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^{13}C Magic-Angle Spinning NMR Studies of Bathorhodopsin, the Primary Photoproduct of Rhodopsin†

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ABSTRACT: Magic-angle spinning NMR spectra have been obtained of the bathorhodopsin photointermediate trapped at low temperature (<130 K) by using isorhodopsin samples regenerated with retinal specifically ^{13}C-labeled at positions 8, 10, 11, 12, 13, 14, and 15. Comparison of the chemical shifts of the bathorhodopsin resonances with those of an *all-trans*-retinal protonated Schiff base (PSB) chloride salt show the largest difference (6.2 ppm) at position 13 of the protein-bound retinal. Small differences in chemical shift between bathorhodopsin and the all-trans PSB model compound are also observed at positions 10, 11, and 12. The effects are almost equal in magnitude to those previously observed in rhodopsin and isorhodopsin. Consequently, the energy stored in the primary photoproduct bathorhodopsin does not give rise to any substantial change in the average electron density at the labeled positions. The data indicate that the electronic and structural properties of the protein environment are similar to those in rhodopsin and isorhodopsin. In particular, a previously proposed perturbation near position 13 of the retinal appears not to change its position significantly with respect to the chromophore upon isomerization. The data effectively exclude charge separation between the chromophore and a protein residue as the main mechanism for energy storage in the primary photoproduct and argue that the light energy is stored in the form of distortions of the bathorhodopsin chromophore.

Absorption of light by the visual pigment rhodopsin initiates a photochemical reaction of the protein's 11-*cis*-retinylidene prosthetic group. The first step in this reaction is an 11-cis ⇒ trans isomerization of the chromophore to form the bathorhodopsin intermediate (Figure 1) (Yoshizawa & Wald, 1963). The light energy absorbed in this process is channeled into the protein, where it initiates a biochemical chain of events leading to the closing of sodium channels in the plasma membrane (Stryer, 1986; Liebman et al., 1987). The amino acid sequence of rhodopsin has been determined and is thought to be folded into seven transmembrane helices (Nathans & Hogness, 1983; Hargrave et al., 1983; Ovchinnikov et al., 1982). The retinylidene chromophore is found in the interior of the protein attached to lysine 296 via a protonated Schiff base linkage. Recent mutagenesis studies have shown that glutamate 113 is the Schiff base counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990). The final step in the rhodopsin photoreaction is hydrolysis of the Schiff base linkage and release of *all-trans*-retinal from the protein.

Various approaches have been taken to study the structure and environment of the retinal chromophore in rhodopsin and bathorhodopsin with the goal of trying to understand how the energy is stored in the primary photoproduct, what factors determine the color change upon photoexcitation, and how retinal isomerization is coupled to the activation of transducin, a GTP-binding regulatory protein. Bathorhodopsin has a 6-s-cis,11-trans chromophore whose ground state energy is ~33 kcal/mol above that of rhodopsin and whose absorption maximum (λ_{max} = 543 nm) is shifted to a longer wavelength relative to rhodopsin (λ_{max} = 498). Several mechanisms have been proposed for the observation that ~60% of the light energy absorbed by rhodopsin is stored in the batho intermediate (Cooper, 1979; Schick et al., 1987) and for the red shift in the visible absorption band of bathorhodopsin. The largest contributions are calculated to come from conformational distortions of the retinal and charge separation between the positively charged Schiff base and its protein counterion [see Birge et al. (1988) for a recent review]. It is thus crucial for the understanding of the primary process of vision to

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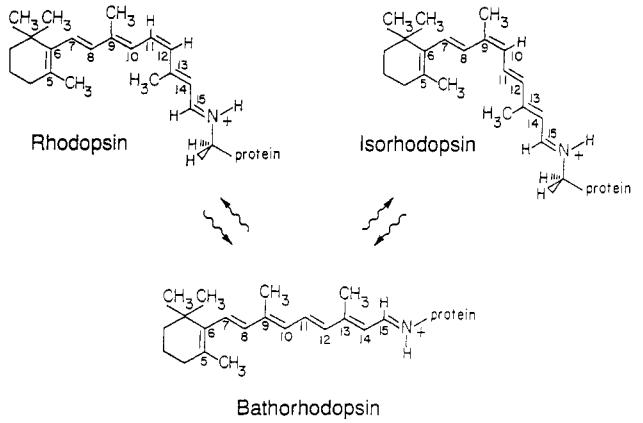

FIGURE 1: Chemical structure and photochemical reaction of the retinal chromophore in rhodopsin. Rhodopsin has an 11-*cis*-retinal chromophore attached to the protein as a protonated Schiff base. The isorhodopsin pigment has a 9-cis chromophore and can be generated by photolysis of the bathorhodopsin intermediate or by removal of the chromophore of rhodopsin and regeneration with exogenous 9-*cis*-retinal. Photolysis of both rhodopsin and isorhodopsin leads to a common intermediate, bathorhodopsin. The thermal decay of bathorhodopsin leads to a series of intermediates designated lumirhodopsin, and metarhodopsin I, II, and III.

characterize experimentally the importance of these two mechanisms for energy storage.

It has been proposed that charged protein residues in the retinal binding pocket and the interaction between the protonated Schiff base and a protein counterion may be involved in charge separation upon isomerization (Kropf & Hubbard, 1958; Blatz & Mohler, 1979; Honig et al., 1979b; Birge et al., 1988). Absorption and resonance Raman studies of bovine rhodopsin and bathorhodopsin have provided evidence for the presence of a protein charge or dipole between positions 12 and 14 of the retinal chromophore (Arnaboldi et al., 1979; Eyring et al., 1982; Palings et al., 1987, 1989), while two-photon absorption studies have concluded that the retinal binding site is neutral, suggesting that this charge is the counterion to the positively charged Schiff base (Birge et al., 1985). However, the importance of at least the location of this perturbation for the energy storage in rhodopsins is questioned by recent resonance Raman experiments on octopus bathorhodopsin, since no evidence was found for a charged protein residue along the polyene chain in this species (Deng et al., 1991). Also, although torsional distortions are observed in both bovine and octopus bathorhodopsin with resonance Raman (Eyring et al., 1982; Palings et al., 1989; Deng et al., 1991), the experiments on octopus bathorhodopsin provide evidence that they do not provide a major contribution to the high ground-state energy of the primary photoproduct (Deng et al., 1991).

NMR[1] provides an excellent method to test the various models in which polarization of the conjugated system contributes to the energy storage in the primary photoproduct. For instance, protonation/deprotonation of a retinylidene Schiff base or its counterion are examples of perturbations that give rise to polarization effects that are easily detected with high-resolution magic-angle spinning (MAS) NMR (Harbison et al., 1985b; de Groot et al., 1990). Recently, MAS NMR of bovine rhodopsin regenerated with retinal specifically ^{13}C-labeled at each position along the chain has provided evidence for the proposed protein charge near position 13 (Smith et al., 1990). The ^{13}C chemical shifts of the chain carbons in rhodopsin exhibit a remarkable deshielding (~6 ppm) near position 13 relative to the corresponding chemical shifts of retinal model compounds in solution. These results are consistent with a negative protein charge in the retinal binding site near this position, possibly the protein counterion to the retinal Schiff base.

A similar NMR approach using ^{13}C-labeled retinals is undertaken in this study to characterize the importance of chromophore polarization and protein–chromophore interactions for the energy storage in bovine bathorhodopsin. We have obtained high-resolution MAS NMR spectra of bovine bathorhodopsin regenerated with [^{13}C]retinal labeled at positions 8 and 10–15. Our data show that the electrostatic interactions between the retinal and the surrounding protein are very similar in bathorhodopsin, rhodopsin, and the artificial pigment isorhodopsin. The results provide evidence against charge separation involving the chromophore as a major mechanism for energy storage in bathorhodopsin and suggest that distortion of the retinal chromophore or protein conformational changes play a larger role in energy storage than previously thought.

Materials and Methods

The synthetic methods for preparing the ^{13}C-labeled 9-*cis*-retinals have been reviewed by Lugtenburg et al. (1985, 1988). The methods for isolating rhodopsin from bovine retinas, purifying the protein, and regenerating the pigment with 9-*cis*-retinal to form isorhodopsin have been described previously (Palings et al., 1987). Briefly, the rod outer segments from ~100 bovine retinas (J. A. Lawson, Lincoln, NE) were purified by density gradient centrifugation. The isolated segments were bleached in 100 mM phosphate buffer containing 10 mM hydroxylamine hydrochloride, washed to remove excess hydroxylamine, and regenerated with 9-*cis*-[^{13}C]retinal for 90 min at room temperature. The regenerated protein was dissolved in 3% Ammonyx-LO (Exciton Chemicals, Dayton, OH) 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 ~2 mL. Excess water was removed from the pigment concentrate by blowing dry N_2 gas over the solution. All of the procedures were carried out under dim red light. Each sample was divided in two for replicate experiments.

^{13}C NMR magic-angle spinning spectra were obtained on a Bruker MSL 200-MHz spectrometer using a home-built double-resonance probe equipped with a 7-mm spinning system from Doty Scientific (Columbia, SC). The ^{13}C and ^{1}H frequencies were 50.3 and 200.0 MHz, respectively. A standard ^{1}H–^{13}C cross-polarization pulse sequence was used with a ^{1}H 90° pulse length of 4 μs, a contact time of 2 ms, and an acquisition time of 40 ms. Data were typically zero-filled to 4K points and line-broadened by ~25 Hz. The 40-ms acquisition time corresponds to a spectral resolution of 0.5 ppm, although the chemical shifts are reported to 0.1 ppm. Several of the rhodopsin spectra (^{13}C-13, -14, and -15) obtained with higher spectral resolution did not exhibit significant changes (>0.3 ppm) in chemical shift. The natural line widths of the retinal resonances are generally about 75–80 Hz (~1.5 ppm). Thus, the errors in line positions are accurate to ±0.5 ppm. The small differences (<1 ppm) between the data in Table I and previous measurements (Smith et al., 1990) can be attributed to different NMR instruments and spectral parameters. Typically, 10000 scans were averaged for each spectrum, corresponding to ~9 h of data accumulation. The

[1] Abbreviations: λ_{max}, absorption maximum, CP, cross polarization; FTIR, Fourier transform infrared; MAS, magic-angle spinning; NMR, nuclear magnetic resonance; PSB, protonated Schiff base.

temperature was maintained near 120 K by using cooled N_2 as the bearing gas. The drive gas was also cooled but was usually above 190 K. The spinning speed was measured by using an optic fiber and was maintained at ~2.5 kHz during all experiments.

For the bathorhodopsin spectra, an isorhodopsin sample was first loaded into an NMR rotor in the dark and spun in the NMR probe to distribute the sample evenly in the rotor. The sample is spread as a thin layer along the wall of the rotor that is easier to illuminate. The rotor was placed in a clear glass dewar containing liquid nitrogen, where it was irradiated with focused light (150 W) from a slide projector passing through a 460-nm interference filter. Irradiation of isorhodopsin at this wavelength yields a steady-state mixture of isorhodopsin, rhodopsin, and bathorhodopsin. We have not been able to obtain back-scattering absorption spectra of the NMR sample in the rotor due to the large amount of scattering from the rotor surface. The amount of photoconversion is best estimated from the NMR spectra that yield distinct peaks for the three components. Measurements on two different samples for both the 8-^{13}C and 12-^{13}C positions gave a range of 46–54% for the amount of bathorhodopsin. The lines were fit with Lorenztian line shapes, and the errors stem largely from low signal-to-noise in the difference spectra. The best agreement between experiments could be obtained if the line width (FWHM) was limited to between 70 and 110 Hz in order to limit fluctuations in noise. After irradiation for ~30 min, the sample was inserted into the precooled NMR probe and data were collected. Difference spectra were obtained during the course of the experiment in order to detect thermal decay of rhodopsin. Depletion of the NMR resonance attributed to rhodopsin was observed if the sample was warmed to above 135 K.

Results

Yoshizawa and Wald (1963) originally demonstrated that bathorhodopsin was stable at <135 K. The present data show that it is possible to perform the MAS NMR experiment under these conditions. Several groups have characterized the quantum yields for the interconversion of rhodopsin, isorhodopsin, and bathorhodopsin at low temperature. These studies have shown that a steady-state mixture of bathorhodopsin (~58%), rhodopsin (~27%), and isorhodopsin (~15%) is formed with 458-nm irradiation (Suzuki & Callender, 1981). On the basis of comparison of model compound data for all-trans, 11-cis, and 9-cis PSBs, the retinal position with the largest dispersion in chemical shifts for these three pigments is C-8 (Smith et al., 1990). The spectrum of isorhodopsin regenerated with 8-[^{13}C]retinal is shown in Figure 2A. This graph displays only the downfield region of the NMR spectrum between 65 and 250 ppm and is dominated by a broad resonance at 175 ppm due to the natural abundance carbonyl resonances of the protein. The broad features at ~225 and 125 ppm are rotational side bands of the 175 ppm resonance. The upfield region (not shown) contains the natural abundance aliphatic resonances of the protein and detergent. In general, this region is difficult to null by using difference methods as the relative intensity of the protein and detergent resonances were variable. Irradiation of the 8-[^{13}C]isorhodopsin sample at low temperature yielded the NMR spectrum of the steady-state mixture in Figure 2B.

In order to obtain the NMR spectrum of the 8-^{13}C resonance without interference from the ^{13}C resonances seen in natural abundance, a difference spectrum was obtained between the ^{13}C-labeled and unlabeled retinal samples (de Groot et al., 1988). The spectrum of an unlabeled sample is shown

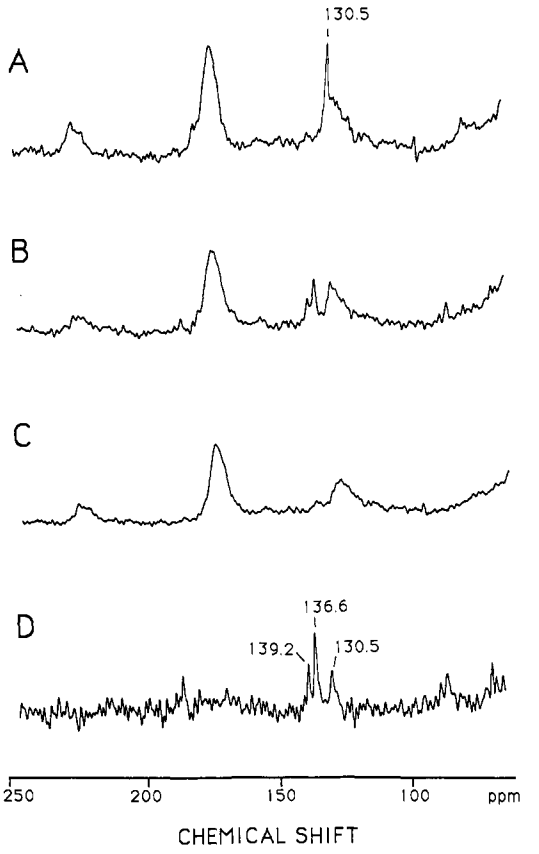

FIGURE 2: MAS NMR spectra of isorhodopsin (A) and a mixture of bathorhodopsin and isorhodopsin (B) containing 8-[^{13}C]retinal. A difference spectrum (D) between spectrum B and a spectrum of unlabeled isorhodopsin (C) yields the spectrum of the 8-^{13}C label alone. The spinning speed was 2.5 kHz.

in Figure 2C, while the difference spectrum is shown in Figure 2D. Three resonances are observed in the difference spectrum, corresponding to rhodopsin, bathorhodopsin, and isorhodopsin. The assignments of these lines are based primarily on the positions of the isorhodopsin and rhodopsin resonances (Smith et al., 1990). The most intense line at 136.6 ppm corresponds closely to that of the all-trans PSB and is assigned to bathorhodopsin. The ratio of the intensities of the three peaks is ~25:50:25, in approximate agreement with the percentages derived from absorption measurements. The signal-to-noise ratio in this spectrum prohibits a better estimate of the peak intensities. The apparent broadening of the 130.5 ppm resonance is most likely due to overlap with the aromatic resonances and poor subtractions. It may be worth noting that the NMR spectrum of this mixture differs in a qualitative sense from spectra obtained by absorption or Raman spectroscopy. In the NMR spectrum, the three components are resolved in the low-temperature mixture, while in the case of absorption and Raman spectra the contributions from the individual components generally overlap.

Difference spectra of isorhodopsin and the steady-state mixture for positions 10–15 were also collected, and the isotropic shifts of the various bathorhodopsin resonances obtained from these data are listed in Table I, together with the corresponding resonances for rhodopsin and isorhodopsin. In the case of position 15, a distinct resonance appears at 163.4 ppm that may be assigned to bathorhodopsin. The integrated intensity of this line is ~45% of the total intensity in the difference spectrum. For positions 10, 11, 13, and 14, the bathorhodopsin resonance is not resolved from both rhodospin and isorhodopsin. In the 10-^{13}C spectrum, the rhodopsin resonance appears at 127.4 ppm and the isorhodopsin reso-

Table I

position of ^{13}C label	^{13}C chemical shift[a] (ppm)			
	batho-rhodopsin	all-trans PSB[b]	rhodopsin[c]	iso-rhodopsin[c]
15	163.4	163.7 (167.0)	165.4	166.7
14	120.0	120.1 (122.6)	121.2	120.0
13	168.5	162.3 (161.8)	168.9	169.2
12	137.7	133.6 (135.0)	132.0	134.2
11	140.0	137.4 (138.9)	141.9	140.0
10	132.0	129.6 (135.0)	127.4	130.8
8	136.6	136.9 (140.8)	139.5	130.5

[a] Errors in chemical shift are estimated to be less than ±0.5 ppm from replicate experiments. [b] Solution data from Shriver et al. (1976). In parentheses, solid-state data from Harbison et al. (1985a). [c] Data from Smith et al. (1990) and present study.

nance appears at ~132 ppm, which corresponds well with the values previously obtained (Smith et al., 1990). The bathorhodopsin resonance is assigned at 132 ppm, coincident with the isorhodopsin resonance, on the basis of the area of this line, which is 4 times as large as the 127.4 ppm resonance. In the 11-^{13}C spectrum, the isorhodopsin and rhodopsin peaks are coincident with the bathorhodopsin peak at 140 ppm on the basis of the values of the rhodopsin and isorhodopsin resonances obtained from the native pigments. The entire 13-^{13}C resonance has shifted to 168.5 ppm in the steady-state mixture, while the 14-^{13}C resonance appears at almost the same chemical shift in all three species (120 ppm). The 13-^{13}C and 14-^{13}C resonances for bathorhodopsin are assigned at 168.5 and 120.0 ppm, although the 13-^{13}C resonance may be slightly lower in frequency if the contributions (based on the 8-^{13}C and 12-^{13}C spectra) from rhodopsin and isorhodopsin are accounted for. The bathorhodopsin resonance is resolved for the 12-^{13}C position at 137.7 ppm and is the dominant component (~50%), similar to what is observed for the 8-position in Figure 2.

For comparison, we list in Table I the isotropic shifts of the corresponding resonances for the PSB model compound, *all-trans*-retinyl-*n*-butylimmonium chloride, in $CDCl_3$ solution and in the solid state. Previously, it was argued that a chloride PSB salt in $CDCl_3$ solution is an appropriate model compound for comparison with rhodopsin (Smith et al., 1990). First, this is because the choice of using PSB solutions rather than PSB powders eliminates the small conformational distortions caused by crystal packing, steric interactions, local charge distributions, and diamagnetic shielding, which make a small difference as is evidenced by the minor differences in solution and MAS chemical shifts in Table I for the all-trans PSB (Harbison et al., 1985a). Second, because the chemical shift of the 15-^{13}C resonance in the visual pigments matches most closely that of the chloride PSB model in solution. Moreover, the frequency of the C=N stretching vibration for the dissolved chloride PSB model at ~1655 cm^{-1} is the same as for the rhodopsins investigated here (Bagley et al., 1985).

Discussion

In previous NMR studies on bovine rhodopsin and isorhodopsin, information about the protein environment was obtained from a comparison of the ^{13}C chemical shifts of the protein-bound chromophore with the corresponding chemical shifts of retinylidene PSB model compounds (Smith et al., 1990). In these studies the largest difference in chemical shift was observed at position 13 and attributed to an interaction between the retinal and the protein at this position. Evidence for a specific protein–chromophore interaction in this region was previously obtained by Nakanishi and co-workers, who postulated a negatively charged protein residue near position

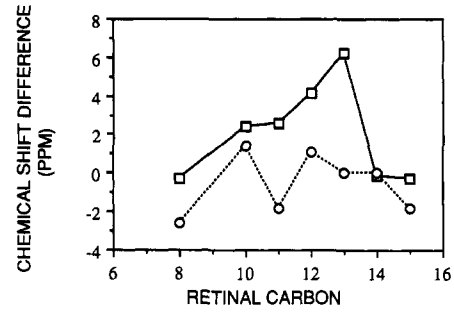

FIGURE 3: (□) Plot of chemical shift differences between bathorhodopsin and the all-trans isomer of the PSB model compound retinyl-*n*-butylimmonium chloride, for several positions along the retinal chromophore. (○) Double difference plot for bathorhodopsin and rhodopsin. Displayed is the difference between bathorhodopsin minus *all-trans*-retinyl-*n*-butylimmonium chloride (this work) and rhodopsin minus 11-*cis*-retinyl-*n*-propylimmonium chloride (Smith et al., 1990).

13 (Arnaboldi et al., 1979). Subsequently, Birge and coworkers concluded from two-photon absorption measurements that the binding site in bovine rhodopsin is neutral (Birge et al., 1985, 1988). In their model, a protein carboxylate serves both as a counterion to the positively charged Schiff base and to stabilize partial positive charge at position 13 of the retinal. This is consistent with the recent identification of glutamate 113 as the Schiff base counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990).

We present a comparison between the rhodopsin and bathorhodopsin NMR results in order to determine whether the protein perturbation previously observed near position 13 changes its location relative to the chromophore as a result of chromophore isomerization. In the context of this comparison, we address the general problem of energy storage in the primary photoproduct and the importance of charge separation involving the chromophore. This discussion begins with a brief description of the NMR data that relates to the chromophore C=N bond structure in bathorhodopsin.

Evidence of a C=N anti Configuration in Bathorhodopsin. The frequency of the 14-^{13}C resonance provides an indication of the C=N configuration in retinal PSBs since it is markedly different in the syn and anti isomers and does not change appreciably with changes in protein environment. For instance, comparisons of retinal PBSs with different counterions show that the 14-^{13}C resonance is virtually insensitive to the counterion size and is seen at ~120 ± 2 ppm, while the 13-^{13}C and 15-^{13}C resonances can shift by 5–6 ppm in going from the chloride (small counterion) to the perchlorate (large counterion) salt (Childs et al., 1987; Harbison et al., 1985a). In contrast, a pronounced shift (~10 ppm) in the 14-^{13}C resonance is observed when the C=N bond is in the syn configuration (Harbison et al., 1984). In bathorhodopsin, the C=N configuration is found to be anti, as in rhodopsin, since we observe the same chemical shift within experimental error. This is in agreement with resonance Raman (Palings et al., 1987) and FTIR (Bagley et al., 1985) results.

Protein Interactions along the Retinal Chain. Figure 3 plots the differences in chemical shift between bathorhodopsin and an all-trans PSB model compound for the positions studied. As in rhodopsin and isorhodopsin, there is a significant deshielding of the ^{13}C resonances from positions 10–13 with the largest difference observed at position 13. *A comparison of these results with those obtained previously (Smith et al., 1990) shows that the protein–chromophore interactions that affect the charge polarization in the conjugated system in bathorhodopsin are surprisingly similar to those in rhodopsin (and isorhodopsin).*

One way to visualize any differences between bathorhodopsin and rhodopsin is to generate a *double* difference plot. Such a plot (Figure 3) is obtained by taking the *difference* between the difference plot shown in Figure 3 (bathorhodopsin minus the all-trans PSB) and the corresponding difference plot previously obtained between rhodopsin and the 11-cis PSB model compound (Smith et al., 1990). In the rhodopsin ⇒ bathorhodopsin transition, the double difference plot indicates that isomerization of the retinal in the formation of bathorhodopsin does not lead to substantial charge polarization in the chromophore, for instance, due to a shift in the location relative to the chromophore of the protein perturbation observed near position 13. Calculations by Honig and co-workers have predicted a red shift and a difference in charge polarization if a negative protein charge is moved away from the Schiff base (Honig et al., 1976). A red shift in the retinal's λ_{max} and a corresponding difference in charge polarization may also result from protonation of the protein counterion. In either case, substantial changes in the chemical shifts of several of the ^{13}C resonances are anticipated, particularly C-13 and C-15, which directly reflect the variations in the local electron density.

As is seen from the double difference plot, only minor shifts are observed in the region from positions 12 to 15 of the chromophore. These shifts are less than 2.5 ppm and correspond to a change of less than ~0.015 electronic equivalents.[2] The errors associated with the double difference plots are in part minimized since a direct difference between the bathorhodopsin and rhodopsin components can be taken from the same spectrum. For instance, a critical position is 15-^{13}C, where the bathorhodopsin peak is observed ~2 ppm upfield from a peak at 165.4 ppm, which is assigned to rhodopsin. Twists in the C_{10}—C_{11}=C_{12} region may be responsible for these (double) differences (Eyring et al., 1982). There have been several studies on the changes in electron density and corresponding ^{13}C NMR chemical shifts due to alterations in the structure and environment of the Schiff base (Shriver et al., 1976; Harbison et al., 1985a; Childs et al., 1987).[3] For instance, upon Schiff base protonation the largest ^{13}C shifts represent up to 0.12 electronic equivalent and are observed in the odd-numbered ^{13}C resonances at positions 13 (18 ppm), 11 (10 ppm), 9 (7 ppm), and 7 (4 ppm) due to stabilization of resonance structures with partial positive charge at these positions.[4] In model compound studies, where the aliphatic amine and counterion used to form the protonated Schiff base are systematically varied (Harbison et al., 1985; Childs et al., 1987), weakening of the Schiff base–counterion interaction results in an effect on the ^{13}C shifts that tapers off rapidly with distance along the chain, i.e., at positions 15 (4 ppm), 13 (3 ppm), and 11 (2 ppm).[5] Furthermore, the ^{13}C double differences (cf. Figure 3) between bathorhodopsin and rhodopsin are smaller than in the retinal model compounds or in bR and do not taper off going down along the chain. Hence, we conclude that there is no substantial charge separation between either the chromophore and Schiff base counterion or the chromophore and the protein perturbation near C-13. The latter essentially maintains its position relative to the retinal.

This conclusion is supported by resonance Raman and FTIR spectra that yield complementary information on the extent of charge separation between the Schiff base and a protein counterion (Bagley et al., 1985; Palings et al., 1987, 1989; Deng et al., 1991). The C=N stretching frequency is sensitive to the Schiff base environment and the nature of the counterion. Since the C=N bond does not change configuration in the primary photochemistry and the retinal isomerizes about only one bond (i.e., the C_{11}=C_{12} in rhodopsin or the C_9=C_{10} in isorhodopsin), a reorientation of the Schiff base proton might be expected and thus would contribute to charge separation between the Schiff base and an associated counterion. Yet, as mentioned above, in rhodopsin, isorhodopsin, and bathorhodopsin, the C=NH frequency is at ~1655 cm^{-1}, similar to that observed for the retinal PSB chloride salts. Together, the Raman and NMR data strongly argue that the hydrogen-bonding environment of the retinal does not change in the primary photochemistry.

The double differences between bathorhodopsin and rhodopsin reflect small changes in the retinal structure or environment and suggest explanations for the red-shifted absorption band in bathorhodopsin. One possible explanation for the double differences and the red shift in bathorhodopsin is a change in out-of-plane distortions of the chromophore due to different steric constraints in the retinal binding pockets of rhodopsin and bathorhodopsin. Figure 3 shows small double differences at C-10, C-11, and C-12, which are in the region of the chromophore undergoing isomerization. An alternative explanation is that glutamate 113 is the sole charged or polar amino acid in the retinal binding site and that isomerization leads to a slight shift of its position toward the ionone ring. In this regard, resonance Raman spectra of bathorhodopsin have argued that interaction with the protein near position 12 is responsible for uncoupling the out-of-plane wagging vibrations of the protons attached to C-12 and C-11 (Eyring et al., 1982; Palings et al., 1989). The Raman spectra of bathorhodopsin also indicate an increase in the bond order of the C_{14}-C_{15} single bond stretch relative to rhodopsin and the all-trans PSB model compound and an increase in the bond order of the C_8–C_9 stretch relative to the all-trans PSB (Palings et al., 1987). These results were taken to indicate that in bathorhodopsin a small amount of electron density is being removed slightly out of the center of the chain (Palings et al., 1987). This would be consistent with the negative double differences (upfield chemical shifts in bathorhodopsin) at C-8 and C-15.

Energy Storage in the Primary Photoproduct. The most important unresolved question involving the primary photochemistry of rhodopsin is how light energy is stored in the bathorhodopsin intermediate. This intermediate is approximately 33 kcal higher in energy than rhodopsin, representing ~60% of the absorbed light energy (Cooper, 1979; Schick et al., 1987). Generally, it is assumed that the major part of this

[2] This is based on the standard relation between ^{13}C chemical shifts and electron density for aromatic compounds and conjugated systems: 1 positive electronic equiv of additional charge corresponds with approximately a +155 ppm ^{13}C shift (Spiesecke & Schneider, 1961; Lauterbur, 1961; Tokuhiro & Fraenkel, 1969). Several groups have shown that this relationship can be extended to the conjugated chain of retinals (Rodman-Gilson & Honig, 1988; Shriver et al., 1976; Inoue et al., 1977).

[3] The largest effects (~150 ppm) are in the ^{15}N chemical shift of the Schiff base nitrogen due to Schiff base protonation (Harbison et al., 1983; de Groot et al., 1989) and result from the loss of one electron from an sp^2 orbital of the nitrogen.

[4] These shifts are derived from a comparison of the solution NMR chemical shifts of the *all-trans*-retinylidenepropylimine Schiff base and the *all-trans*-retinylpropylimmonium chloride PSB (Shriver et al., 1976). In this comparison, the λ_{max} of the Schiff base (370 nm) shifts ~4300 cm^{-1} in the formation of the PSB (440 nm).

[5] These shifts are derived from a comparison of the solution NMR chemical shift of the *all-trans*-retinylpropylimmonium chloride (above) with those of the *all-trans*-retinyl-*tert*-butylimmonium perchlorate (Childs et al., 1987). In this case, the λ_{max} of the chloride PSB (440 nm) shifts ~1540 cm^{-1} in comparison with the perchlorate PSB (472 nm).

energy is stored in the form of charge separation between the chromophore and the counterion and as conformational distortions of the chromophore (Honig et al., 1979a; Birge et al., 1988). Birge et al. (1988) estimated on the basis of molecular orbital calculations that 60% of the stored energy was in the form of conformational distortions of the protein and chromophore and 40% in the form of charge separation.

The MAS NMR data show, however, that the electronic interactions between the protein and the chromophore in rhodopsin do not change substantially in the conversion to the primary photoproduct. The changes in charge density on the retinal carbons studied by NMR, as reflected by the double differences between bathorhodopsin and rhodopsin, represent at most 0.015 electronic equiv at any one position. One approach for estimating how the charge density changes with a change in Schiff base–counterion separation is to use semiempirical electronic calculations. For example, the calculations of Rodman-Gilson and Honig (1988) predict a change of ~0.04 electronic equiv at C-15 with a change in charge separation of 0.2 Å when the counterion is in the plane of the retinal and hydrogen bonded to the Schiff base proton. A smaller change (~0.012 electronic equiv) is calculated for position 13. Both the C-13 and C-15 positions have a large partial positive charge in retinal PSBs that is sensitive to the position of the counterion. The relative magnitude of the changes at C-13 and C-15 depend on the position of the counterion and the method of calculation. Our calculations with the program MNDO (M. J. S. Dewar, University of Texas at Austin) yield shifts of 0.04 and 0.01 electronic equiv for the C-13 and C-15 positions, respectively, if the counterion is placed beneath the plane of the chromophore and is moved by 0.5 Å. In either of the calculations above, the calculated shift in charge density is more than twice that experimentally observed, yet a separation of 0.5 Å corresponds to a difference in stored electrostatic energy of at most 5 kcal/mol.[6] Storage of more energy by a charge separation mechanism would involve larger separations and correspondingly larger changes in charge density. The NMR observations are therefore consistent with less than ~3 kcal/mol stored via charge separation.[7] This is small compared to the energy of ~33 kcal/mol stored in the batho photoproduct.

Conformational distortions are a second mechanism that has been proposed for energy storage in bathorhodopsin. Although resonance Raman spectra of bathorhodopsins have demonstrated that the retinal indeed contains conformational distortions between C-10 and C-14 with an unusual influence of the protein near C-12 (Eyring et al., 1982; Palings et al., 1989; Deng et al, 1991), it has not been possible to model the perturbed frequencies of the hydrogen out-of-plane wags solely on the basis of conformational distortions. This has suggested that other factors are responsible for the perturbed frequencies and only a small part of the energy is stored in the form of torsional distortions (Palings et al., 1989; Deng et al., 1991). The amount of energy that might be stored in out-of-plane distortions of the retinal can be estimated from small molecule studies and electronic calculations. For example, the activation barrier between the s-cis and s-trans isomers of 1,3-butadiene is 3.9 kcal/mol (Squillacote et al., 1979). No comparable experimental results are available for a retinal PSB, although calculations for a retinal PSB without a counterion yield activation barriers of 6–13 kcal/mol (Tavan et al., 1985). Inclusion of a counterion would decrease both electron delocalization and the bond order of C–C single bonds, yielding barriers closer to that observed in the small model compounds. Birge et al. (1988) calculates a total of ~10 kcal/mol for conformational distortions in the retinal chain and lysine.

Together the NMR and Raman data suggest that additional mechanisms play a part in energy storage beyond the out-of-plane deformations and electrostatic interactions implicated in wavelength regulation. Approximately 20 kcal/mol of stored energy remains unaccounted for. Two processes that have not been considered and would be consistent with the experimental data are (1) in-plane bends of the retinal and (2) storage of energy in protein conformational changes. The original studies on rhodopsin demonstrated that the retinal binding site does not accommodate an all-trans chromophore (Hubbard & Wald, 1952; Matsumoto & Yoshizawa, 1978; van der Steen et al., 1989). Since the geometry of a molecule is determined in large part by considerations of steric hinderance [see, e.g., Hendrickson et al. (1970)], photoisomerization to the all-trans isomer must result in either a change in protein binding pocket geometry or a distortion in the retinal structure. Furthermore, since structural changes in the protein are restricted at the low temperatures where bathorhodopsin is stabilized in its ground state, absorbed energy is most likely stored in the retinal. This argument can be extended to room-temperature structural changes by considering the translational and rotational correlation times of groups (helices, large aromatic side chains) in membranes that might be involved in energy storage. For such groups, the correlation times range from 0.01 to 100 μs (Smith & Oldfield, 1984), whereas batho forms in picoseconds. Due to the presence of the methyl groups along the polyene chain and the ionone ring in the retinal molecule, conformational distortions might be expected to give rise to severe steric interactions with the static protein environment. In the calculations of Birge et al. (1988), ~10 kcal/mol was attributed to steric constraints of the retinal with the surrounding protein. Further NMR experiments that target the methyl groups and ionone ring are necessary to characterize the steric interactions at these positions in order to establish the role of in-plane distortions in energy storage.

Conclusions. The low-temperature ^{13}C NMR spectra of bathorhodopsin reveal that the electrostatic protein–retinal interactions stay essentially the same in the primary photochemistry, since a comparison of bathorhodopsin, rhodopsin, and isorhodopsin with appropriate PSB models shows essentially the same general chemical shift differences. The largest perturbation is at position 13. However, the absence of large changes in the average electron density suggests the same location for this protein perturbation in all three species.

These data directly address the mechanism of energy storage in bathorhodopsin. The absence of large chemical shift changes at C-13 and C-15 rules out charge separation between the chromophore and a protein residue as a significant mechanism for energy storage. Torsional distortions of the retinal chromophore may contribute to the energy stored in batho, but resonance Raman studies along with considerations of C–C torsional barriers suggest that these play only a minor role. The involvement of in-plane distortions of the retinal is suggested as a mechanism for energy storage and would be

[6] The stored electrostatic energy can be calculated by using $E = (q_1q_2)4\pi\epsilon_0 rD$, where $D = 1$ (in a "worst-case" approach), the nitrogen has a partial charge of $q_1 = 0.5$ ($\times 1.6 \times 10^{-19}$), the charge on the counterion is taken as $q_2 = 0.5$ ($\times 1.6 \times 10^{-19}$), and r changes either from 3.0 to 3.2 Å or from 4.0 to 4.2 Å [see Honig et al. (1979a)]. These changes correspond to ~2 and 4 kcal/mol, respectively. Alternatively, the MNDO calculations yield a difference in the heat of formation of ~5 kcal/mol, if a negative charge is in line with the Schiff base proton and moved from a distance of 3.0 to 3.5 Å.

[7] More detailed quantum chemical calculations are in progress to explore the changes in charge density with charge separation and chromophore torsion.

consistent with the absence of large changes in electron density along the retinal chain between rhodopsin and bathorhodopsin.

The NMR data are also consistent with the location of the counterion beneath the plane of the retinal between C-13 and the Schiff base nitrogen as proposed by Birge and co-workers. In this position, the counterion would be able to generate the same pattern of chemical shift changes with respect to the solution model compounds in rhodopsin, isorhodopsin, and bathorhodopsin and may play a major role in wavelength regulation.

This work sets the stage for studying the other photointermediates of rhodopsin with NMR. Of particular interest are the meta I and meta II intermediates. Transducin is activated at the meta II stage and is thought to involve a large protein conformational change. In a more general way, this study illustrates the potential of low-temperature MAS NMR for studying enzymatic reactions.

Supplementary Material Available

Two figures showing difference spectra of 10-, 11-, 12-, 13-, 14-, and 15-[^{13}C]isorhodopsin and -bathorhodopsin (2 pages). Ordering information is given on any current masthead page.

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