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Solid-State NMR Studies of the Mechanism of the Opsin Shift in the Visual Pigment Rhodopsin[†]

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ABSTRACT: Solid-state ¹³C NMR spectra have been obtained of bovine rhodopsin and isorhodopsin regenerated with retinal selectively ¹³C labeled along the polyene chain. In rhodopsin, the chemical shifts for ¹³C-5, ¹³C-6, ¹³C-7, ¹³C-14, and ¹³C-15 correspond closely to the chemical shifts observed in the 11-*cis* protonated Schiff base (PSB) model compound. Differences in chemical shift relative to the 11-*cis* PSB chloride salt are observed for positions 8 through 13, with the largest deshielding (6.2 ppm) localized at position 13. The localized deshielding at C-13 supports previous models of the opsin shift in rhodopsin that place a protein perturbation in the vicinity of position 13. Spectra obtained of isorhodopsin regenerated with ¹³C-labeled 9-*cis*-retinals reveal large perturbations at ¹³C-7 and ¹³C-13. The similar deshielding of the ¹³C-13 resonance in both pigments supports the presence of a protein perturbation near position 13. However, the chemical shifts at positions 7 and 12 in isorhodopsin are not analogous to those observed in rhodopsin and suggest that the binding site interactions near these positions are different for the two pigments. The implications of these results for the mechanism of the opsin shift in these proteins are discussed.

The 11-*cis* protonated Schiff base (PSB)¹ of retinal (Figure 1) is the photoreactive group responsible for light transduction in the visual pigment rhodopsin [for reviews, see Ottolenghi (1980) and Birge (1981)]. The retinal chromophore lies within the interior of the protein and isomerizes from an 11-*cis* to an all-*trans* geometry upon absorption of light (Wald, 1968). Retinal protonated Schiff bases also serve as light transducers in several bacterial membrane proteins, including bacteriorhodopsin (a proton pump), halorhodopsin (a chloride pump), and sensory rhodopsin (a phototactic pigment). In both the visual rhodopsins and bacterial pigments, the protein induces shifts in the absorption spectrum of the retinal. Retinal PSB model compounds absorb at ~440 nm, while the absorption maxima (λ_{\max}) for retinal pigments vary from ~420 to 620 nm (Liebman, 1973). The red-shift (or "opsin shift") in λ_{\max} between the retinal model compounds and the pigments allows the chromophore to absorb a wide range of wavelengths in the visible spectrum. The importance of protein regulation of the retinal absorption band is probably best appreciated in the cone pigments, where distinct absorption profiles are required for color vision.

The visible absorption band of retinal results from electronic excitation of the conjugated π electrons in the retinal polyene chain. Several mechanisms for delocalizing these electrons and inducing red-shifts in retinal pigments have been discussed (Blatz et al., 1972; Honig et al., 1976; Kakitani et al., 1985),

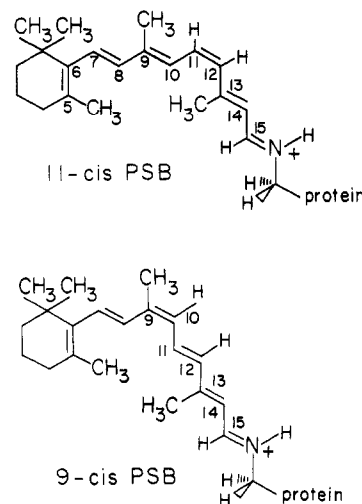


FIGURE 1: Structure of the 11-*cis*-retinal protonated Schiff base in rhodopsin and the 9-*cis* protonated Schiff base in isorhodopsin.

including (1) charge separation between the protonated Schiff base and its protein counterion, (2) electrostatic interactions between the chromophore and a charged amino acid residue, and (3) twisting about double bonds or about single bonds with significant double bond character.

Varying the distance between the Schiff base nitrogen and its protein counterion represents an effective mechanism for modulating the visible absorption band of retinal pigments. Charge separation from close contact to infinity is calculated to shift the λ_{\max} from 440 to 600 nm (Blatz et al., 1972). The λ_{\max} for rhodopsin is at 498 nm, and the absorption band of PSB model compounds with large and easily polarizable

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¹ Abbreviations: bR, bacteriorhodopsin; HOOP, hydrogen out-of-plane; λ_{\max} , absorption maximum; MAS, magic-angle spinning; NMR, nuclear magnetic resonance; ppm, parts per million; PSB, protonated Schiff base; cw, continuous wave.

counterions has been observed to red-shift to 480 nm (Blatz et al., 1972; Childs et al., 1987). In fact, the opsin shift in bR is attributed in large part to a weak electrostatic interaction between the Schiff base and its associated counterion (Harbison et al., 1983; Lugtenburg et al., 1986; Spudich et al., 1986; de Groot et al., 1989).

The second mechanism for red-shifting the absorption band in retinal pigments involves stabilizing delocalized electronic structures by placing protein charges near the conjugated retinal chain, a mechanism proposed originally by Kropf and Hubbard (1958). Nakanishi, Honig, and co-workers found evidence for a negative protein charge, $\sim 3 \text{ \AA}$ from C-12 and C-14 above the plane of the retinal, based on rhodopsin regenerated with dihydroretinal derivatives (Arnaboldi et al., 1979). However, Baasov and Sheves (1985) studied modified retinals containing nonconjugated charges and found that a charge located in the vicinity of C-12 and C-14 had only a minor effect on the λ_{max} . Birge and co-workers (Birge et al., 1985, 1988) proposed that this charge was the carboxyl group of an aspartate or glutamate residue which also served as the counterion to the PSB nitrogen. The amino acid sequences of human cone visual pigments have been determined (Nathans et al., 1986a,b) and potential charged perturbants identified. Recently, mutagenesis experiments have established Glu-113 as the Schiff base counterion in rhodopsin (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). Also, recent resonance Raman microscope experiments on blue-absorbing 440-nm photoreceptors have shown that these pigments contain a protonated Schiff base that is unperturbed by protein charges, consistent with the sequence data (Loppnow et al., 1989).

Conformational distortions of the planar retinal chain may also generate red-shifts in the retinal absorption band. In general, in conjugated polyenes, twisting about double bonds induces red-shifts, while twisting about single bonds induces blue-shifts. However, calculations on polyenes, in the presence of external charges that delocalize the conjugated π system, show that single-bond twists can also induce spectral red-shifts (Kakitani et al., 1985). In resonance Raman spectra of rhodopsin, strong hydrogen out-of-plane wagging vibrations of the C₁₁-H and C₁₂-H protons indicate conformational distortion in this region of the chromophore (Eyring et al., 1980). These vibrations most likely originate from some distortion in the C₁₀—C₁₁=C₁₂—C₁₃ bonds. Thus, in the presence of an external charge believed to be located between C-12 and C-14 (Honig et al., 1979), a twist in the C₁₂—C₁₃ single bond may contribute to the opsin shift.

¹³C NMR is a sensitive spectroscopic method for studying the structure and protein environment of the retinal prosthetic group in rhodopsins. NMR studies of rhodopsin regenerated with [¹³C]retinal have been reported using both solution (Shriver et al., 1977; Mateescu et al., 1984) and solid-state NMR techniques (Smith et al., 1987; Mollevanger et al., 1987). In solution, octyl β -glucoside was employed to solubilize the membrane protein in an effort to shorten its rotational correlation time, while in the solid-state either low temperature (Smith et al., 1987) or lyophilization (Mollevanger et al., 1987) was necessary to quench rotational diffusion of the protein in order to observe its NMR spectrum. In our previous work, we have discussed some of the advantages of the solid-state NMR approach for obtaining high-resolution spectra of rhodopsin using magic-angle spinning (MAS) and cross-polarization techniques (Smith et al., 1987). MAS produces a sharp centerband at the frequency of the isotropic chemical shift together with sets of rotational sidebands spaced at the spin-

ning frequency of the sample. The rotational sidebands provide information on the principal values (σ_{11} , σ_{22} , σ_{33}) of the chemical shift tensor. The chemical shift tensor elements can be extracted from the intensities of these sidebands (Herzfeld & Berger, 1980; de Groot et al., 1989). Both the isotropic and anisotropic chemical shifts are sensitive to the configuration of the retinal, as well as to charged protein residues in the retinal binding site (Harbison et al., 1984a,b, 1985a; Smith et al., 1987; Mollevanger et al., 1987).

In this paper, we present solid-state NMR spectra of rhodopsin and isorhodopsin regenerated with retinal containing ¹³C labels at each position along the conjugated chain of the chromophore. Comparison of the ¹³C chemical shifts observed in the pigment with the corresponding chemical shifts of retinal PSB model compounds allows us to examine the mechanism of the opsin shift in these pigments. The chemical shifts for ¹³C-5, ¹³C-6, ¹³C-14, and ¹³C-15 correspond closely to the chemical shifts observed in the 9-*cis* and 11-*cis* PSB model compounds, while differences in chemical shift are observed for positions 8 through 13, with the largest differences in both pigments localized at position 13. These data provide support for the model of the opsin shift which relies on a protein perturbation in the vicinity of C-13. Concurrently, the results argue that the factors regulating the absorption wavelength in another well-studied retinal protein, bacteriorhodopsin, namely, protein perturbations near the β -ionone ring (C-5...C-7), a 6-*s-trans* single bond, and a weak hydrogen-bonding interaction with the Schiff base counterion, are not important in rhodopsin and isorhodopsin. Finally, in isorhodopsin, a substantial shift is observed at C-7, and only a small shift is present at C-12. Taken together with resonance Raman results (Palings et al., 1987), these observations suggest that 9-*cis*- and 11-*cis*-retinals reside differently in the opsin binding pocket, consistent with their significantly different opsin shifts ($\sim 1000 \text{ cm}^{-1}$ less for isorhodopsin).

MATERIALS AND METHODS

The procedures for synthesizing the [¹³C]retinals have recently been reviewed (Pardoen et al., 1984, 1985; Lugtenburg et al., 1985, 1988). The methods for isolating and regenerating rhodopsin were described in detail previously (Palings et al., 1987). Briefly, the rod outer segments from ~ 100 bovine retinas were purified by density gradient centrifugation. The isolated segments were then bleached in 100 mM phosphate buffer containing 10 mM hydroxylamine hydrochloride, washed to remove excess hydroxylamine, and regenerated with 9-*cis* (isorhodopsin) or 11-*cis* (rhodopsin) [¹³C]retinal for 90 min at room temperature. The regenerated protein was dissolved in 3% Ammonyx-LO and purified by hydroxylapatite chromatography to remove excess retinal and nonregenerated protein. The purified pigment was concentrated with Amicon Centriflo membrane cones (CF25) (Danvers, MA) to $\sim 2 \text{ mL}$. Excess water was removed from the pigment concentrate by blowing dry N₂ gas over the solution. The resulting moist gel was then loaded into the NMR rotor and placed into the NMR probe. All of the procedures were carried out under dim red light. Absorption spectra obtained after the NMR experiment verified that bleaching of the rhodopsin had not occurred.

¹³C NMR spectra were obtained by using the methods previously discussed (Smith et al., 1987). The ¹³C and ¹H frequencies were 79.9 and 317 MHz, respectively, and standard ¹H—¹³C cross-polarization was employed to increase the ¹³C sensitivity and shorten the effective ¹³C T₁. Typically, the mixing time was 2 ms, and the ¹H 90° pulse length was 3.0 μs . All chemical shifts were referenced to external TMS, and no correction was made for bulk susceptibility effects,

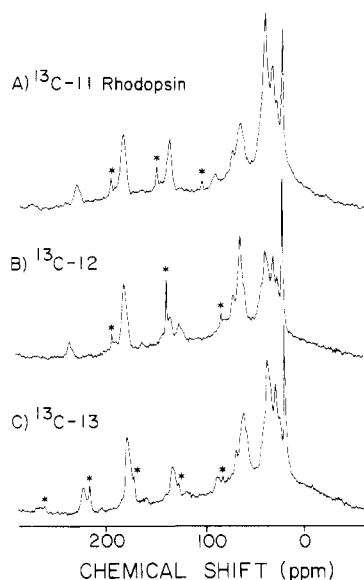


FIGURE 2: MAS ^{13}C NMR spectra of (A) ^{13}C -11, (B) ^{13}C -12, and (C) ^{13}C -13 rhodopsin. Centerbands and rotational sidebands of the retinal resonances are marked with asterisks.

which are expected to be small. The samples were maintained between -35 and -15 $^{\circ}\text{C}$ with cooled N_2 as the spinning gas. The low temperature quenches protein rotational diffusion (Cone, 1972) which can interfere with ^1H - ^{13}C decoupling and MAS line narrowing and can reduce cross-polarization efficiency by reducing the proton $T_{1\rho}$ of the protein. ^{13}C shift tensor elements were determined with a computer program that fits the relative intensities of the rotational sidebands and centerband in the MAS spectra, and subsequently calculates the shift anisotropy according to the method of Herzfeld and Berger (1980).

Solution NMR spectra of the PSB model compounds were obtained at -30 $^{\circ}\text{C}$ in CDCl_3 . Spectral assignments were made by off-resonance cw decoupling, by on-resonance selective decoupling, and by comparison with chemical shift data of other retinal isomers. The Schiff base was prepared with *tert*-butylamine in diethyl ether and protonated with HCl or HClO_4 using modified procedures of Blatz et al. (1972) and Shriver et al. (1979).

RESULTS

The general strategy for establishing the sites of protein-chromophore interactions in rhodopsin involves introduction of selective ^{13}C labels at each position along the length of the retinal chromophore. Differences in the ^{13}C chemical shifts between rhodopsin and retinal PSB model compounds reveal the regions of the chromophore where changes occur in the retinal's-structure or environment. Figure 2 presents several solid-state ^{13}C NMR spectra of rhodopsin that illustrate the resolution and sensitivity that can be obtained by using MAS methods. In these spectra, the protein has been regenerated with retinal specifically ^{13}C labeled at positions 11, 12, and 13, and in each case, the retinal resonance exhibits a sharp centerband at the isotropic chemical shift and is flanked by rotational sidebands. Other lines in the spectrum are the natural-abundance ^{13}C resonances of the protein carbonyls (~ 175 ppm) and aliphatic carbons (0–100 ppm). Contributions from the Ammonyx-LO detergent in these spectra are seen in the different intensities in the 0–100 ppm region. Ammonyx-LO does not exhibit NMR resonances above 100 ppm. Spectra of the 9-*cis* pigment isorhodopsin are similar.

Table I summarizes the isotropic chemical shifts from solid-state NMR spectra of rhodopsin regenerated with retinal

Table I: ^{13}C Chemical Shifts (ppm)^a for Rhodopsin and 11-*cis* PSB Model Compounds

	rhodopsin	11- <i>cis</i> PSB ^b	
		Cl^- , <i>n</i> -propyl ^d	ClO_4^- , <i>tert</i> -butyl
5- ^{13}C	130.3	131.7	132.0
6	137.7	137.2	137.5
7	132.3	132.3	132.2
8	139.2	137.2	137.0
9	148.5	146.6	146.8
10	127.8	126.4	127.7
11	141.6	137.5	139.1
12	132.1	129.0	129.4
13	168.9	162.7	166.0
14	121.2	121.3	119.5
15	165.4	163.9	160.0
19	12.0	12.5	12.5
20	168.8	18.8	18.0

^aChemical shifts are referenced to TMS. ^bThe data for the 11-*cis* PSB chloride salt are from Shriver et al. (1979). ^cDesignates the counterion to the PSB. ^dAmine used to form the Schiff base.

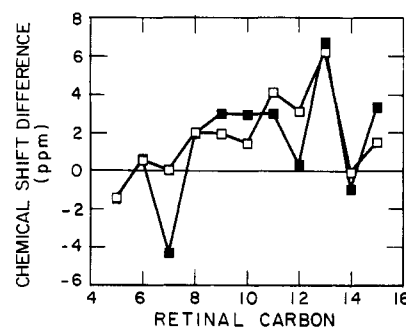


FIGURE 3: Plot of differences in chemical shift observed between rhodopsin (open squares) and the 11-*cis*-retinal PSB chloride salt, and between isorhodopsin (closed squares) and the 9-*cis*-retinal PSB chloride salt, for retinal carbons along the polyene chain.

^{13}C labeled at each position along the conjugated polyene chain. These data are compared with chemical shifts from two 11-*cis* PSB salts which differ in the amine and acid used to form the protonated Schiff base. The λ_{max} for the *all-trans*-retinal PSB perchlorate salt is ~ 470 nm in CCl_4 compared to ~ 440 nm for the chloride salt (Blatz et al., 1972; Childs et al., 1987). Thus, the bulky ClO_4^- counterion and *tert*-butyl group may be inducing a weaker hydrogen bond at the Schiff base.

The differences in chemical shift observed between rhodopsin and the 11-*cis* PSB chloride salt are plotted in Figure 3. The shifts of ^{13}C -5 through ^{13}C -7, ^{13}C -14, and ^{13}C -15 in rhodopsin are close to their values in the 11-*cis* PSB model compound, while larger shift differences are observed at ^{13}C -11 (4.1 ppm), ^{13}C -12 (3.1 ppm), and ^{13}C -13 (6.2 ppm).

Table II summarizes the isotropic chemical shifts from the ^{13}C NMR spectra of isorhodopsin along with chemical shift data from the 9-*cis* PSB chloride salt. The differences in chemical shifts between isorhodopsin and the 9-*cis* PSB are qualitatively similar to the differences observed between rhodopsin and the 11-*cis* PSB with the exception of C-7, where a 4.3 ppm chemical shift difference is observed, and C-12, where the difference, amounting to about 3 ppm in rhodopsin, has now vanished.² Some possible explanations for these shift

² The shift differences reported in Tables I and II are dependent on the assignments of spectra of model compounds. In the case of 9-*cis*-retinal, there is an ambiguity in the assignment of the C-7 and C-12 resonances. With the current assignment, the shift differences amount to -4.3 ppm (C-7) and 0.3 ppm (C-12). If the assignments are reversed, these numbers change to -5.4 and 1.4 ppm, respectively.

Table II: ^{13}C Chemical Shifts (ppm)^a for Isorhodopsin and the 9-cis PSB *n*-Butylimmonium Chloride Salt

	isorhodopsin	9-cis PSB
5- ^{13}C	130.5	132.0
6	137.0	136.4
7	128.2	132.5 ^b
8	131.1	129.2
9	147.5	144.5
10	130.8	127.9
11	139.3	136.3
12	133.9	133.6 ^b
13	169.2	162.5
14	119.0	120.0
15	166.5	163.2
19	19.8	20.9
20	13.6	14.3

^aAll chemical shifts are referenced to TMS. ^bThe assignments of the C-7 and C-12 resonances in the 9-cis PSB may be reversed since on-resonance decoupling affects both resonances (see footnote 2).

differences in isorhodopsin will be considered below. In both rhodopsin and isorhodopsin, the largest chemical shift difference is observed at C-13.

Recently, an analysis of the solid-state NMR spectrum of ^{13}C -12 rhodopsin suggested that both the isotropic chemical shift and individual shift tensor elements moved relative to their values in bR (Mollevarer et al., 1987). These results were interpreted as indicating a strong protein interaction at C-12. We have reexamined these shifts and found that, although the isotropic chemical shift moves slightly in comparison to the PSB model compounds (129 → 132 ppm), the principal values of the chemical shift tensor are largely unperturbed ($\sigma_{11} = 58$ ppm, $\sigma_{22} = 133$ ppm, $\sigma_{33} = 212$ ppm). This is illustrated in Figure 4, where difference spectra between labeled and unlabeled rhodopsin highlight the retinal resonance, and are compared with simulations using the shift tensor values obtained from the all-trans PSB model compound (Figure 4B) and from the previous ^{13}C -12 rhodopsin spectra of Mollevanger et al. (1987) (Figure 4C). In these spectra, the centerband (at the isotropic chemical shift) is at ~130 ppm and is flanked by rotational sidebands spaced at the spinning frequency. The relative intensities of the centerbands and sidebands determine the chemical shift tensor. Comparison of the intensity of the first sideband with the centerband intensity clearly shows the difference between the two simulations. The experimental data closely resemble the simulations based on the all-trans PSB values. The discrepancy between the two solid-state NMR studies on rhodopsin arises in part from a difference in signal-to-noise, and in part from possible problems associated with a fatty acid resonance which overlaps with the centerband in the previous study. The simulations illustrate the sensitivity of the sideband intensities to changes in the chemical shift tensor, as well as the quality of data necessary to accurately determine the shift tensor values.

DISCUSSION

Retinal proteins have evolved mechanisms for accomplishing a variety of functions ranging from the transport of ions across biological membranes to the detection of single photons in the initial process of vision. Key components of these mechanisms involve the structure of the retinal chromophore and its interaction with the surrounding protein. Differences in protein-chromophore interactions among retinal pigments are manifested in the range of visible absorption spectra exhibited by the protein-bound chromophore, where λ_{max} may vary from ~420 to ~620 nm. One of the most intensely studied retinal proteins is the light-driven proton pump, bR, where solid-state

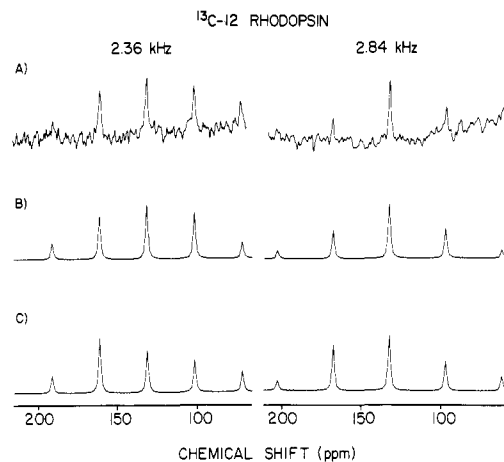


FIGURE 4: Solid-state NMR spectra of the ^{13}C -12 retinal resonance in rhodopsin (A) obtained by taking the difference between ^{13}C -12 rhodopsin and unlabeled rhodopsin spectra. Spectra were obtained at two spinning speeds, 2.36 kHz (left) and 2.84 kHz (right), and are compared with simulations (B and C) based on different chemical shift tensors. The simulations in (B) are for a chemical shift tensor having the principal tensor elements of the all-trans PSB chloride salt ($\sigma_{11} = 58$, $\sigma_{22} = 133$, $\sigma_{33} = 212$), while the simulations in (C) use the shift tensor values ($\sigma_{11} = 41$, $\sigma_{22} = 149$, $\sigma_{33} = 209$) obtained for 12- ^{13}C rhodopsin by Mollevanger et al. (1987).

NMR studies have demonstrated that several factors contribute to red-shifting the visible absorption band to 568 nm. These include (1) a 6-*s-trans*-retinal conformation (Harbison et al., 1985a; van der Steen et al., 1986), (2) a weak interaction between the Schiff base nitrogen and its associated counterion (Harbison et al., 1983; de Groot et al., 1989), and (3) protein perturbations near the β -ionone ring (Honig et al., 1976). In this paper, we use solid-state NMR methods to examine the mechanism of the opsin shift in rhodopsin and isorhodopsin, and compare our results with those obtained previously on bR.

C₆-C₇ Conformation. The conformation of the C₆-C₇ bond can influence the visible absorption band of retinals either by extending π -electron conjugation to the C₅=C₆ double bond in planar conformations or by breaking the conjugated π system in twisted conformations. The C₆-C₇ bond in most retinal model compounds adopts an *s-cis* conformation, with the ring twisted (40–70°) relative to the retinal chain (Honig et al., 1971). A strictly planar *s-cis* conformation is prevented by steric interaction between the C-18 methyl group and the C-8 hydrogen. In contrast, a planar 6-*s-trans* conformation occurs in some crystal forms of retinoic acid (Stam & McGillivray, 1963) and 13-*cis*-retinal (Simmons et al., 1981), in the *tert*-butyl perchlorate PSB (Childs et al., 1987), and in the retinal chromophore of bR (Harbison et al., 1985a).

We have previously used a comparison of the ^{13}C -5 chemical shift between rhodopsin and 6-*s-cis*- and 6-*s-trans*-retinoic acid to argue that the C₆-C₇ bond in rhodopsin is twisted *s-cis* (Smith et al., 1987). The ^{13}C -5 resonance was observed at 130.3 ppm in rhodopsin and at 130.5 ppm in isorhodopsin, close to the characteristic shift of 6-*s-cis*-retinals (~132 ppm). Furthermore, analysis of the ^{13}C -5 chemical shift tensor in rhodopsin revealed that the σ_{33} tensor element was at ~210 ppm, a result expected for the 6-*s-cis* form. In 6-*s-trans*-retinal, this element shifts downfield by about 20 ppm. Similar conclusions were reached by Mollevanger et al. (1987) using samples of lyophilized rhodopsin. However, it was not possible in these studies to determine the C₆-C₇ dihedral angle. This is important since calculations indicate that the λ_{max} of the retinal PSB is sensitive to C₆-C₇ twist (Honig et al., 1976; Birge et al., 1982). Consequently, protein binding may change the C₆-C₇ dihedral angle relative to the retinal model com-

pounds and contribute to the opsin shift. A more planar conformation about the C₆-C₇ bond would increase the opsin shift, while twisting the ring out-of-plane may lead to a decrease in the shift.

On the basis of retinal model compound studies, Harbison et al. (1985b) noticed that the ¹³C-8 chemical shift is also sensitive to the C₆-C₇ conformation, moving from 138.9 ppm in 6-*s-cis*-retinoic acid to 130.9 ppm in the 6-*s-trans* isomer. The upfield shift in the *s-trans* geometry is due to a steric interaction between the C-16,17 methyl groups and the C-8 hydrogen. The ¹³C-8 chemical shift in rhodopsin of 139.2 ppm (Table I) supports the previous conclusion of a 6-*s-cis* structure, and also indicates that the steric interaction at C-8, and thus the C₆-C₇ dihedral angle, is similar to that in the retinal model compounds.

A somewhat similar conclusion regarding the C₆-C₇ angle can be drawn in the case of isorhodopsin, since the C-5 and C-8 shifts in isorhodopsin are essentially identical with those observed in rhodopsin. However, a 4.3 ppm upfield shift is also observed in the ¹³C-7 resonance of isorhodopsin relative to the 9-*cis* PSB. A possible explanation of this shift involves an additional twist about the C₆-C₇ bond and increased steric interaction between the proton attached to C-7 and the methyl groups at C-1. However, if such a twist about C₆-C₇ occurs, it moves C-18 in a direction away from C-8, while not perturbing the ¹³C-5 or C-8 chemical shifts. At the same time, this out-of-plane movement would lead to reduced coupling of the C₅=C₆ bond with the remainder of the polyene chain, and therefore to a reduction in the opsin shift as is observed in isorhodopsin (λ_{\max} = 480 nm). Alternatively, the shift at C-7 may result from increased steric interactions with the protein and may simply reflect the fact that 9-*cis*-retinal is not a completely isomorphous replacement of 11-*cis*. It should be noted that Palings et al. (1987) observed perturbations of the C₁₀-C₁₁ stretching mode, and Eyring et al. (1982) found an enhanced HC₇=C₈H A_u HOOP mode in isorhodopsin. In rhodopsin, neither the perturbation nor the enhancement was present. This provides further evidence that the 9-*cis* chromophore does not occupy the binding site in the same manner as does the 11-*cis*. Further, the observation of unique vibrational perturbations in the C₇-C₁₁ region of the isorhodopsin chromophore, especially the enhancement of the HC₇=C₈H HOOP mode, provides support for the idea that the conformation of the C₆-C₈ region of the isorhodopsin chromophore is perturbed by protein binding. This is not surprising because the *cis* bond has been moved from the 11-position to the 9-position, and this new chromophore conformation must interact differently with the protein.

Schiff Base Environment. An important contribution to the opsin shift in bR appears to be an extremely weak interaction between the Schiff base nitrogen and its protein counterion. This conclusion is based in large part on unusual ¹⁵N chemical shifts observed in ϵ -¹⁵N-labeled lysine bR₅₆₈ (144.9 ppm) and bR₅₄₈ (151.6 ppm) (Harbison et al., 1983). For comparison, the ¹⁵N chemical shifts for the halide salts of the all-*trans* PSB are 172 ppm (Cl⁻), 166 ppm (Br⁻), and 154 ppm (I⁻), where the ¹⁵N chemical shift is observed to decrease as the counterion size is increased. More recently, measurements of the ¹⁵N Schiff base tensor elements in a series of model compounds, and in bR itself, have confirmed this trend (deGroot et al., 1989). Unfortunately, ¹⁵N NMR spectra of the retinal Schiff base in rhodopsin have not been obtained since it is not possible to isotopically label the protein. However, on the basis of studies of bR and several PSB model compounds, it is possible to extract some information on the Schiff base environment

from the ¹³C-15 chemical shift.

The ¹³C-15 chemical shifts in bR₅₆₈ and bR₅₄₈ are at 160 and 163 ppm, respectively, while the ¹³C-15 resonance in the all-*trans* PSB salts occurs at 167 ppm (Cl⁻), 165 (Br⁻), and 163.5 ppm (I⁻). The relative ordering of these shifts parallels the larger ¹⁵N shifts and suggests that the electron density at C-15 is sensitive to electrostatic interactions between the Schiff base and its associated counterion. Polarization of the Schiff base is expected to extend over several bonds [see, e.g., Hofacker (1983)]. Table I presents data on two PSB salts that differ in the Schiff base counterion and the amine used to form the Schiff base. The *tert*-butyl group and perchlorate counterion serve to decrease the mutual polarization with the Schiff base and increase the electron density at the Schiff base end of the conjugated system. This is reflected in the λ_{\max} of the retinal PSB, which shifts from ~440 nm in the *n*-butylretinal PSB chloride salt in CCl₄ to ~470 nm in the *tert*-butyl PSB perchlorate salt (Blatz et al., 1972; Childs et al., 1987). In solution NMR spectra of the 11-*cis-tert*-butyl perchlorate salt (Table I) and in solid-state NMR spectra of the all-*trans* isomer (Childs et al., 1987), the ¹³C-15 resonance is observed at ~160 ppm, 4-7 ppm upfield of its position in the chloride salt. The upfield shift of the ¹³C-15 resonance in the *tert*-butyl perchlorate salt is associated with a slight upfield shift of the ¹³C-14 resonance and a downfield shift of the ¹³C-13 resonance.

These comparisons of the ¹³C-15, ¹³C-14, and ¹³C-13 chemical shifts in the retinal model compounds and bR provide a framework in which to consider the chemical shifts observed in rhodopsin. First, comparison of the ¹³C-15 chemical shift in rhodopsin with the 11-*cis* PSB model compounds indicates that the ¹³C-15 resonance is shifted 1-2 ppm downfield of the chloride salt. The direction of this shift is opposite to that expected for a weak Schiff base-counterion interaction. Also, the chemical shift of the ¹³C-14 resonance in rhodopsin is similar to that of the chloride salt and slightly downfield from its position in the perchlorate salt. These data suggest that the Schiff base environment in rhodopsin is similar to that in the 11-*cis* PSB chloride salt. This conclusion is supported by resonance Raman spectra of rhodopsin that exhibit a C=N stretching frequency at 1657 cm⁻¹, very close to that in the 11-*cis* PSB chloride salt (1658 cm⁻¹) (Palings et al., 1987). The C=N stretch in the perchlorate salt (all-*trans* isomer) is at 1636 cm⁻¹ (R. Mathies and S. Fodor, unpublished results), well below the frequency observed in the chloride salt, but close to that in bR (1640 cm⁻¹). Consequently, it seems unlikely that a weak interaction between the Schiff base and its counterion is responsible for the opsin shift in rhodopsin (or isorhodopsin where the same arguments apply).

Protein Perturbations Adjacent to C-13. Our interpretation of the ¹³C-15 and ¹³C-14 chemical shifts in rhodopsin and the 11-*cis* PSB model compounds suggests that the Schiff base environment in rhodopsin is comparable to that in the 11-*cis* PSB chloride salt. In particular, the fact that the ¹³C-15 and ¹³C-14 resonances do not shift upfield indicates that the ¹³C-13 shift is not a response to a weakened Schiff base interaction. This indicates that the protein induces a large change in the chemical shift of C-13 (Table I) and there appear to be two possible sources for this shift. The first is a protein-induced conformation change in the C₁₁=C₁₂-C₁₃ region of the chromophore. In 11-*cis*-retinal, it is known that the C₁₂-C₁₃ single bond cannot adopt a planar conformation due to steric interaction between the C-20 methyl group and the C-10 hydrogen across the *cis* C₁₁=C₁₂ double bond. If protein binding added an intermolecular contribution to the intramolecular twist already present, this might move the ¹³C

resonance position. From Figure 3, we note an unusual ^{13}C -13 shift in isorhodopsin, which has a similar absorption spectrum to rhodopsin, and presumably a similar mechanism is responsible for its opsin shift. However, the ~ 3 ppm perturbation present at C-12 is now smaller. The general deshielding of the C-8, C-9, C-10, C-11, and C-13 positions in isorhodopsin suggests that this reduction at C-12 may be due to two compensating effects. First, we infer that the deshielding due to charge perturbations may be compensated by a protein-induced conformational change. In solution, the $\text{C}_{12}\text{-C}_{13}$ bond in the 9-cis chromophore will be planar, but in isorhodopsin the chromophore, like the 11-cis chromophore in rhodopsin, it must have rotated about the $\text{C}_{12}\text{-C}_{13}$ bond to accommodate the dimensions of the binding site. This could alter the conformation of the C-11-C-13 region of the chromophore and the chemical shift at C-12.

The second possible source of the opsin shift is a protein charge near C-13 of the retinal. Such a mechanism was proposed by Nakanishi, Honig, and co-workers based on dihydroretinal rhodopsin absorption spectra (Arnaboldi et al., 1979). These studies indicated that a charge interacts with C-12, C-13, and C-14. Our previous solid-state NMR studies of ^{13}C -14 rhodopsin (also Table I) showed that no significant change occurs in the ^{13}C -14 chemical shift, thus providing no support for a protein charge near this position. However, the solid-state NMR spectra presented here indicate that the largest influence of the protein is localized at position 13 both in rhodopsin and in isorhodopsin. Thus, the two pigments may be interacting in a similar fashion with protein charges. However, the different configurations of the 9-cis- and 11-cis-retinals, together with the geometry of the binding pocket, clearly lead to different molecular distortions of the chromophores.

CONCLUSIONS

In bR, three well-defined contributions to the rather large (5100 cm^{-1}) opsin shift have been identified and studied in some detail—(1) a weak hydrogen bond at the Schiff base, (2) a 6-s-trans-retinal, and (3) a perturbation near the β -ionone ring portion of the chromophore. Rhodopsin and isorhodopsin exhibit a much smaller opsin shift ($2000\text{--}3000\text{ cm}^{-1}$), and the NMR spectra indicate a number of differences are present in the chromophore which lead to this reduction. First, the configuration about the 6-7 bond in both rhodopsin and isorhodopsin is 6-s-cis. Since in this configuration the 5-6 double bond is decoupled from the polyene chain, this leads to a substantial reduction in the shift. The perturbation present in the C-7 shift of isorhodopsin, which is possibly steric in origin, might indicate a further decoupling of this bond and the chain, and thus partially explain the lower 2100 cm^{-1} shift observed in this pigment. Second, the ^{13}C data along the polyene chain suggest that the hydrogen-bond strength at the Schiff base is similar to that in strongly H-bonded model compounds. This is true in both rhodopsin and isorhodopsin. Third, there are protein-induced perturbations clearly present in the C-10-C-13 region of the polyene, the dominant effect being a general deshielding in the C-8-C-13 region with the largest effect at C-13. This supports the presence of a protein perturbation which causes the opsin shift. In isorhodopsin, a similar trend is observed, with the exception that the shift at C-12 is reduced, perhaps because the conformations of the 9-cis and 11-cis chromophores are perturbed in different fashions by protein binding.

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Effect of Phosphorylation on Hydrogen-Bonding Interactions of the Active Site Histidine of the Phosphocarrier Protein HPr of the Phosphoenolpyruvate-Dependent Phosphotransferase System Determined by ^{15}N NMR Spectroscopy[†]

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ABSTRACT: The phosphocarrier protein HPr of the phosphoenolpyruvate-dependent sugar transport system of *Escherichia coli* can exist in a phosphorylated and a nonphosphorylated form. During phosphorylation, the phosphoryl group is carried on a histidine residue, His15. The hydrogen-bonding state of this histidine was examined with ^{15}N NMR. For this purpose we selectively enriched the histidine imidazole nitrogens with ^{15}N by supplying an *E. coli* histidine auxotroph with the amino acid labeled either at the N δ 1 and N ϵ 2 positions or at only the N δ 1 position. ^{15}N NMR spectra of two synthesized model compounds, phosphoimidazole and phosphomethylimidazole, were also recorded. We show that, prior to phosphorylation, the protonated His15 N ϵ 2 is strongly hydrogen bonded, most probably to a carboxylate moiety. The H-bond should strengthen the nucleophilic character of the deprotonated N δ 1, resulting in a good acceptor for the phosphoryl group. The hydrogen bond to the His15 N δ 1 breaks upon phosphorylation of the residue. Implications of the H-bond structure for the mechanism of phosphorylation of HPr are discussed.

HPr is a general component of almost all bacterial phosphoenolpyruvate- (PEP)¹ dependent sugar transport systems (PTS). It mediates the transfer of a phosphoryl group from EI, another general PTS component, to the sugar-specific component EII or EIII (Figure 1). HPr from *Escherichia coli* was first characterized by Anderson et al. (1971) and has since been the subject of several reports. The enzyme can exist in a phosphorylated (P-HPr) and a nonphosphorylated form.

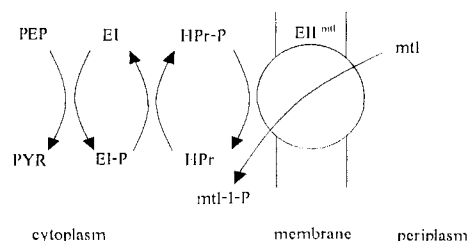


FIGURE 1: Schematic representation of the mannitol- (mtl) specific PTS. For some sugars an additional sugar-specific component, EIII, is present between HPr and the sugar-specific EII.

It was shown by Anderson et al. (1971) that the phosphorylation very probably takes place at the N δ 1 position of a

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