Solid-State ^{13}C NMR Detection of a Perturbed 6-s-trans Chromophore in Bacteriorhodopsin†

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ABSTRACT: Solid-state ^{13}C magic angle sample spinning NMR spectroscopy has been used to study the ionone ring portion of the chromophore of bacteriorhodopsin. Spectra were obtained from fully hydrated samples regenerated with retinals ^{13}C labeled at positions C-5, C-6, C-7, C-8, and C-18 and from lyophilized samples regenerated with retinals labeled at C-9 and C-13. C-15-labeled samples were studied in both lyophilized and hydrated forms. Three independent NMR parameters (the downfield element of the C-5 chemical shift tensor, the C-8 isotropic chemical shift, and the C-18 longitudinal relaxation time) indicate that the chromophore has a 6-s-trans conformation in the protein, in contrast to the 6-s-cis conformation that is energetically favored for retinoids in solution. We also observe an additional 27 ppm downfield shift in the middle element of the C-5 shift tensor, which provides support for the existence of a negatively charged protein residue near C-5. Evidence for a positive charge near C-7, possibly the counterion for the negative charge, is also discussed. On the basis of these results, we present a new model for the retinal binding site, which has important implications for the mechanism of the "opsin shift" observed in bacteriorhodopsin.

A major class of biological pigments contain the polyene aldehyde retinal, Schiff base linked to a surrounding protein via a lysine residue (Wald, 1967; Schreckenbach et al., 1977). These rhodopsins include both signal transducers (rhodopsins, iodopsin, porphyropsin, slow rhodopsin) and energy transducers (bacteriorhodopsin, halorhodopsin), found in mammals, invertebrates, and bacteria. Rhodopsins absorb over a wide range of wavelengths. The pigments of human color vision absorb at 447, 540, and 577 nm (MacNichol, 1964) while vertebrate rhodopsins absorb as low as 417 nm (Fager & Fager, 1982) and as high as 620 nm (Liebman, 1973). Bacteriorhodopsin absorbs close to the upper limit at 568 nm. In contrast, simple protonated retinal Schiff bases absorb at 440–450 nm and unprotonated ones at 390 nm. Thus, the spectral properties of the rhodopsins reflect the sensitivity of the chromophore to the environment provided by the surrounding apoprotein. In addition to shifting the absorption to an advantageous range, intimate interactions between the chromophore and the protein presumably serve to channel absorbed energy according to the specific functions of the particular pigment.

The mechanisms that have been proposed to account for the ability of a protein to significantly perturb the absorption maximum of a protonated Schiff base divide roughly into two classes—conformational and electrostatic. A red shift induced by twisting about double bonds (Yoshizawa & Wald, 1963) has been observed in bianthrones (Korenstein et al., 1973) but is unlikely in rhodopsins because it requires a thermodynamically unfavorable chromophore configuration and because no evidence for it is seen by vibrational spectroscopy. On the other hand, twisting about single bonds is, on both theoretical and experimental grounds, an eminently resonable means by which the λ_{max}[1] of a retinal-containing pigment might be shifted. If a single bond in a conjugated chain is rotated significantly away from a torsion angle of 0 or 180°, it acts as a break in the conjugation, terminating or partially terminating the chain at the point of the break. Since the λ_{max} is dependent on the effective number of conjugated bonds in the chain (Salem, 1967), it is clear that a purely s-trans chain will have an absorption to the red of a chain with one or more twisted single bonds. This is particularly relevant for retinoids, where steric constraints make twisting about single bonds favorable. Specifically, the 6–7 single bond is found to be s-cis and twisted 40–70° out of plane in the majority of retinoid crystal structures [reviewed in Simmons et al. (1981)] and to a predominant extent in solution (Honig et al., 1971). This was calculated to cause a blue shift from a purely s-trans conformation (Honig et al., 1976), an effect that may be greatly amplified by other factors (see below).

An electrostatic perturbation of the chromophore by charged protein residues may also drastically affect the λ_{max} of rhodopsins. The first such charge that must be considered is the Schiff base counterion, presumably a negatively charged amino acid side chain of the protein. In theory, moving this from close contact (3 Å) to infinity can shift the λ_{max} of a protonated retinal Schiff base from 440 to 600 nm (Blatz et al., 1972). However, it has been pointed out that such a removal is

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[1] Abbreviations: bR, bacteriorhodopsin; MASS, magic angle sample spinning; CP, cross-polarization; PM, purple membrane; λ_{max}, absorption maximum.

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thermodynamically not feasible, and it is unlikely that the counterion could be more than 4 Å or so away in the ground state (Honig et al., 1976). This distance could, however, red shift the absorption maximum by approximately 30 nm (Blatz et al., 1972). We have already shown that the Schiff base ^{15}N chemical shifts of bR_{568} and bR_{548} are much closer to those of model compounds in which the interaction between counterions and $N-H^+$ is relatively weak (iodide salt) than those where it is strong (e.g., chloride) (Harbison et al., 1983). Thus, it is reasonable to suppose a weakly hydrogen-bonded counterion.

A more potent means of red shifting the λ_{max} would be to place another negative charge adjacent to the conjugated chain in the neighborhood of the β-ionone ring. This would have the effect of stabilizing the strongly dipolar excited state (Mathies & Stryer, 1976) and thus lowering the excitation energy. Such a red-shift mechanism was first proposed by Kropf & Hubbard (1958) for rhodopsin, and Honig, Nakanishi, and co-workers have more recently presented evidence for such a charge in bacteriorhodopsin (Nakanishi et al., 1980; Kakitani et al., 1983). By replacing the retinal moiety with modified chromophores containing either breaks in the conjugation (Nakanishi et al., 1980) or a pair of nitrogens at either end of the conjugated chain (Derguini et al., 1983), they inferred that the binding site contains an approximately symmetrical external charge distribution with one negative charge near the counterion and another interacting with the polyene in the vicinity of C-5. An additional factor discussed by Honig et al. (1976) is that a point charge interacting with C-5, coupled with a 6-s-trans conformation, could have a synergistic effect on the red shift. Specifically, a perturbation of C-5 is most efficiently coupled to the remainder of the chain when the β-ionone ring and the polyene chain are coplanar. Thus, to understand the red-shifted λ_{max} of rhodopsins in general and bacteriorhodopsin in particular, it is desirable to know both the conformation and the local charge environment of the retinal Schiff base. This requires a spectroscopic probe that is sensitive to both.

Previously, vibrational spectroscopies—Raman and Fourier-transform infrared—have been the major tools for investigating the in situ retinal structure in bacteriorhodopsin (Smith et al., 1985; Bagley et al., 1982; Rothschild et al., 1982). More recently, we have demonstrated that magic angle sample spinning (MASS) NMR experiments can also provide detailed structural information. Using ^{15}N solid-state NMR of [ϵ-^{15}N]Lys bR, we confirmed the protonation of the Schiff base linkage in dark-adapted bR and reported that its ^{15}N chemical shift was consistent with weak hydrogen bonding to the counterion (Harbison et al., 1983). Subsequently, ^{13}C MASS experiments on bacteriorhodopsin regenerated with specifically ^{13}C-enriched retinals showed that the configuration of the Schiff base end of the chromophore was a mixture of 13-cis,15-syn and all-trans,15-anti isomers (Harbison et al., 1984a) while the geometry of the central part of bR chromophore was very similar to that of retinal Schiff bases in solution (Harbison et al., 1984b). These results were obtained with lyophilized purple membrane and have since been confirmed in many cases with hydrated samples.

In this paper we report the ^{13}C MASS NMR spectra of bR labeled on the olefinic carbons C-5 through C-9, C-13, and C-15 and the methyl group C-18. Our results demonstrate that the configuration about the 6–7 bond of retinal is s-trans. Furthermore, the results are consistent with the presence of an external negative charge interacting with C-5 and suggest another positive charge close to C-7, C-8, and C-19. The latter could be the counterion to C-5 and might also play a significant role in determining the bR absorption maximum.

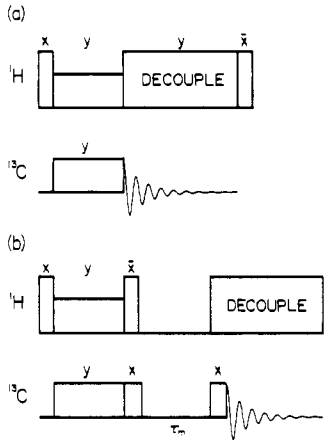

FIGURE 1: Pulse sequences used in this work: (a) standard cross-polarization/flipback sequence used for obtaining spectra of ^{13}C-5-, ^{13}C-6-, ^{13}C-9-, and ^{13}C-13-labeled bR; (b) sequence used to obtain T_1-filtered spectra of ^{13}C-7-, ^{13}C-8-, and ^{13}C-18-labeled bR, as well as for T_1 measurements. Except for inversion–recovery spectra, the phase of the first low-frequency pulse is phase-cycled to eliminate signals from relaxed ^{13}C.

Materials and Methods

^{13}C-Labeled retinals were synthesized and incorporated into white membrane as described elsewhere (Smith et al., 1983). Reconstituted PM was lyophilized at 0.1 Torr and packed under ambient humidity into cylindrical alumina rotors (Doty Scientific, Columbia, SC). Hydrated PM samples were obtained by adding 50 wt % water to the lyophilized materials and mixing mechanically until homogeneous. Samples regenerated with ^{13}C-5-, ^{13}C-6-, ^{13}C-7-, ^{13}C-8-, and ^{13}C-18-labeled retinals were studied in fully hydrated form, while the ^{13}C-9- and ^{13}C-13-labeled PM samples were studied in lyophilized form. ^{13}C-15-labeled PM was studied in both hydrated and lyophilized forms. ^{13}C MASS spectra were obtained at rotor speeds between 3.1 and 4.5 kHz, with a ^{13}C frequency of 79.9 MHz.

The pulse sequences used in this work are shown in Figure 1. Figure 1a is the standard cross-polarization with flipback sequence (Tegenfeldt & Haeberlen, 1979) used to obtain spectra of ^{13}C-5-, ^{13}C-6-, ^{13}C-9-, and ^{13}C-13-labeled retinal PM. Figure 1b shows the sequence used for T_1 measurements and for T_1 filtering. This sequence was also employed for cross-polarization inversion–recovery experiments.

The T_1 filter, which is based on an idea initially proposed by Torchia (1978), operates as follows. After cross-polarization, the ^{13}C magnetization along y has intensity $M_{CP} = \epsilon_{CP}(\gamma_H/\gamma_C)M_B$, where γ_H and γ_C are the respective gyromagnetic ratios of the proton and carbon-13 nuclei, ϵ_{CP} is the cross-polarization efficiency (a dimensionless factor, typically 0.75–0.8), and M_B is the equilibrium Boltzmann magnetization of ^{13}C. If we then apply an $\bar{x}$ pulse to ^{13}C, the magnetization is now directed along Z, resulting in a Z magnetization of $-M_{CP}$. Sampled with a final $\bar{x}$ pulse, this relates to an equilibrium value of M_B by T_1 processes, approximately according to

$$M_1 = -M_{CP}e^{-\tau/T_1} + M_B$$

On the other hand, if after cross-polarization we apply an x pulse, the initial magnetization is M_{CP} and after time τ becomes

$$M_2 = M_{CP}e^{-\tau/T_1} + M_B$$

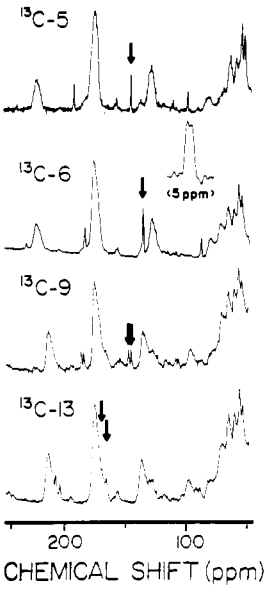

FIGURE 2: Downfield region of the ^{13}C MASS spectra of bR labeled on the quaternary retinylic carbons. The centerbands in all cases are arrowed. The inset shows the centerband of ^{13}C-6-labeled bR with 30-Hz resolution enhancement. Spectra of ^{13}C-5 and ^{13}C-6 were obtained from fully hydrated samples while the ^{13}C-9 and ^{13}C-13 samples were lyophilized.

sampled with an x pulse. If we phase cycle the acquisition to subtract these two experiments, the net magnetization $M = M_2 - M_1$ becomes

$$2M_{CP}e^{-\tau/T_1}$$

which declines monotonically to zero at $\tau \gg T_1$. Thus, by setting τ much greater than T_1 of rapidly relaxing residues, we can filter them out, retaining only those with $T_1 \geq \tau$. The magnetization is placed alternately along Z and $\bar{Z}$ by phase cycling the first low-frequency 90° pulse. A minor addition is the x proton pulse after the cross-polarization period. This returns the proton magnetization to Z, preventing ^{1}H saturation by dephasing of xy magnetization during the decay time and thus eliminating transient Overhauser effects that may distort the T_1 measurements.

Chemical shift tensors of ^{13}C-5-labeled retinal PM were calculated from several spectra taken at different spinning speeds by the method of Herzfeld & Berger (1980), with the computer program described in that publication. Errors in the tensor components are estimated to be ±2 ppm. Spectra of model compounds were assigned as described elsewhere (Harbison et al., 1985b). T_1's were measured by obtaining MASS spectra of these compounds by pulse sequence 1b, with delay times between 62.5 ms and 64 s. The intensities decay nearly exponentially (Naito et al., 1983) from those obtained by cross-polarization to zero at infinite delay time. Logarithms of these intensities were fitted by a linear least-squares program; the time constant of this decay is T_1.

Results

Figure 2 shows the downfield region of the MASS spectrum of bR labeled on the nonprotonated olefinic carbons of retinal—C-5, C-6, C-9, and C-13. These were obtained by standard techniques. Spectra of C-9 and C-13 clearly exhibit two centerbands due to the coexisting 13-cis and all-trans isomers in dark-adapted bR. The spectrum of ^{13}C-6-labeled bR is not obviously split; however, the resolution-enhanced inset (line broadening = −30 Hz) reveals a small splitting of ~0.5 ppm. Only a single line is observed from ^{13}C-5-labeled

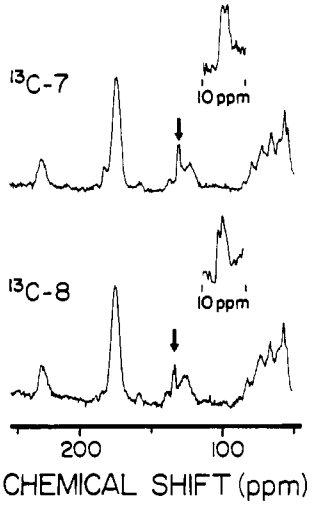

FIGURE 3: Downfield region of the ^{13}C MASS spectra of bR labeled on C-7 and C-8, obtained with the T_1-filtered expeeriment with a delay time τ_D of 2 s. Samples for both spectra were fully hydrated.

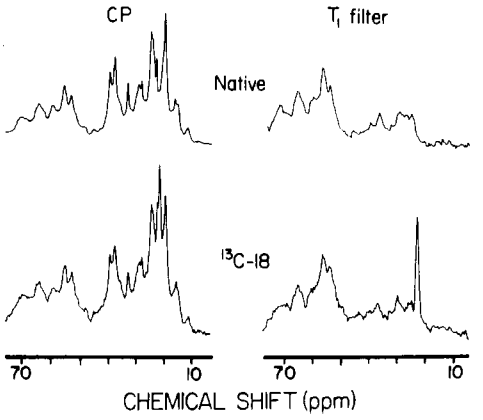

FIGURE 4: Upfield (aliphatic) region of the ^{13}C MASS spectra of unlabeled and ^{13}C-18-labeled bR (fully hydrated). Spectra on the left were taken with standard cross-polarization. The labeled retinylic resonance (arrowed) is visible but highly overlapped with natural-abundance carbon. The T_1-filtered experiment (right) with τ_D = 2 s shows the ^{13}C-18 much more clearly.

bR. As noted previously, the ^{13}C-5- and ^{13}C-6-labeled PM samples were fully hydrated whereas C-9 and C-13 were lyophilized. Thus, the traces shown in Figure 2 permit a direct assessment of the effects of hydration on the ^{13}C spectra. Figure 3 shows the same region of the ^{13}C-7-labeled and ^{13}C-8-labeled bR spectra. These were obtained by the "T_1 filtering" experiment discussed in the previous section, with a 2-s time delay to allow the underlying aromatic natural-abundance carbon magnetization to decay. Again, two lines are observed.

The utility of the T_1 filtering experiment is more clearly demonstrated in Figure 4, which shows the upfield region of native and ^{13}C-18-labeled bR with and without the technique. The cross-polarization spectra on the left barely betray the presence of an extra line due to the labeled retinylic carbon. However, with the pulse sequence of Figure 1b and a 2-s delay, native PM shows no strong upfield resonances, while the ^{13}C-18-labeled bR signal is still strong.

Figure 5 shows an inversion–recovery sequence of ^{13}C-6-labeled bR, which confirms the short T_1's (~150 ms) of the aliphatic signals and the long relaxation times of the C-α (~25 s), C-6 (~25 s), and C=O (~50 s) magnetization.

Table I gives the isotropic chemical shifts of the ^{13}C-labeled bRs studied in this work and compares them with those of protonated Schiff bases in the solid and in solution. Table II

Table I: Chemical Shifts of the 13-Cis and All-Trans Isomers of bR, Compared with Those of *all-trans*-Retinylidenebutylimmonium Chloride, in the Solid State and in Solution

	bR_{568}	bR_{548}	$NRBH^+Cl^-$ solid[d]	$NRBH^+Cl^-$ solution[e]
C-5	144.8	144.8[a]	128.7	131.8
C-6	135.4	134.9[a,f]	138.8	137.4
C-7	129.5	130.7[a]	128.8	132.0
C-8	132.7	131.6[a]	140.8	136.9
C-9	146.4	148.4[a]	142.1	145.3
C-10	133.0	129.7[b]	135.0	129.5
C-11	139.1	135.4[b]	138.9	137.4
C-12	134.3	124.2[b]	135.0	133.6
C-13	169.0	165.3[a,f]	161.8	162.3
C-14	122.0	110.5[c]	122.5	120.1
C-15	163.2	160.4[a,f]	167.0	163.6
C-18	22.0	22.0[a]	23.3	21.9
C-19	11.3	11.3[b]	14.0	13.2
C-20	13.3	13.3[b]	14.0	14.3

[a] This work. [b] Harbison et al. (1984b). [c] Harbison et al. (1984a). [d] Harbison et al. (1985b). [e] Shriver et al. (1976). [f] Assignment to bR_{548} or bR_{568} is not known.

Table II: Chemical Shifts of ^{13}C-5-Labeled and ^{13}C-8-Labeled bR, Compared with Those of Various 6-*s*-*cis*- and 6-*s*-*trans*-Retinal Derivatives[a]

compound	^{13}C-5	^{13}C-8
6-s-cis		
all-trans-retinal	128.5	138.2
13-*cis*-retinal (6-s-cis conformation)	126.7	138.9
triclinic retinoic acid	128.8	138.9
N-retinylideneethylimine	127.2	139.1
N-retinylidenebutylimmonium bromide	129.4	140.6
β-carotene	128.7	139.1
retinyl acetate	127.5	140.0
6-s-trans		
13-*cis*-retinal (6-s-trans conformation)	136.8	<133.0
monoclinic retinoic acid	135.9	130.9
N-retinylidenemethylimine	134.6	133.4
[^{13}C]retinylbacteriorhodopsin	144.8	131.6, 132.7

[a] Harbison et al. (1985b).

compares the ^{13}C-5 and ^{13}C-8 chemical shifts in bR with those of several crystalline 6-*s*-*cis*- and 6-*s*-*trans*-retinal derivatives. Table III gives the methyl group ^{13}C T_1's for a series of crystalline retinal derivatives and compares them with those of ^{13}C-18-labeled bR. Finally, Table IV gives the shift anisotropies of ^{13}C-5- and ^{13}C-8-labeled retinal bacteriorhodopsin, obtained by the method of Herzfeld & Berger (1980).

It was mentioned above that two of the PM samples (^{13}C-9 and ^{13}C-13) were studied in lyophilized form and that ^{13}C-15 was examined in both lyophilized and hydrated states. Chronologically, ^{13}C-9 and ^{13}C-13 were examined first when we thought it was difficult to spin fully hydrated membranes.

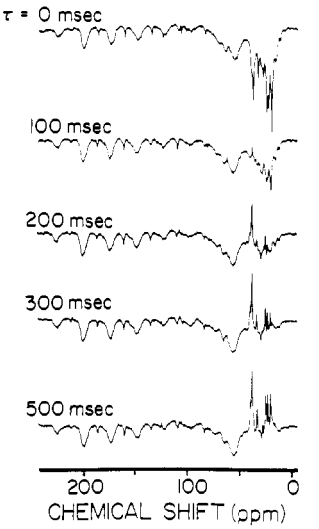

FIGURE 5: Cross-polarization inversion–recovery spectra of hydrated ^{13}C-6-labeled bR, illustrating the short T_1 of the narrow aliphatic amino acid side-chain signals (10–50 ppm) and the much longer T_1 of the rigid α (~60 ppm), carbonyl (centerband at ~175 ppm), and retinylic (centerband at 135 ppm) carbons.

During our studies of ^{13}C-15, methods were developed for circumventing this problem, and thus, the remaining five samples were examined in fully hydrated form. Results shown in Figure 2 illustrate that there are only minor differences between spectra of hydrated and lyophilized samples and these have to do exclusively with the line widths. For example, in the region around 50 ppm somewhat better resolution is apparent in the ^{13}C-5 and ^{13}C-6 spectra because they are hydrated. Otherwise, the shape of, for instance, the C=O centerbands and sidebands is roughly the same. Identical results—a decrease of the line widths on hydration—have been observed for lyophilized and hydrated ^{13}C-15-labeled PM and for [ε-^{15}N]Lys-labeled PM where the Schiff base lines and the line due to the six Lys $-NH_3^+$'s narrow on hydration (Harbison et al., 1985b). These latter samples permit us to assess the effect of hydration on retinal chemical shifts, and as expected, we have observed none. Specifically, the ^{13}C-15-labeled and the [ε-^{15}N]Lys-labeled bR spectra both show lines due to the coexistence of 13-cis and all-trans isomers, and the chemical shifts of both components are unchanged by lyophilization (hydration). Similar results for hydrated and lyophilized proteins have been observed in ^{113}Cd spectra of Cd-substituted metalloproteins, parvalbumin and concanavalin A (Marchetti et al., 1985). In these two cases, the ~1-kHz line widths observed in lyophilized samples narrow by a factor of 4 upon hydration. Apparently, in both bR and the Cd-substituted metalloproteins, lyophilization freezes in a certain amount of

Table III: ^{13}C T_1 of ^{13}C-18-Labeled bR (Obtained Using the Pulse Sequence 1b) Compared with Methyl Group T_1's of a Series of 6-*s*-*cis*- and 6-*s*-*trans*-Retinal Derivatives

	position				
compound	16[a]	17[a]	18	19	20
6-s-cis					
β-carotene	0.25	0.25	0.41	24.2	25.4
triclinic retinoic acid	0.30	0.25	2.00	21.9	25.5
N-retinylidenecyclohexylemine[b]	0.15	0.18	1.63, 2.01	13.59	15.12
β-ionylideneketazine[b]	0.31, 0.28	0.28, 0.21	2.50, 3.69	16.84, 16.92	
6-s-trans					
monoclinic retinoic acid	1.05	1.32	25.6	18.3	26.4
N-retinylidenemethylimine	1.05	15.33	31.7	22.1	19.9
^{13}C-18-labeled bR			16.6		

[a] C-16 and C-17 are chemically identical and only conformationally distinct: Their designation is purely arbitrary. [b] Two molecules per asymmetric unit.

Table IV: Chemical Shielding Tensors of ^{13}C-5- and ^{13}C-8-Labeled Retinal Bacteriorhodopsins[a]

	σ_{33}	σ_{22}	σ_{11}
^{13}C-5-labeled bR	237 ± 2	170 ± 1	27 ± 3
^{13}C-8-labeled bR	216 ± 5	131 + 2	49 ± 6

[a] ppm from trimethylsilane.

local disorder. The rehydration process relaxes this heterogeneity, and the lines in the MASS spectra narrow.

Discussion

Conformation of the 6-s Bond in Bacteriorhodopsin. The conformation about the 6–7 bond joining the β-ionone ring to the olefinic chain in retinals and rhodopsins has long been the subject of attention. Early theoretical calculations and proton NMR studies (Honig et al., 1971) suggested a predominantly 6-s-cis conformation in solution. X-ray diffraction studies of crystalline retinals and carotenoids confirmed a natural bias toward 6-s-cis conformers in the solid state, with a smaller representation of 6-s-trans compounds (Simmons et al., 1981). The conformation about the 6-s bond of crystalline retinoids may also be studied with solid-state NMR, as we have recently shown (Harbison et al., 1985b). The evidence regarding the conformation in a native pigment is indirect. The ability of the planar synthetic chromophores phenylretinal and "naphthylretinal" (7-naphthyl-4-methylheptatrien-1-al) to bind to bleached bR has been used to infer a planar binding site in bR (Bayley et al., 1981; Akhtar et al., 1982), and energetic considerations dictate that this be of a 6-s-trans geometry. In addition, the optical spectrum of bacteriorhodopsin-bound retinal has been interpreted to indicate a planar conformation (Schreckenbach et al., 1977).

Our case for a 6-*s-trans*-retinal chromophore in native bR rests on three entirely independent NMR parameters, each of which individually would be highly suggestive and which together convincingly demonstrate a planar 6-s-trans conformation. These are as follows:

(A) Chemical Shift Tensor and Chemical Shifts of ^{13}C-5-Labeled bR. In our recent study of ^{13}C chemical shifts and shift tensors in crystalline retinal derivatives, we showed that the ^{13}C chemical shifts at the C-5 position differ profoundly in 6-s-cis and 6-s-trans derivatives (Harbison et al., 1985b). The monoclinic modification of retinoic acid (Stam, 1972) and one of the two molecules in the unit cell of 13-*cis*-retinal (Simmons et al., 1981), both known by X-ray diffraction to be 6-s-trans, shows ^{13}C-5 chemical shifts of 136 and 137 ppm, respectively. These shifts are 7–9 ppm downfield of crystalline 6-s-cis derivatives [β-carotene (Sterling, 1964), *all-trans*-retinal (Hamanaka et al., 1972), triclinic retinoic acid (Stam & McGillavry, 1963), retinyl acetate (Oberhansli et al., 1974)] and 5–6 ppm downfield of solution C-5 chemical shifts. Additionally, two of the seven Schiff bases studied also showed shifts in the range 134–136 ppm, in contrast to the others, which had typical 6-s-cis shifts. These data are summarized in Table II. Thus, a strongly downfield shifted C-5 resonance is indicative of a 6-s-trans conformation and vice versa. This phenomenon is quite different from the γ effect, noted on isomerization about double bonds, in that it does not appear to be steric in origin. This distinction is manifested in the distribution of the isotropic chemical shift change between the three principal values of the shielding tensor. Cis–trans isomerizations, as we (Harbison et al., 1985b) and others (Mehring et al., 1983; Terao et al., 1984) have noticed, affect primarily the upfield (σ_{11}) tensor element, while in contrast we showed that the differences between the ^{13}C-5 shift of

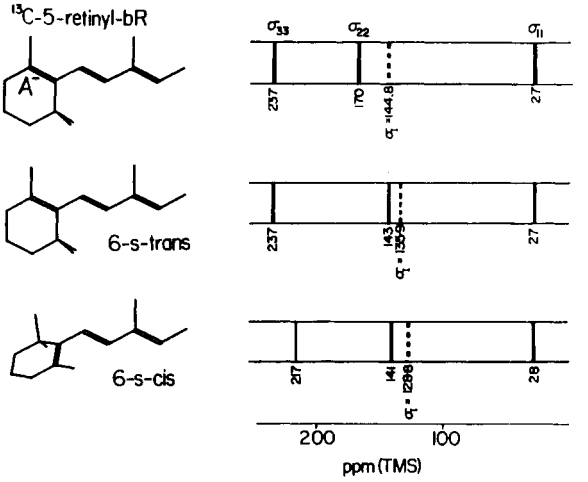

FIGURE 6: Chemical shielding tensor elements of hydrated ^{13}C-5-labeled bR, obtained by the method of Herzfeld & Berger (1980), compared with those of ^{13}C-5 carbons at 6-*s-cis*- (triclinic) and 6-*s-trans*- (monoclinic) retinoic acid. The position of the anion, A^-, is arbitrary. The 6-s-cis conformer is shown in a skewed conformation.

6-*s-cis*- and 6-*s-trans*-retinoic acids resides primarily in σ_{33}.

It is obvious that the chemical shift of ^{13}C-5-labeled bR (144.8 ppm) is much closer to the 6-s-trans derivatives studied than to the 6-s-cis; this suggests the former conformation is present in bR and also clearly illustrates that there is a strong additional downfield shift from even 6-s-trans model compounds (discussed below). The shift tensor further illuminates this phenomenon. Figure 6 shows the ^{13}C-5 shielding tensors of 6-*s-cis*-retinoic acid, 6-*s-trans*-retinoic acid, and ^{13}C-5-labeled bR. (Since the carboxyl group is 10 bonds away from C-5, the use of retinoic acids rather than protonated Schiff bases as model compounds is of little consequence.) σ_{11} is nearly identical in all three compounds, showing little variation in steric effects at C-5. σ_{22} in bR is strongly downfield-shifted from both retinoic acids, a phenomenon that will be discussed below. σ_{33} (the element primarily affected by the 6–7 torsion angle) of ^{13}C-5-labeled bR is 20 ppm downfield from the 6-s-cis compound at a chemical shift essentially identical with that of 6-*s-trans*-retinoic acid. Thus, the chemical shifts of ^{13}C-5-bR support a 6-s-trans conformation for the chromophore.

(B) Chemical Shift of ^{13}C-8-Labeled bR. The other ^{13}C chemical shift affected by 6-s-cis to 6-s-trans isomerization is that of C-8, which in retinoic acid and in unprotonated Schiff bases is upfield-shifted by 8.0 and 5.7 ppm, respectively. Because of overlap of the ^{13}C-8 lines with other lines in the spectrum, it was not possible to obtain a shift tensor for a 6-s-trans retinoid, and thus, the origin of the shift is less clear. However, it is reasonable to assume it to be at least partly steric in view of the close (~2.0 Å) contacts between the C-8 proton and the C-16 and C-17 methyls observed by X-ray diffraction in the structure of 6-*s*-trans-retinoic acid. These contacts are not present in 6-s-cis structures. Again, in bacteriorhodopsin the ^{13}C-8 chemical shift is much closer to those of 6-s-trans derivatives than to 6-s-cis compounds (Table II). Additionally, the shielding tensor of ^{13}C-8-labeled bR shows an upfield-shifted σ_{11} element (Table IV) from those observed for 6-s-cis compounds, which are typically 60–70 ppm (Harbison et al., 1985b). Since σ_{11} appears to be primarily affected by steric interactions, this again leads to an inference that bR possesses the more sterically hindered 6-s-trans structure.

(C) ^{13}C T_1 of ^{13}C-18-Labeled Bacteriorhodopsin. As shown in Figure 4 the retinal line in ^{13}C-18-labeled bR is severely overlapped with the signals from natural abundance methyls of the protein amino acid side chains. Since these methyl

groups have short longitudinal relaxation times (as seen in the inversion–recovery spectra in Figure 5), of the order of 150 ms, we applied the pulse sequence of Figure 1b, which is phase cycled in such a manner to constitute an effective T_1 filter, cancelling the signal from rapidly relaxing carbons but retaining that from slowly relaxing residues. As is seen in Figure 4, applying the pulse sequence with a delay time of 2 s to an unlabeled sample of PM resulted in removal of almost all of the sharp resonances in the 10–45 ppm range, while conserving the intensity of the essentially immobile α-carbons. In ^{13}C-18-labeled bR, an additional line is visible even in the ordinary MASS spectrum. However, its presence is graphically obvious in the τ_D = 2 s T_1-filtered spectrum, being the only intense resonance remaining in the aliphatic methyl region. By plotting the log residual intensity of the signal against time, we obtain a T_1 of 16 ± 2 s, approximately 100 times longer than the amino acid side-chain methyls.

Methyl group relaxation in the solid is usually dominated by the modulation of the ^{13}C-^{1}H dipole–dipole interaction by hops about the C_3 symmetry axis. The resulting T_1 has been approximated by Naito et al. (1983) under conditions of magic angle spinning. Their expression, at our field (7.4 T), leads to correlation times of 2×10^{-10} or 7×10^{-9} s for 3-fold hops of the bulk protein methyls and 3×10^{-12} s for the retinal ^{13}C-18 methyl.[2]

In Table III we compare the measured T_1 of ^{13}C-18-labeled retinal PM with those of the methyl groups of a series of retinal derivatives. It is apparent that the T_1's divide into two classes. Those of methyls attached to the conjugated chain (C-19 and C-20) are uniformly long, while those attached to C-1 (C-16 and C-17) are rather short and, in some cases, close to the T_1 minimum. The T_1's of the C-18 depend strongly on the conformation about the C-6–C-7 bond, being long where the bond is planar 6-s-trans (and where there is a strong steric interaction with the proton on C-7, similar to those experienced by the C-19 and C-20 methyls) and short where the system is skewed 6-s-cis and where C-18 is out of the plane of the hydrogen atoms of the conjugated system. As is seen from the table, ^{13}C-18-labeled bR has a long T_1, in the range of the 6-s-trans derivatives. Furthermore, because in bR there exist many more fast relaxing methyls with chemical shifts similar to those of ^{13}C-18 to act as relaxation sinks for spin–diffusion, the measurement of the bR ^{13}C-18 T_1 is likely to be an underestimate of the "true", i.e., ^{13}C–^{1}H dipolar, T_1. Thus, the relaxation time of the ^{13}C-18 methyl in bR also points to a 6-s-trans linkage.

Charge Distribution in the Vicinity of ^{13}C-5. Since ^{13}C-5-labeled bR is strongly downfield-shifted even from 6-s-trans derivatives, it is apparent that its chemical shift cannot be explained solely by a 6-s-trans conformation, and another origin must be postulated for the additional ~9 ppm shift. This shift is rather singular in that it is not accompanied by a similar perturbation of the directly bonded ^{13}C-6 carbon and thus appears to be a highly localized effect at C-5.[3] The shift is far too large to be due to ring currents. Nor, for example, can it be due to some hypothetical transient covalent linkage to C-5, since this would inevitably eliminate the sp^2 hybridization and produce a significant upfield shift. The phenomenon cannot be steric: first, because strong sterically induced shift changes are not generally experienced by quaternary carbons (Harbison et al., 1985b); second, because no change is observed in the σ_{11} element of the ^{13}C-5-labeled bR shielding tensor (which would be expected to change in such a case); third, because a *downfield* shift would be induced only by relief of a previously strong steric interaction and no such interaction is apparent in the structure of a 6-s-trans retinoid.

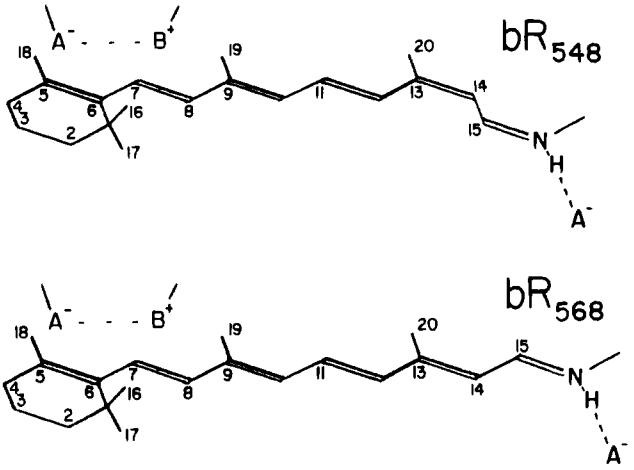

FIGURE 7: Structure of the retinal chromophores in bR_{548} and bR_{568} derived from this and previous work. Note that these are drawn in perspective in order to emphasize the approximate locations of the charges. The configuration about the C-13=C-14 bond has been shown to be trans in bR_{568} and cis in bR_{548} by Raman (Braiman & Mathies, 1982), chemical extraction (Pettei et al., 1977), and ^{13}C NMR (Harbison et al., 1984a) experiments. Solid-state NMR (Harbison et al., 1984a) and Raman (Smith et al., 1984) results indicate that the C-15=N bond is anti in bR_{568} and syn in bR_{548}. Results presented here demonstrate that both bR_{548} and bR_{568} contain perturbed 6-s-trans conformers.

It appears that the only reasonable explanation of our experimental observations is to place a negative charge adjacent to C-5 as is illustrated schematically in Figure 7. Here, the anion, A^-, repels electrons from that part of the conjugated chain and thus increases the positive charge density. This decreased shielding present at C-5 then leads to the observed downfield chemical shift. This sort of interaction has also been hypothesized by Honig and Nakanishi (Nakanishi et al., 1980) to explain the optical spectra of bR. The ^{13}C-5 chemical shift tensor data provide support for the presence and location of this negative charge and, in addition, further constrain the exact form of any molecular model. In particular, a feature of any successful model must be that the charge affects primarily the σ_{22} element of the shift tensor.

It is not easy to correlate the magnitude of this inductive effect with those observed in simple molecules since one can seldom introduce a pure "external charge" without otherwise disturbing the geometry of the system. However, the size of the downfield shift is not unreasonable given the much greater changes in chemical shift induced elsewhere in the chain, by perturbations that have a comparable effect on λ_{max}. For example, protonation of the Schiff base (Shriver et al., 1976) brings about a change in excitation energy comparable to that provided by the postulated external charge and leads to a ~20 ppm change in the chemical shift of ^{13}C-13. It is thus anticipated that the observed phenomenon will be shown to be consistent on theoretical grounds with the absorption maximum changes also induced by the point charge.

It is noteworthy that the 13-cis and all-trans isomers in dark-adapted bR give rise to a single C-5 resonance and thus differ in chemical shift by no more than 0.5 ppm. There is, in fact, a consistent decrease in chemical shift differences

[2] ^{13}C relaxation times, in general, correspond with two possible correlation times for 3-fold methyl hops. Deuterium NMR spectroscopy of deuterated retinal derivatives has been used to discriminate between the two possibilities (G. S. Harbison, J. Herzfeld, and R. G. Griffin, unpublished data).

[3] Studies of the ^{13}C spectra of other positions in the β-ionone ring (^{13}C-1 through ^{13}C-4 and ^{13}C-16 and ^{13}C-17) are in progress.

between the two isomers from C-10 to C-5, such that the two lines are barely resolvable in ^{13}C-7 and ^{13}C-6 bR and coincident in ^{13}C-5-bR. The fact that bR_{548} and bR_{568} possess identical 9 ppm perturbations at C-5 suggests that the spatial relationship between ionone ring and point charge is identical in both species. Since the point of attachment of the Schiff base is also identical, bR_{568} must compensate in some way for the shorter chromophore of bR_{548}. This may be accomplished either by twisting the polymethylene chain of lysine-216, by twisting the point charge residue, or by bodily displacing the lysine-216 and the helix carrying the putative point charge relative to each other.

Charge Distribution about the Conjugated Chain. Almost as remarkable as the charge-induced shift at C-5 is the fact that the shift is not manifested elsewhere in the chain. Usually a perturbation at a single position in a conjugated system is not localized in its effects but rather affects the chemical shift carbons quite distant from the perturbation. For example, protonation of the retinal Schiff base, which brings about a 20 ppm shift at C-13, shifts C-11 by 10 ppm, C-9 by 7 ppm, and C-7 by 3 ppm (Shriver et al., 1976; Harbison et al., 1985b). A corresponding, although proportionally smaller, effect might be expected at C-7 from the point charge at C-5. On the contrary, however, C-7 in bR is somewhat *upfield*-shifted from protonated Schiff bases in solution and is slighly downfield from the solid. C-9 in bR also manifests a similar chemical shift in bR as in protonated Schiff bases.

One possible explanation for this anomalously ordinary chemical shift in C-7 is that the downfield change induced by the point charge at C-5 is being offset by an opposite, but somewhat weaker, perturbation acting at C-7. This would take the form of a positively charged amino acid side chain, probably an arginine. This is depicted schematically in Figure 7 where the positively charged side chain is represented as B^+. There are several attractive features to this idea. First, it has been argued on thermodynamic grounds that the point charge, being an internal charge in a hydrophobic region of a protein, must be associated with a counterion. The expected ion–counterion distance of 2.5–3 Å corresponds nicely with the distance between C-5 and C-7. Second, we have previously noted (Harbison et al., 1984b) an upfield shift at C-19, which might be due to the same counterion, particularly if it is a guanidinium group whose positive charge may be distributed over three nitrogens. Finally, recent studies on bacteriorhodopsin containing dihydroderivatives of retinal indicate that there is a larger opsin shift in the 7,8-dihydroderivative (3500 cm^{-1}) than in 5,6-dihydro-bR (2300 cm^{-1}), which supports the placement of a positive charge near C-7 (J. Lugtenburg and M. Muradin-Szweykowska, unpublished results).

Opsin Shift. Both the occurrence of an ion pair near the ionone ring and the presence of a 6-s-trans conformation in the chromophore greatly influence the absorption maximum in bR. It is generally assumed that retinal-protonated Schiff bases in solution, with $\lambda_{max} \sim 440$ nm, have a 6-s-cis structure with a C-6–C-7 torsion angle of 40–70° (Honig et al., 1971). Honig et al. (1976) calculated that a 6-s-cis (twisted) → 6-s-trans (planar) conversion produces an ~800–1500-cm^{-1} shift in λ_{max}. Thus, planarization of the chromophore in bR could account for nearly one-third of the opsin shift. Much of the remaining ~3600–4300-cm^{-1} shift presumably arises from interactions of the 6-s-trans conformer with protein charges at the two ends of the chromophore. The dipolar pair of charges near $C_5 \cdots C_7$ will interact electrostatically less effectively with the chromophore than a single negative charge and attenuate the opsin shift. The observation of a large opsin shift in the 7,8-dihydro derivative, in which both the ionone ring charges and the C-6–C-7 conformation are isolated from the conjugated π system, suggests that weak hydrogen bonding of the Schiff base to its protein counterion is predominantly responsible for the opsin shift.

The detection of a 6-s-trans conformation may shed light on some anomalous results of previous studies, using modified or photoaffinity-labeled chromophores. For example, Sheves et al. (1984) noted an equilibrium between two species, one with a "normal" opsin shift and one with a blue-shifted λ_{max}, on reconstituting bR with retinals substituted at the 4-position. One possible explanation for this finding is that some of the modified bR is being forced into an unnatural 6-s-cis conformation by substitution on the ring, thus displacing C-5 from its usual place near the point charge. Thus, we suggest that any studies conducted with bR's modified on or near the ionone ring should be carefully examined to ensure that they do not force the chromophore into an unnatural 6-s-cis conformation, which, in the case of photoaffinity labels, could displace the active residue several angstroms from its position in the 6-s-trans derivative. The anomalously low λ_{max} obtained by Huang et al. (1982) using (diazirinophenyl)retinal, for example, may be a result of an unnatural 6-s conformation in that derivative.

Conclusions

The absorption maximum of bR occurs at the extreme red end of the range of λ_{max} values displayed by retinal-containing pigments. As such, it is not surprising that the opsin shift in bR is a result of a combination of several influences of the protein on the polyene chain. In this study we have shown that retinal in bacteriorhodopsin is 6-s-trans and verified the existence of a perturbation acting at C-5, two effects that act synergistically to decrease the excitation energy and increase the λ_{max} (Honig et al., 1976). We have also found evidence for an additional charge at C-7, which could be the counterion for the negative charge and might also play a role in the determination of the color in bR. Finally, previously published (Harbison et al., 1983) and recently obtained ^{15}N NMR data (Harbison, Roberts, Herzfeld, and Griffin, unpublished results) support the hypothesis of a weakly hydrogen-bonded Schiff base counterion, which would also tend to increase the λ_{max}. Thus, three different mechanisms are of importance in the determination of the absorption maximum of bacteriorhodopsin, and some or all of these may also play a role in λ_{max} regulation of other retinal-containing pigments.

This work also completes the spectroscopic determination of the structure of a significant part of the "active site" of a membrane protein. The in situ configuration of the bR polyene chain about C-13=C-14 was first obtained by Raman spectroscopy (Braiman & Mathies, 1982) and agreed with chemical extraction results (Pettei et al., 1977), that about C-15=N by ^{13}C solid-state NMR (Harbison et al., 1984a) and by Raman (Smith et al., 1984), and the conformation about C-6–C-7 by ^{13}C solid-state NMR (this work). ^{13}C NMR data presented here, together with studies of model compounds (Harbison et al., 1985b), also rule out unusual configurations about other bonds in the chain. These structural aspects of the retinal chromophore in bR are summarized in Figure 7. The success of this endeavor points the way to spectroscopic characterizations of other proteins and in particular emphasizes the usefulness of solid-state NMR in determining structures of biological entities that are intractable by other techniques.

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