor solvation (Baasov & Sheves, 1986), the C=N stretching shift increased to 11 cm^{-1}. Similar effects are observed by comparing deuteriated chromophores.

Discussion

Model Compounds in Solution. Model compounds carrying nonconjugated positive charges in the vicinity of the chromophore ring, or those having a planar s-trans structure, demonstrate that environmental perturbations in the vicinity of the ring moiety, although shifting the absorption maxima, do not significantly change the C=N stretching mode (Figure 5). This insensitivity may be rationalized if one assumes that the nonconjugated charges or the planar conformation mainly affects the excited state, while producing only small changes in π-electron delocalization along the ground-state C=N bond. Thus, a small change is observed in the C=N stretching, which is associated with the ground state. By contrast, perturbations in the vicinity of the C=N bond lead to significant changes in its stretching frequency, an effect that we studied by varying the strength of hydrogen bonding with the N–H bond and by changing π-electron delocalization of the C=N bond.

Coupling between C=N stretching and N–H rock has been suggested previously to explain the high frequency of C=N stretching (Aton et al., 1980; Kakitani et al., 1983; Smith et al., 1983). Recently, it was also proposed that protonation increases the C=N force constant of retinal Schiff base, due to rehybridization of the C=N bond (Lopez-Garriga et al., 1986a,b). The present results clearly demonstrate that the C=N mode is strongly influenced by hydrogen-bond formation with the N–H bond. The importance of the hydrogen bonding to the N–H bond was stressed previously (Kakitani et al., 1983; Maeda et al., 1985; Sugihara & Kitagawa, 1986; Mathies et al., 1987). Our results point to a decrease of 11 cm^{-1} in $\nu_{C=N}$, following substitution of Cl^- ($CHCl_3$) by excess TFA in

CH_2Cl_2. The chloride anion strongly hydrogen bonds due to its small ionic radius and high negative charge density. Excess TFA hydrogen bonds to the anion associated with the positive ion and diminishes ion pairing, thereby weakening the hydrogen bond between the N–H and the counteranion and leading to the lower energy of C=N vibration. A possible explanation to the influence of the hydrogen bonds is that strong hydrogen bonding to the N–H bond shifts the C=N—H bend vibration to higher energy (Nakanishi, 1962), resulting in effective coupling with the C=N mode, due to close proximity of the energy levels. A weak hydrogen bond, on the other hand, shifts the C=N—H bend to lower energy, weakening the interaction with the C=N mode and shifting the C=N frequency closer to its "pure" value. A similar explanation might also account for the smaller isotope effect observed when the hydrogen bonding is weakened. Since deuteriation quenches C=N/N—H coupling, which in turn affects C=N stretching, the isotope effect will be smaller in cases where coupling has already diminished because of weak hydrogen bonding. Similar trends were observed by varying solvents. Methanol, for example, forms strong hydrogen bonding with the N–H bond, pushing the C=N mode to a higher frequency (1656 cm^{-1}) and evincing a large isotope effect (23 cm^{-1}), due to effective C=N/N—H coupling. Fluorinated alcohols, such as HFIP, or excess TFA in methylene chloride, however, forms weak hydrogen bonds with N–H, lowering C=N stretch and reducing the isotope shift (Table II).

π-Electron delocalization along the C=N bond might also influence its stretching frequency. However, experimental results reveal that changes in hydrogen bonding at N–H do not significantly affect C=N stretching through charge redistribution at that bond but mainly through changes in C=N/C=N—H coupling effectiveness, probably due to modification of vibrational energy level proximities. This conclusion is borne out by measuring C=N stretching in deuteriated chromophores. For example, $\nu_{C=N}$ of the deuteriated compound is shifted by only ca. 6 cm^{-1} in the presence of excess TFA in methylene chloride relative to methanol, whereas in the protonated form a shift of ca. 16 cm^{-1} is observed. These C=N shifts in both the deuteriated and protonated forms are accompanied by an identical 70-nm shift in the absorption (2500 cm^{-1}). Further support for the importance of C=N/C=N—H coupling in C=N stretching is gained from pyrrolidinium perchlorate salts, a chromophore that lacks C=N/C—N—H mode coupling and in which environmental perturbations do not significantly change the C=N frequency. Thus, dissolving chromophore **8** in methylene chloride containing excess TFA instead of methanol produced only a slight shift of 5 cm^{-1} (1630 to 1625 cm^{-1}) despite a ca. 40-nm shift in absorption (Table III).

These results strongly support the assumption that the C=N mode is coupled with the C=N—H bend. Nevertheless, possible coupling to N–H or N–D stretching should still be considered. However, the vastly different energies of N–H and C=N stretching should result only in weak coupling between the two. Interaction between the N–D stretch and the C=N mode seems more plausible. However, comparison of the C=N vibration of pyrrolidinium perchlorate salts lacking C=N/N—H interaction with that of deuteriated Schiff bases reveals a similar sensitivity to environmental perturbations (Tables II and III), despite possible C=N/N—D coupling in the latter. In addition, their behavior is quite different from that of protonated Schiff bases. These facts point to the importance of C=N/N—H coupling relative to that of C=N/N—D, supporting the suggestion that strong coupling exists between the C=N stretch and the C=N—H bend (Aton et al., 1980; Kakitani et al., 1983; Smith et al., 1983).

Introducing a nonconjugated positive charge in the vicinity of the Schiff base linkage significantly perturbates C=N stretching due to a redistribution of electrostatic charge within this bond. Thus, chromophore **9** exhibits a shift of 6 cm^{-1} in its C=N band, relative to **1** (in $CHCl_3$). The similar isotope effects of **9** and **1** observed upon deuteriation of the nitrogen reveal that C=N/N—H coupling is not affected by the nonconjugated charge. In summary, the experimental results demonstrate that the C=N stretching of protonated retinal Schiff base is affected by C=N/N—H coupling as well as by charge distribution in the C=N bond. In addition, our results reveal that a change in the absorption maximum of $RSBH^+$, occurring without C=N stretching alteration, implies a change in the ring region or along the polyene part close to the ring (approximately C_5–C_{10}). Alteration of both the absorption spectrum and the C=N mode points to perturbation in the vicinity of the Schiff base linkage (Figure 5).

Bacteriorhodopsin and Rhodopsin. The C=N stretching frequency provides a probe for evaluating the environmental conditions experienced by the protonated Schiff base linkage in bR. The retinal–protein interactions in the vicinity of the chromophore ring induce a red shift in the absorption maximum. These interactions, however, should not affect the C=N stretching frequency, as deduced from our above-described studies in model compounds. Thus, the C=N shift of bR, relative to $RSBH^+$ in methanol (1640 vs. 1656 cm^{-1}), must be due to weak hydrogen bonding (relative to methanol) of the N–H with its counteranion, with protein dipoles, or with residual water. This conclusion is directly associated with the observation that the shifts in the C=N mode observed in our model compounds due to diminishing hydrogen bonds are of the order of 12 cm^{-1}, accompanied by a shift of ca. 2400 cm^{-1} in the absorption maximum [see Table II for methanol vs. HFIP or $CHCl_3$ (Cl^-) vs. CH_2Cl_2 + 1 M TFA]. In bR, a shift in the absorption maxima of ca. 3000 cm^{-1} relative to methanol solution was suggested to originate from weak hydrogen bonding with the N–H bond (Harbison et al., 1983; Muradin-Szweykowska et al., 1984; Sheves et al., 1985; Lugtenburg et al., 1986; Spudich et al., 1986). This shift is indeed accompanied by a ~16-cm^{-1} shift in C=N stretching (relative to methanol solution). In addition, the deuterium isotope effect observed in bR (ca. 17 cm^{-1}) is similar to that found in instances where weak hydrogen bonding exists, such as in retinal protonated Schiff base in methylene chloride with excess TFA or perchlorate as a counteranion. This supports the existence of weak hydrogen bonding with the N–H bond in bR (Harbison et al., 1983) and cancels the possibility of other kinds of environmental effects in the region of the Schiff base, such as electrostatic interactions.

A change in C=N stretching points to a perturbation of its environment. For example, a shift from 1640 to 1650 cm^{-1} was measured in 5,6-dihydrobacteriorhodopsin artificial pigment, accompanied by a marked decrease in the opsin shift to 2300 cm^{-1} (Schiffmiller et al., 1985a,b). This shift in the C=N mode indicates an environment that should cause a blue shift (relative to native bR) in the visible spectrum. Thus, part of the abnormal decrease in the opsin shift of the 5,6-dihydro pigment may originate from environmental perturbations near the C=N bond and not merely from chromophore–protein interactions in the vicinity of the ring.

The C=N stretching frequency of bovine rhodopsin (1660 cm^{-1}) indicates a C=N/opsin interaction differing from that

in bR. Here, solvation of the positively charged nitrogen is even stronger than that of protonated retinal Schiff base in methanol solution. Thus, the absorption maximum of rhodopsin expected from this C=N mode should be lower than 440 nm. The isotope effect of ca. 30 cm^{-1} following deuteriation supports this suggestion. Therefore, the red-shifted absorption found in bovine rhodopsin (498 nm) must originate from interactions that do not affect the C=N stretching. Our results reveal that ring-chain planarity will induce a red shift relative to a twisted conformation in solution, without affecting the C=N stretching. However, experiments with 5,6-dihydroretinal (Honig et al., 1979a), as well as with an artificial rhodopsin pigment derived from bicyclic chromophore (Ito et al., 1985), reveal a substantial opsin shift despite the incapability of ring-chain planarity to contribute to the opsin shift. These observations eliminate the possibility that ring-chain planarity exists in bovine rhodopsin and support the assumption that the red shift originates from a different chromophore–opsin interaction. These arguments support the external point-charge model (Honig et al., 1979), which proposes an interaction with nonconjugated negative charge as the source of the red shift.

The present and previous (Baasov & Sheves, 1985) results point to the importance of the negative charge or protein dipole location along the polyene skeleton. Positioning in the vicinity of carbons 9 and 10 will avoid C=N perturbation, leading to a high C=N stretching frequency and to a red shift due to electrostatic interaction mainly with the excited state. A negative charge present in the vicinity of carbons 12–14 will probably affect the C=N stretching and will, moreover, interact strongly with the ground state (relative to the excited state), inducing a blue shift in the spectrum (Baasov & Sheves, 1985). Thus, we suggest that in bovine rhodopsin a nonconjugated negative charge (or protein dipole) may be located closer to carbons 9 and 10 than to carbons 12–14.

Following light absorption, bR is converted to a red-shifted intermediate K_{610}. C=N stretching was measured by several groups using resonance Raman and FTIR techniques. While the exact frequency is still a controversial subject, both methods detect a substantial shift in stretching (1626 cm^{-1} by Raman [see Rothschild et al. (1984a) and references cited therein] and 1609 cm^{-1} (Rothschild et al., 1984b) or 1613 cm^{-1} (Gerwert & Siebert, 1986) by FTIR), indicative of C=N environmental perturbation. Weakening of hydrogen bonding between the N–H bond and its counteranion or, alternatively, alteration of N–H solvation by protein dipoles or residual water (Warshel & Barboy, 1982; Hildebrandt & Stockburger, 1984; Baasov & Sheves, 1986) should induce the observed shift. These changes could occur following isomerization of the C_{13}=C_{14} bond as a consequence of light absorption. Still, an approaching positive charge to the C=N linkage (Hanamoto et al., 1984; Baasov & Sheves, 1986) can also shift the C=N stretch but will not affect the deuterium isotope effect.

In bovine rhodopsin other spectroscopy phenomena are observed. The red-shifted intermediate, bathorhodopsin (generated following light absorption), and the pigment itself exhibit similar C=N stretching frequencies. The model compounds studied in solution demonstrate the possibility of inducing red shifts not associated with changes in C=N vibration by introducing chromophore–opsin interactions in the C_5–C_{10} region of the chromophore. Still another explanation that may account for the insensitivity of the C=N mode to generation of bathorhodopsin may involve separation of the positively charged nitrogen from its counteranion (Honig et al., 1979b) and/or reduction of the positive charge stabilization by protein dipoles (Warshel & Barboy, 1982) while maintaining strong hydrogen bonding to N–H. This charge separation or reducing stabilization might lead to a red shift in the absorption maximum (Baasov & Sheves, 1986) but does not affect the C=N stretching, due to strong hydrogen bonding at N–H. Our above-described results with deuteriated chromophores or pyrrolidinium perchlorate salts support this suggestion. The C=N stretch is significantly less sensitive to environmental perturbations in cases where C=N/N–H coupling alterations do not take place. The underlying cause of the behavior of the C=N mode in bovine rhodopsin still needs further investigation.

Registry No. **1** (X = Cl), 28448-64-8; **1** (X = ClO_4), 28448-69-3; **1** (X = CF_3CO_2), 64161-99-5; **2**, 103383-50-2; **3**, 107615-96-3; **4**, 107615-97-4; **5**, 107615-98-5; **6a** (X = Cl), 89071-49-8; **6a** (X = ClO_4), 107616-01-3; **6a** (X = CF_3CO_2), 89071-57-8; **7a**, 103383-58-0; **8**, 23369-82-6; **9**, 107615-99-6; **10**, 107616-00-2; deuterium, 7782-39-0; *unsym*-dimethylethylenediamine, 108-00-9; retinal (all-trans), 116-31-4; pyrrolidine perchlorate, 22401-44-1.

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